THORLABS
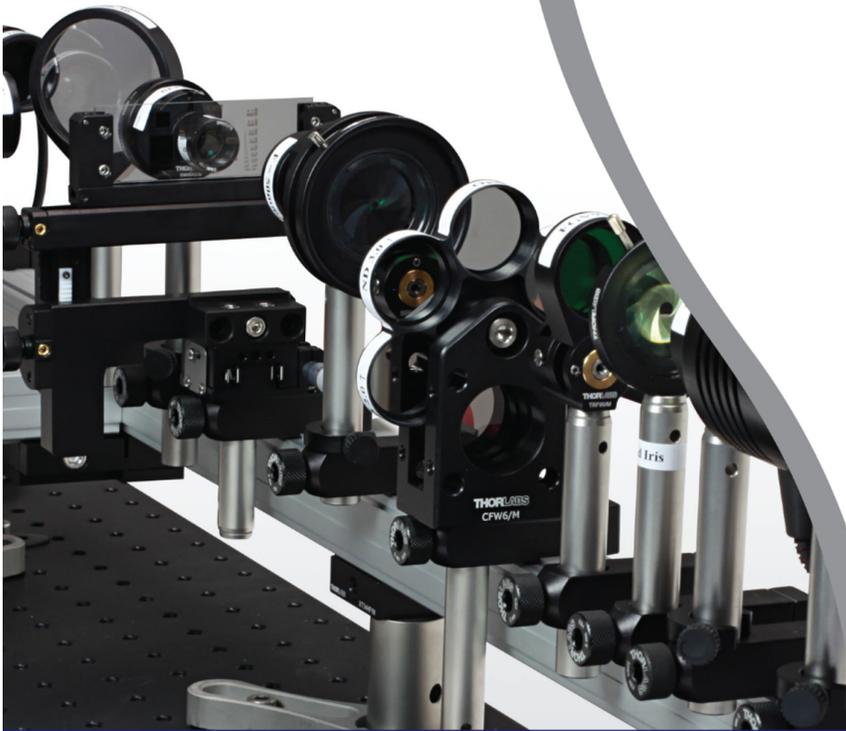
Optical Microscopy Course

Neil A. Switz ● Daniel A. Fletcher

Based on the Course at
UC Berkeley

Lab Notes

Optical Microscopy Course

Lab Notes Preface

Optical microscopy is extremely powerful – witness the vast number of microscope systems in research labs, schools, hospitals, and companies, not to mention the recent Nobel Prizes (2008, 2014) awarded for advances in it. Microscopy is a part of fundamental discoveries in physics, chemistry, biology, and other natural sciences, and it is a central tool in many fields of engineering. Using microscopes properly (or being able to build what you need!) is often the key to a successful experiment, research project, or product.

The past few years have seen a proliferation of new capabilities that build on the fundamentals of optical microscopy. Confocal microscopy allows micron-scale 3D reconstruction of cells and other objects, and two-photon and second-harmonic generation microscopy, using pulses of laser light more intense than the surface of the sun, extend this further. Advanced fluorescence techniques are now enabling ~3 nanometer resolution, far below the traditional "Rayleigh limit". Optics is a hot field, and there are new, sophisticated techniques being developed regularly.

Yet a course on the fundamentals of optical microscopy – one that introduces the true beginner to the field and the regular user to a microscope's inner workings – has not yet become standard in any of the disciplines that rely on the data produced by optical microscopy. This course is intended to fill that gap. The idea is to introduce the main concepts that optical engineers and microscopy experts use to design, specify, and build their own systems.

Why a laboratory course? Optics presented in standard textbooks as a series of ray or wave diagrams and formulas can be remarkably dry. The very same optics, discovered with a lenses and cameras (or your eyes!) can be unbelievably exciting, not to mention beautiful. Concepts that can be so hard to understand on paper come alive when you can simply see them, and watch what happens as you adjust the system yourself. One can accomplish an enormous amount using a fairly small number of basic concepts and rules of thumb, especially if one has practical understanding based on working with real optical systems. So do not hold back! Dive in, and get your (gloved) hands on the equipment; it is the only way to truly learn how things work, and it is fun!

These laboratory notes are based on a Practical Light Microscopy course taught periodically at UC Berkeley beginning in 2010. Despite multiple iterations of the laboratory modules and many student and instructor eyes on each page, we have undoubtedly missed some errors. Please let us know of any you find, so that we may correct them and save others from confusion. Also, should you find one, understand that these notes – in fact, the entire effort to make the lab and the associated parts available to the optics community – is not a commercial venture on the part of the authors. Both of us benefitted from great lab courses put together by others, and we hope our effort here contributes in turn to that cycle.

Lastly, we wish to acknowledge a spiritual debt to Paul Horowitz and Thomas Hayes, for their beautiful lab manual for The Art of Electronics, and for their ability (along with Winfried Hill) to make transfer-function-based techniques accessible using only algebra. If this lab course makes optical microscopy even a fraction as tractable (and fun) as their labs have made electronics, then we will be very satisfied.

Neil Switz and Dan Fletcher

Berkeley, CA

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Introduction to Optical
Imaging (I)

**Optical Microscopy
Course**

Goals

The goals for this laboratory course are to introduce you to:

1) **Optical prototyping** using standard "optical breadboarding" parts, so that you develop some comfort with them and the confidence that you can build a (simple) custom optical system if one would be helpful for your own project.

2) **The Abbe theory of image formation** and, more generally, to help you develop an intuitive feel for the manipulation of "spatial frequencies" in an optical system. For those of you interested in pursuing optics further, this will give you a good base to understand optics from (the unbelievably elegant and powerful) Fourier perspective. We approach this from a conceptual and experimental standpoint, rather than a heavily mathematical one.

3) **Fluorescence imaging**, especially the details of filter choice. It is often easy to buy $500 worth of optical filters and double the sensitivity of a $250,000 microscope if you know what you are doing. You will learn how it is done.

4) **Optical rules of thumb**. It is rare for anyone to do a complicated calculation while building a basic optical system. Rather, if one knows the basics and a few rules of thumb used by the pros, one can quickly get an idea of what should work. We want to introduce you to, and give you practice with, those simple formulae – so that by the end of the course you have the confidence and skillset to try things on your own.

This is not a mathematically difficult course – only once do we use any math beyond algebra (we do a single integral). However, it is conceptually dense, and if you are not up on the course reading, and **especially if you have not read – in advance – the Lab Notes covering what you will be doing in lab, and the Course Notes explaining why, you will get lost and run out of time. There are few, if any, opportunities to catch up. This is NOT a course where you can skip the reading, then cram for the final.**

You should be prepared for a **quiz on the lab instructions each lab.** If you have read them carefully, you will be fine – no trick questions. This is solely to make sure you read the lab instructions ahead of time, and know how to handle the (expensive and delicate) equipment properly.

Be sure to keep up on the readings for each lab!

There will be weekly quizzes on the lab instructions.

Lab 1 Notes: Introduction to Optical Imaging (I)

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Overview

Lab 1

You will be introduced to the basics of digital cameras, optical spectra and simple spectrometers, lenses and measuring focal length, and some of the optomechanical equipment you will be using. These things will underlie the entire class, and you will go into all of it in much more depth over time.

Lab 2

During the 2nd lab, you will explore imaging further with your camera and a lens, set your system up to be the collection side of an "infinity-corrected optical system," and get further experience with the optomechanics by building the collection side of your optical train and the mechanical rail system, which you will use for the rest of the course. Using this system, you will examine the imaging quality of different lens orientations and types and in different colors of light, as well as familiarize yourselves with the resolution test target and use it to calibrate your magnification. This latter set of tasks will likely spill over into Lab 3.

Lab 1 Reading & Preparation:

1. Lab 1 Lab Notes (this document).

2. Lab 1 Course Notes – be sure to read these for review, even if your class schedule is such that you have done the lab first.

Lab 2 Reading & Preparation:

1. Lab 2 Lab Notes.

2. Lab 2 Course Notes.

3. Install ImageJ on your personal computer, or whatever you will use to write your lab reports. See *ImageJ* under the *Reference Links* tab at www.thorlabs.com/OMC to download it.

4. Always bring a USB drive to the lab class so you can copy your saved images for use in your lab reports (or upload those images to your own cloud storage at the end of class).

5. "Equations to Memorize," Appendix A of the Course Notes.

   a. Read the entire document.
   b. For the Lab 2 class/quiz, you are responsible only for memorizing the following equations on the first page:

      - NA (all forms)
      - Magnification (all forms)
      - Thin Lens Formula
      - Depth of Field

6. Wikipedia section on Lens Basics; see *Lens Basics* under the *Reference Links* tab at www.thorlabs.com/OMC for the link. Skim to get the ideas, do not worry about the math.

7. Watch the (very short – 4 minutes each) videos on using the camera and spectrometer software; see the *Videos* tab at www.thorlabs.com/OMC.

   a. *DCC Quick Start Guide* video on using the camera software.
   b. *OSA Quick Start Guide* video on using the spectrometer software.

Supplementary Reading (Not Required):

8. *IDEX / CVI / Melles Griot Fundamental Optics Guide* (under the *Reference Links* tab at www.thorlabs.com/OMC). For now, skim through the first 34 pages of the PDF, and read any bits that look particularly interesting to you. There will be a number of assigned readings from this over the next few labs (particularly, the sections on Performance Factors, Lens Shape, and Lens Combinations). Note: Though the content is identical, the older version of the "CVI/Melles Griot Fundamental Optics Guide" is better laid-out; if you can find a copy, it is easier to read.

9. MicroscopyU section on CCD cameras. Skim this <u>very</u> quickly to get the idea – do not get caught up in confusing bits or subtleties. See *CCD Cameras* under the *Reference Links* tab at www.thorlabs.com/OMC.

10. Not required, but helpful for reference: Wikipedia articles on "Color," "Phase (waves)," and "Snell's Law." Also, the section on refraction and the interactive tutorial. See *Snell's Law* and *Interactive Tutorial on Snell's Law* under the *Reference Links* tab at www.thorlabs.com/OMC.

Lab 1 Tasks

The first lab will be an introduction to the equipment and methods (both technical and teaching) that underlie this course. Typically lab will be a mixture of work on your own (with your instructor to answer questions) and brief lecture segments to introduce the next steps, explain things that are confusing to multiple teams, etc.

Because you are just familiarizing yourself with the system, there is more detail here than there will be in subsequent labs. As you become used to the equipment, instructions will shift to more of an outline of what to set up or measure, rather than exact step-by-step instructions.

> **You may not finish all of this today. Do not worry; some can be completed during Lab 2.**

I.   CMOS Camera Use & Imaging

Handling & Use:

1. Log onto your computer and make a folder for yourself and your documents.

    a. *Bring a USB drive to copy your folder or new files after each lab, or upload to your own cloud storage.*

    b. Please use the following directory structure so that we can find your images as well:

        i. Your main folder (on c: drive): **Coursename_Year_YourGroupLastName(s)** (keep short to avoid filename length issues).

        ii. Place shortcut to this folder on computer desktop.

        iii. Inside your folder, make a subfolder for "Lab 1". This is where you will store all data and images for this lab. Similarly, at the start of each lab, make a new subfolder for that lab and use it to store data for that day.

2. Put on gloves.

3. Connect and start using a camera.

    a. Take the monochrome camera (Thorlabs DCC1545M), and plug it into the computer via the USB cable.

        i. **BE CAREFUL inserting the mini-USB connector into the camera – the connection is delicate, and the camera is ruined if the connection breaks.**

            1. **NEVER lift or handle a camera by holding the USB connector – ALWAYS hold the main camera body.**

            2. **NEVER yank/pull the USB cable when it is attached to the camera – be careful that it is never under strain.**

        ii. The camera may need the full bandwidth of the USB port; sometimes not all ports are separate (e.g. all the front panel ports may be routed through the same bus to the motherboard, so that they cannot all run at full bandwidth simultaneously). As a result, you may be unable to run two cameras off the front panel USB ports – if so, plug one into the front and one into the back of the computer.

Blue text indicates important material that will be helpful for the lab.

Be sure to read the side blurbs as the arrows appear in the text!

REMEMBER! It will be your responsibility to back up your folder and documents.

CAUTION! Always use gloves (nitrile lab gloves) when handling the optics (including cameras!) to protect THEM, not you. It is very hard to properly clean optics, and once they are dirty they are never as good again.

b. To test the camera, go to the computer 'start' menu, find the "ThorCam" program and run it (or use the shortcut on the computer desktop).

c. If you have only one camera connected, the main camera window should open.

    i. If you see only the following window:

          T ThorCam   —   □   ✕

          File   Help

          1 Camera(s) Found

    then click on the camera icon (on the left) and select a camera (with a number ending in "M" for Monochrome) from the drop-down menu.

Live Capture Icon

d. Once the main camera window is open (it may start automatically if there is only one camera connected), go to the ThorCam tool bar and select the Live Capture (film) icon. This will start the camera image acquisition. The screen will likely be black, since you still have the lens cap on.

    i. **RIGHT NOW: go look over the pictures and description of the camera controls (at the end of the Lab 1 Notes) and familiarize yourself with the software.**

e. Holding the camera **FACE DOWN** (so lens cover is down), and wearing gloves, remove the lens cover; place the lens cover **FACE DOWN** on a kimwipe (kimwipes are made of lint-free paper).

    i. **NOTE: you hold the camera FACE DOWN so that dirt from the threads does not fall on the camera face and get stuck there forever.** Always take the cap off with the camera face down (or horizontal; never face up). Similarly, put the lens cap face down on a kimwipe to prevent crud from the table from sticking to it and getting on the camera when you replace the lens cap. If no kimwipes are available, put it face up to avoid contact with the table surface.

    ii. **NOTE: If your camera already has a lens tube on it (e.g. from a previous class), then hold it with the tube horizontal – to prevent any debris from the anodized threads from getting on the camera sensor – and carefully unscrew the lens tube from the camera, without putting too much force on the camera body (but DO hold the camera body, NOT a post, if the camera is attached to a steel post.) Carefully put the tube somewhere secure so the lens in it will not get damaged (e.g. by rolling off the table.)**

CAREFUL!
Cameras are very hard to clean, and any dust/crud you get on the surface will be in every image you take for the rest of the semester. Treat it carefully, and try to keep it closed!

f. Turn the camera face-up; the screen should go white (if it has not already); wave your hand over the camera and you should be able to verify that it is light sensitive.

4. **While the lens cap is off the camera, take a look at the sensor so you know what the camera looks like inside. Which part actually captures the image?**

Lab 1 Notes: Introduction to Optical Imaging (I)

Imaging

Part I: Camera

5. Hold the camera against some feature (other than the camera image window) on your monitor screen, and adjust the exposure-time slider until your image is gray.

   a. Do this by selecting the "Settings" icon on the toolbar, then adjusting the exposure time slider.

      i. You may have to turn down the frame rate, or the pixel clock, or both (they are on the same tab in the Settings window) in order to get sufficiently short exposure time.

      ii. If you see lines moving across the image, they are probably due to beating between the acquisition time for each image (the exposure time) and the 120 Hz flicker frequency of fluorescent lights in the room (or fluorescent backlight in your monitor). LED back-lit monitors will not show this effect.

   b. Open a line-scan profile to see if the pixels are saturating ("overflowing" – if the line is all the way at the top, and not noisy, then you are saturating – i.e., overwhelming the pixels with light). Turn down the exposure until the line is at about 50 - 80% on the y-axis (about 125 - 200 counts).

      i. Do this by clicking on the toolbar Line Profile icons.

      ii. Move your cursor over the image and click where you want the line to cross the image. The pixels on this line will have their values plotted in the scan window.

      iii. You can open one horizontal and one vertical line profile at the same time. Keep track of which is which to avoid getting confused!

6. Take the corner of a (clean!) bit of lens paper (we will give this to you- it is lint-free, non-scratching paper. Never touch an optical surface – lens or camera – with anything else).

   a. Touch the edge of the paper to the camera surface, <u>while holding the LED flashlight maybe 6 inches (15 cm) above it to illuminate it well</u>. **This is the only time you will ever touch the camera face with anything.**

   b. Put the corner of the paper down onto the lens (very gently); adjust the exposure until you get an image of the paper edge.

   c. **Move the paper up and down a little. How far away from the camera face can you have the paper and still see any detail?**

7. **What does this imply about what the camera senses? Why does the paper have to be right up against the camera in order for you to see the paper edge at all?**

   a. **In a camera-based microscope, where will the "image plane" be?**

8. Take an image. Do this by clicking "Quick Save" icon.

   a. Check your Quick Save Setup Settings so you know where the images will get saved. The image will save with sequential numbers (e.g. image_1, image_2, etc.).

   b. **ALWAYS save images in this course as lossless TIFF or bitmaps (BMP) format – this saves a numerical value for the light intensity at each pixel.**
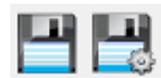
Settings Icon

Line Profile Icons

CAREFUL!
Only use lint free non-scratching paper to touch the camera lens!

Quick Save and Quick Save Setup Icons

i. Do not use JPG (saves file as .jpg); this format uses "lossy compression" (i.e., throws away image information to make the image file smaller), and so you will lose critical image detail and be unable to do parts of your lab reports.

9. Check that you can find your image, and that you can open it.

> a. **NOTE** that Windows 10 does not natively open TIFF (TIF is the same as TIFF) files – it will open an image program and just display black. Instead:
>
> i. Open the ImageJ program using the start menu or desktop icon. (We will use ImageJ for the whole course.)
>
> ii. Drag and drop your image file onto the ImageJ toolbar. This should open it.

Part II: Imaging & Lens

10. Hold a sheet of paper up to your computer monitor, far enough away that you can see the side the light is hitting, and look at the "image" on the paper. Do you see anything at all? (Try this with the room lights low, and use contrasting features – like a white window open on a dark desktop background.)

11. With the room lights on, hold an inexpensive lens (your instructor will give you one) above the table, until you get an image of the overhead lights on the table.

    a. If your classroom has very diffuse ceiling lighting, the instructor will provide another source of light for you to image.

12. Place the camera face up on the table under that image.

    a. Switch the camera back to "live capture" by clicking the ThorCam live capture icon.

    b. Can you get a decent image of the lights, or some part of them?

        i. **Look right on the camera surface – you should be able to see the image of the lights there as you move the lens around above the camera.**

        ii. **Where is the "image plane" of this system you have just made?**

        iii. **What does the screen look like; is the image the same size as the one on the desk? If not, why not?**

            1. **Can you see more detail in the camera image than you can when you look at the image on the desk? If so, why do you think that is?**

        iv. **Why could you not just hold a camera in front of the computer screen and get an image?**

    c. **Take a (properly exposed – the line profile should not show saturation) snapshot of the lights, and save it.**

13. **Put the cap back on the camera.**

II. Lenses & Focal Length

14. Take your ruler and the inexpensive lens (ask your instructor which lens this is!) and determine the focal length of the lens by making an image of the ceiling lights on the table, just as you did a minute ago. Then use the "thin lens formula" from the "Equations to Memorize" document (see Course Notes Appendix A).

   a. **Record how you did this: the distance from the lens to the image, the distance from the lens to the object (the light) that you are imaging, and the uncertainty in each of these measurements (how well can you read the ruler?)**

   b. **Would it be better if the ceiling lights were higher up/farther away?**

      i. **Why?**

      ii. **Estimate the error in your measurement of the focal length.**

◀ **Use a formula for 14 (b) part ii. Do not guess!**

III. Spectra

Cameras & Spectra

Comment: **How do color cameras work?**

Most color cameras (including yours in this lab) use the same sensors as monochrome (grayscale, also called "black and white") cameras. As you might guess, somehow the camera has to get information on the different colors in the image. The typical way this is done is to put a color filter over each pixel, with some pixels having green filters, some having red filters, and some having blue filters. This is called a color filter array (CFA), and the usual colors chosen are red, green, and blue (RGB). That way in red light, only some of the pixels will detect the light; in green light a different set will detect light, etc. Then the various values are combined (in a process called "de-mosaicking") to get red, green, and blue values at each point, which is what is sent to a monitor (which usually has a group of red, green, and blue elements clustered at every "pixel" point). One way of laying out the different color filters on top of the pixels is known as a Bayer array, and looks as follows:
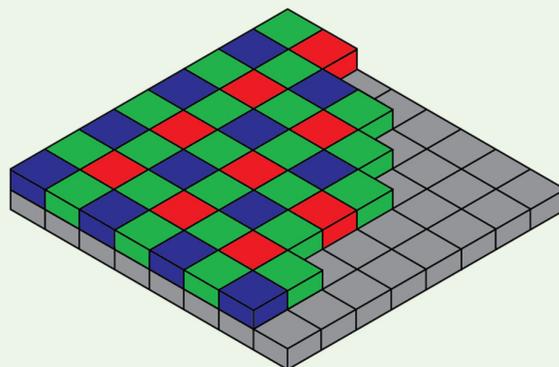
Figure 1: A Bayer array featuring RGB filters on top of an underlying monochrome sensor. Image after Wikipedia (2006).

15. Take the cap off your monochrome camera (if it is still on).

16. Attach the color camera to a different USB cable (so both cameras are attached to the computer).

   a. Take the lens cap off of the color camera (or the lens tube, if it has a tube on it).

   b. **Does the camera face look different from the monochrome camera in any way?**

17. Using the ThorCam program menu, click on the camera icon on the left and choose your second camera from the drop-down menu.

   a. Start live capture (as you did for the monochrome camera; we will no longer give explicit instructions for every step).

   b. Set the exposure to mid-level.

   c. The image displayed from the camera won't be gray, since it is a color camera. Use a line-scan to figure out what is midrange (there will be a line on the plot for each color: Red, Green, and Blue – in general, you do NOT want the one(s) you are interested in to be saturating.)

18. **With the room lights out,** and cameras facing away from your monitor:

   a. Adjust the exposure so both of the camera images are pretty dark, almost black.

   b. Point the IR LED (from your equipment rack (plugs into a USB cable; not the LED flashlight) at the **monochrome** camera while watching the video on the screen.

      i. **Does the *monochrome* camera respond to this LED?**

      ii. **Turn <u>down</u> the exposure setting until the image gets dim (if you can turn it down enough; using a higher frame rate may help; this may also require a higher pixel clock setting).**

         1. **Record the exposure time used.**

      iii. What does this imply about the sensitivity of the camera? Does it respond to things you cannot see?

   c. Point the IR LED at the **color** camera while watching the video onscreen.

      i. **Does the *color* camera respond to this LED?**

      ii. **Turn the exposure <u>up</u> until you see something.**

         1. **Record the exposure setting used.**

      iii. What does this imply about the sensitivity of the camera? Does it respond to things you cannot see? How well?

   d. **If the cameras respond differently, think about why that might be.**

      i. Consider what happens if you take a picture under fluorescent lights (which have little or no infra-red emission) **and a regular lightbulb (which has a lot of infra-red emission). Would you expect the pictures to look different? Would you <u>want</u> them to?**

      ii. **What is the ratio between the color camera exposure and the one for the monochrome camera? I.e., how much more sensitive is the monochrome camera to the IR LED than the color camera is?**

19. Put the caps back on your cameras, and close the ThorCam camera software.

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Spectrometers & Measuring Light Spectra

20. Plug your CCS Compact Spectrometer into a USB port.

   a. If it is not already attached, carefully attach an optical fiber to the spectrometer. Ask your instructor for assistance.

      i. Unscrew the red cover from the spectrometer entrance port.

      ii. Carefully remove the black protective cap covering the fiber face.

         1. Do this by pulling the edge with your fingernails; squeezing causes it to stick.

         2. Put the cap in the spectrometer box for safekeeping.

      iii. **DO NOT touch the end of the fiber! That will irreparably damage it.**

      iv. CAREFULLY insert the fiber end into the spectrometer entrance port, and screw it on.

      v. Remover the rubber cap from the other end of the fiber.

      vi. Carefully store the red spectrometer cap and rubber fiber caps in the spectrometer box for later use.

21. Open the "Thorlabs OSA" software (icon on your desktop, or using the Start menu).

   a. **Look at the pictures of the software interface, further down in this document.**

   b. **You may find it useful to watch the (4 minute) "OSA Quick Start Guide" video on using the spectrometer software; see *OSA Quick Start Guide* under the *Reference Links* tab at www.thorlabs.com/OMC for the link.**

22. Click "Scan USB."

   a. If a menu pops up (it may not, if it recognizes only one spectrometer), then select your spectrometer.

23. Click the "repeat" play button, or use Ctrl+R to start continuous data acquisition.

24. You can choose to average your measurements to get a smoother-looking spectrum. To do this, take the cap off the spectrometer fiber, very carefully.

25. Point the spectrometer fiber at the overhead fluorescent lights.

26. Click "Auto Setup."

   a. **DO NOT** click 'Auto Setup' unless there is light on the spectrometer fiber face or entrance port, otherwise Auto Setup will take an extremely long time as it tries to optimize for a small signal (using a very long integration time). If this happens, use the Task Manager to stop the OSA software, and then restart it and try again with light on the spectrometer.

   b. Right-click the screen area to autoscale so you can see everything.

   c. You should see some bumps appear on the spectrometer trace on the screen.

   d. Adjust the integration time (this is the same as "exposure" for the camera) until the trace is almost, but not quite, full-scale.

      i. **An integration time of a few milliseconds (ms) is a good starting point. Aim the fiber at the room lights, and right-click**

the spectrometer window area periodically to keep the system autoscaling. Keep turning up the integration time until the trace clips (goes flat / horizontal) at the top of the screen even after clicking to autoscale, then turn it down a bit (so it does not clip).

e. Save your spectrometer data, and then import into a spreadsheet. There are two ways to do this:

    i. Copy and paste:

        1. Right-click the "Trace" letter for the trace you want, and choose "Copy to clipboard," then "Tab Separated."

            a. See the final figure at the end of the Lab 10 Lab Notes if confused; alternatively watch the OSA Quick Start Guide video mentioned previously.

        2. Click on an open cell of a spreadsheet (e.g. Excel) and use "Ctrl+V" to paste the data in.

        3. Label the data columns! One column is wavelength, in nm, the other is raw data counts. Make sure you also include WHAT the data is from (e.g., the fluorescent lights, LED, etc.)

        4. SAVE this file with a descriptive filename.

    ii. Via 'Export Trace':

        1. Click the "Stop" button under the Sweep tab to stop data acquisition.

            a. If the spectrometer is no longer collecting data, this button should change to say "Repeat".

        2. Under the "File" tab, select "Export Trace" – this button is to the right of the other buttons to save data.

            a. Name your file and select to save as a zipped comma-separated (CSV) file.

            b. You will then be prompted to select a delimiter to separate the columns in your data file (comma, semicolon, or tabs). Select "OK" after choosing your delimiter.

            c. Import your data into a spreadsheet (you may be able to do this just by double-clicking the file; if not, open MS Excel and go to Data/Import from Text and select the file.

    iii. **La**bel the data columns! **O**ne column is wavelength, in nm, **and** the other is raw data counts. Make sure you also include WHAT the data is from **(e.g., the fluorescent lights in this case).**

    iv. SAVE this file with a descriptive filename.

REMEMBER! All software crashes sometimes. Be sure to save (Ctrl+S) often!

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f. Copy and paste an image of the spectrum into a word processor (e.g. MS Word) or presentation (e.g. PowerPoint) file.

> i. **Note**: On Microsoft Windows computers, Ctrl+PrintScreen copies the whole screen; Alt+PrintScreen copies only the active window.
>
> ii. Alternatively, you may save (and even add notes to) the full spectrum using the spectrometer software.

iii. Write a note about what the image is:

1. What integration time did you use? What type of spectrometer? What is the spectrum of? Save it with a descriptive filename.

iv. **Get in the habit of doing this – copying images and data into files is a major way you will keep your data during the class, and you will need the extra information when you are doing your lab write-ups:**

1. **You will save your images as bitmaps, but also paste screen-captures (use Ctrl+Alt+PrintScreen or Alt+PrintScreen to capture an image, and paste it into your document with Ctrl+V) with notes to remind you of experimental details.**

g. Similarly, take **and save in your MS Word document** spectra from:

i. A white LED flashlight, or the clip-on white LED lights above your monitor.

ii. Several other LEDs, including the clear (IR) one you used before.

iii. A white area on your computer screen.

iv. A halogen lamp with no filter or glass in front.

v. A halogen lamp with a (e.g. green) color filter held in front of it.

vi. A halogen lamp with the light blue BG40 Schott-glass (Thorlabs item # FGB37M) filter held in front of it.

1. **Compare this with the bare lamp. What do you think this filter is for? Why would you use it?**

2. You could try taking the spectrum of clear (IR) LED again, and putting the BG40 Schott-glass (FGB37M) filter in between to see what happens.

a. The color camera has BG40 glass (or something equivalent) built onto it. **Contemplate why that might be.**

WATCH OUT!
Be careful: the lamps can get quite hot. Only turn them on when you are taking the data, then turn them back off. Only hold them by the post they are attached to, not the back housing.

IV. Clean Up

27. Put the cap back on the spectrometer.

28. **Carefully disconnect the USB cable from your spectrometer by gently pulling the connector straight out.**

29. **Carefully disconnect the USB cable from your camera(s) by gently pulling the connector straight out.**

30. Put the caps on the cameras.

31. Put everything back in the cabinets.

32. Save all your data, and shut down the computer.

Administrative Details:

- Your instructor will notify you of:
    - o The due date.
    - o How to submit your report.
    - o How to name your report.

- **Be sure to look at Appendix A, "Lab Write-Up Template" so you know what is expected.**

1. **Focal Length Measurement:**

    Give the focal length of the lens you measured, along with a *brief* description of how you did it, noting particularly relevant details. Obvious question: what are the relevant details? Please give a (ballpark) error level on your measurement, and explain how you arrived at it.

    > **Note**: The error in your measurement is NOT the difference between using the equation with infinity as the object distance and with some finite distance plugged in there; the error will be due to the uncertainty in the image and object distances you measured. There are several ways to approximate that (quantitatively); you can use standard propagation-of-errors techniques, or alternatively compute (e.g. using a spreadsheet) the focal length for all combinations of your measurements and errors (i.e., of $S_i \pm$ error in S_i, and $S_o \pm$ error in S_o, and get a rough estimate of your uncertainty that way.

2. **Which way will the lens move?**

    If you have put a lens on your camera and focused at an object across the room, which direction (toward or away from the camera) will you need to move the lens to get it to image an object infinitely far away? Look at the appropriate equation to figure this out. Explain in your report.

3. **Spectra:**
    a. Paste into your lab report your spectra for the LEDs, halogen lamp, and the white area of your computer screen. Be sure to label and caption them appropriately.

    b. Use Excel (or a similar spreadsheet) to graph your spectra data from the fluorescent lights. Plot this with axes properly labeled and a suitable title and caption.

    c. Explain what type of light the backlight for the LCD computer monitor is (LED, incandescent, or fluorescent), and why you think that.

4. **Worked exercise – how far is "infinity"?**

    In Lab 2, you will set up your camera with a lens, imaging at infinity. This exercise is intended to help prepare you for that; Lab 2 will make more sense if you have done this problem, properly, first:
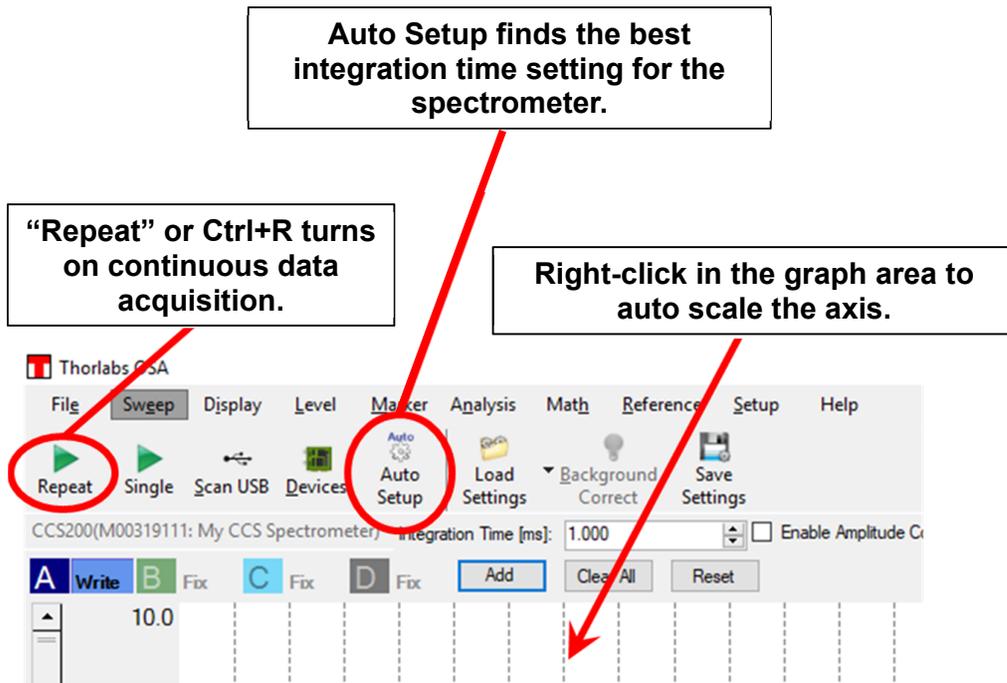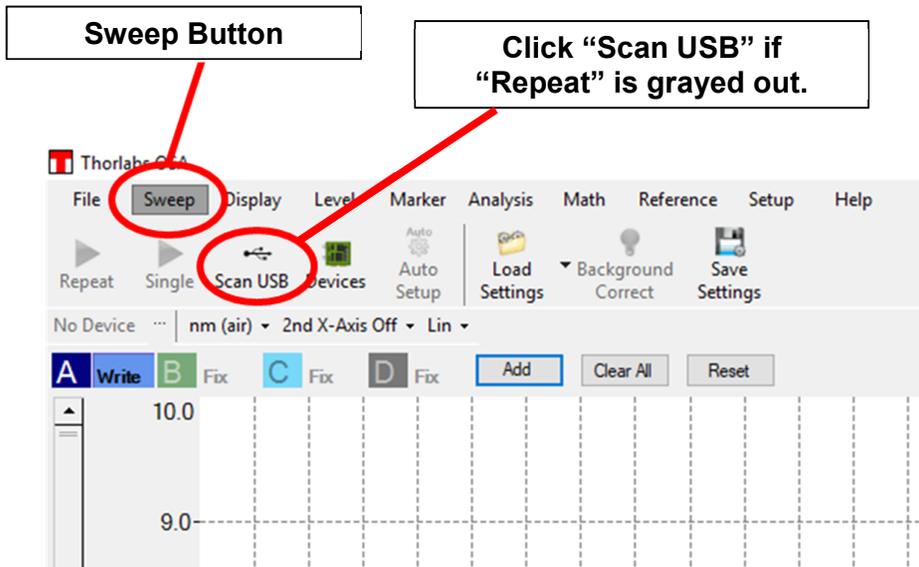
Some Data:

- o Focal length of the achromatic lens ('achromat") you will use: 200 mm
- o Diameter of the achromat: 25 mm
- o Threads per inch of the lens tube you will use to adjust the focus: 40
- o This means one turn is one 40th of an inch (0.635 mm).
- o Wavelength of green light: 550 nm.
- o Use n = 1 in the Depth of Field formula, since air has an index very close to 1.

1. Calculate the NA of the lens imaging onto your camera, if the lens is placed one focal length from the camera sensor (use the formula on the Equations to Memorize sheet, Course Notes Appendix A).

2. Calculate the depth of field using the formula on the Equations to Memorize sheet and the NA you just calculated.
   a. Assume you are imaging in green light.

3. The lens can be mis-positioned by <u>half</u> a depth of field from the point of perfect focus before it makes a visible difference in the image. Assume the lens is positioned a total of 200 mm **plus** ½ a depth of field from the camera.
   a. Using the thin lens equation calculate the object distance corresponding to this new distance from the lens to the camera.
      i. Note that everything from this distance to infinity will be in focus; in photography this distance is called the *hyperfocal* distance.
      ii. To get a sense of scale, how many focal lengths is this distance? I.e., if it is 400 mm, then it would be 400 mm/200 mm = 2 focal lengths. Note that this ratio will vary for lenses of different focal lengths and diameters.
   b. Using Google Maps, find some landscape features (building, etc.) approximately that distance from your lab building; in Lab 2 you will want to focus on something **at least** that far away when setting your camera lens up for the rest of the semester, so that you are focused at a distance appropriately close to "infinity".

5. **Worked Exercise – Focusing Intuition:**
   a. If your lens (f = 200 mm) is exactly one focal length away from the camera, how far away is the object which will be in best focus on the camera?
   b. If your lens is exactly 5 mm *farther than a focal length* from the camera, can you get an object in good focus on the camera? If so, where would that object be located (how far from the camera)?
   c. If your lens is exactly 5 mm *closer than a focal length* from the camera, can you get an object in good focus on the camera? If so, where would that object be located (how far from the camera)?

Thorlabs CCS200 Spectrometer with Thorlabs OSA Software (The Basics)

Sweep Button

Click "Scan USB" if "Repeat" is grayed out.

Auto Setup finds the best integration time setting for the spectrometer.

"Repeat" or Ctrl+R turns on continuous data acquisition.

Right-click in the graph area to auto scale the axis.

Note: DO NOT use Auto Setup unless you have light going into your spectrometer. If you have the cap on the spectrometer or fiber when you click Auto Setup, it will take forever since it is optimizing for very low light.

Camera Control Software (ThorCam)

Display Pixels 1:1 (Prevents Aliasing, but Cuts Off Image in Small Windows)

Fit Image to Screen (See Everything, But May Get Aliasing on Fine Patterns)

Toggle Profiles On/Off

Make a Histogram of Pixel Values

Camera Settings (Adjust Exposure, Frame Rate)

Set Up Image Saving – Pick a Folder You Can Find and Set to Save As TIFF or BMP

Note: The Mouse Scroll Wheel allows you to zoom in on screen features. Be careful not to zoom in and forget!

Take a Single Snapshot (It Does Not Save!)

Start or Stop Capture (Take a Live Video)

Live - C1285R12M   sn:4002723749

Camera Control Software (ThorCam)

You may need to reduce the pixel clock in order to lower the frame rate/exposure time far enough (try this last, however). If you cannot get a high enough frame rate (your video is slow), turn the pixel clock up.

If you are getting "Dropped Frames", or the video is not live when it should be, try turning down the pixel clock until the problem goes away.

Turning down the frame rate allows you to set higher exposure times.

Turning up the frame rate allows you to set shorter exposure times.

The video is smoother at higher (>8 fps) frame rates.

Camera Settings

Camera | Image | Image Enhancement | Input/Output
Shutter | Trigger | Binning/Subsampling

* To minimize image noise, use lower sensor gains.
* Using live capture mode, reduce the pixel clock to minimize dropped frames.

Pixel Clock (MHz)   5   43   19

Dropped Frames: 0.0%

Frame Rate (FPS)   3.86   11.95   9.22
☑ Hold
☐ Max

Exposure Time (ms)   0.1   108.3   29.68
☑ Hold
☐ Max
☐ Auto
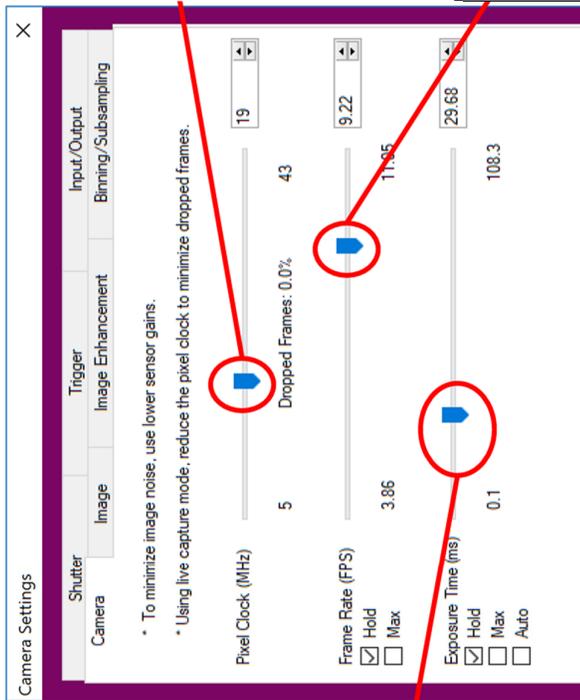
Use this slider 1st. Turn down the exposure time to make the image dimmer. Turn up the exposure to make the image brighter.

Use the line-profiles to tell when pixels are saturating.

Camera Settings Panel

Camera Gain is available from the "Image" tab. You only need to work with the "Master Gain" for now. Unless needed, always set to "0", and do not use "Auto" or "Gain Boost".

Leave "Black Level" unchecked.

For quantitative work, always set "Gamma" to 1.
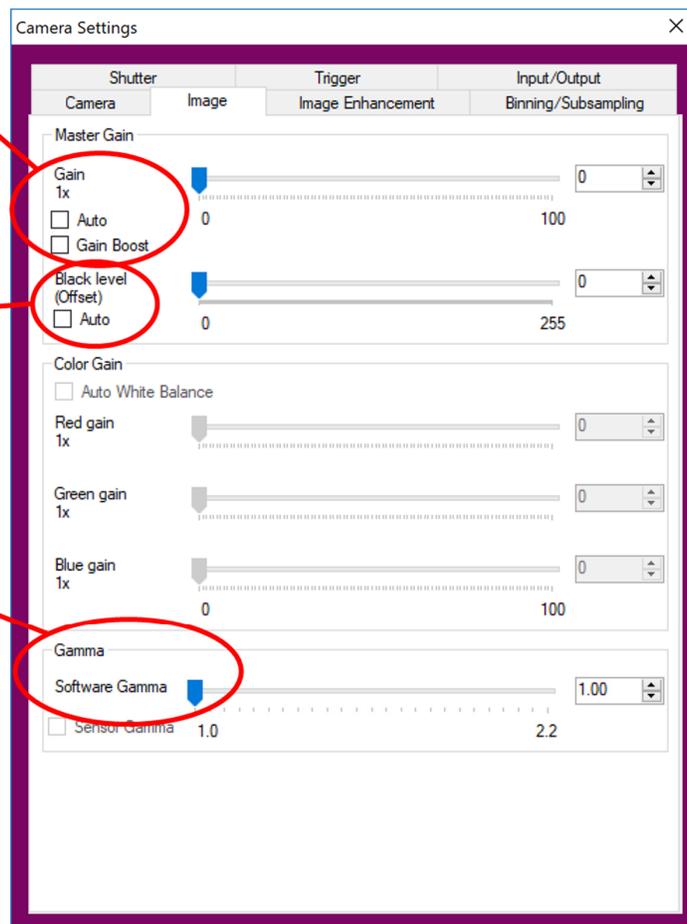
ImageJ
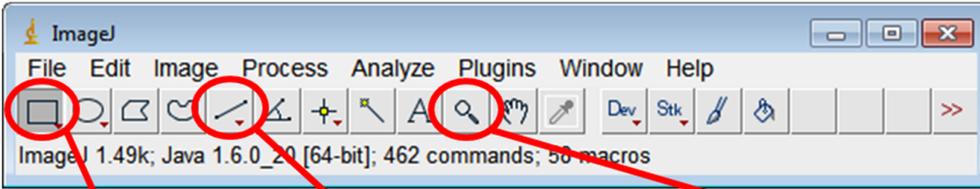
Select Region Tool

Select Line Tool

Zoom Tool:
Move the cursor over the image and click " - " or " = " to zoom out or in. Ctrl+minus and Ctrl+plus also work.
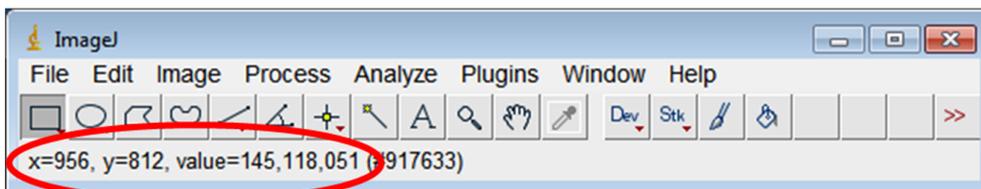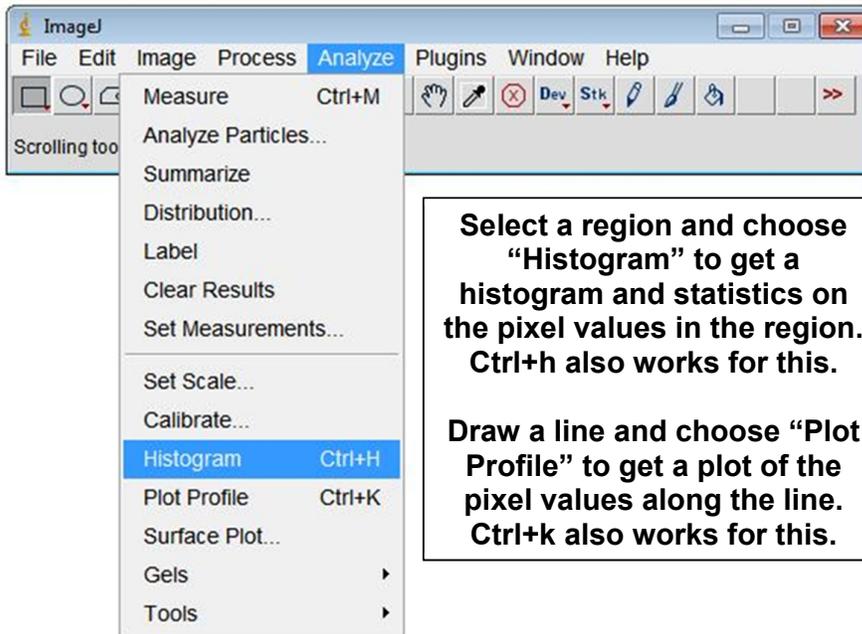
Analyze
Measure   Ctrl+M
Analyze Particles...
Summarize
Distribution...
Label
Clear Results
Set Measurements...
Set Scale...
Calibrate...
Histogram   Ctrl+H
Plot Profile   Ctrl+K
Surface Plot...
Gels ▸
Tools ▸

Select a region and choose "Histogram" to get a histogram and statistics on the pixel values in the region. Ctrl+h also works for this.

Draw a line and choose "Plot Profile" to get a plot of the pixel values along the line. Ctrl+k also works for this.

x=956, y=812, value=145,118,051 (917633)

Move your cursor over the image and notice that the pixel x,y position and RGB (or monochrome, depending on the camera) pixel intensity at the cursor position are listed here.

Lab 2 Notes:
Introduction to Optical Imaging (II)

Optical Microscopy Course

Overview

During the 2nd lab, you will explore imaging further with your camera and lens, set your system up to be the collection side of an "infinity optical system," and gain experience with the optomechanics by building the collection side of your optical train and the mechanical rail system that you will use for the rest of the course. Using this system, you will examine the imaging quality of different lens orientations and types, and in different colors of light, as well as familiarize yourselves with a resolution test target and use it to calibrate your magnification; this latter set of tasks may spill over into Lab 3.

Lab 2 Reading & Preparation:

1. Lab 2 Lab Notes (this document).

2. Lab 2 Course Notes.

3. Install ImageJ on your personal computer, if you have one. See www.thorlabs.com/OMC to download it.

4. Always bring a USB drive to the lab class so you can copy your saved images for use in your lab reports (or upload those images to your own cloud storage at the end of class).

5. "Equations to Memorize," Appendix A in the Course Notes.

    a. Read the entire document.

    b. For the Lab 2 class/quiz, you are responsible only for memorizing the following equations on the first page:

        - NA (All Forms)

        - Magnification (All Forms)

        - Thin Lens Formula

        - Depth of Field

6. Lens Basics, in the Wikipedia page on lenses; See *Lens Basics* under the *Reference Links* tab at www.thorlabs.com/OMC. Skim to get the ideas, do not worry about the math.

Supplementary Reading (Not Required):

7. *IDEX / CVI / Melles Griot Fundamental Optics Guide* (under the *Reference Links* tab at www.thorlabs.com/OMC). For now, skim through the first 34 pages of the PDF, and read any bits that look particularly interesting to you. There will be a number of assigned readings from this over the next few labs (particularly, the sections on Performance Factors, Lens Shape, and Lens Combinations). Note: Though the content is identical, the older version of the "CVI/Melles Griot Fundamental Optics Guide" is better laid-out; if you can find a copy, it is easier to read.

8. MicroscopyU section on CCD cameras. Skim this <u>very</u> quickly to get the idea – do not get caught up in confusing bits or subtleties. See *CCD Cameras* under the *Reference Links* tab at www.thorlabs.com/OMC.

9. Not required, but helpful for reference: Wikipedia articles on "Color," "Phase (waves)," "Snell's Law". Also, the section on refraction and the interactive tutorial. See the links to *Snell's Law* and *Interactive Tutorial on Snell's Law* under the *Reference Links* tab at www.thorlabs.com/OMC.

Lab 2 Tasks

> **You may not finish all of this today.**
> **Do not worry: if necessary, there is extra time during Lab 3.**

I. Setting Up Camera Imaging

1. **Wearing gloves** (!), take your 200 mm focal length Achromatic lens in its SM1 focus tube with 1" long threaded section. The lens should already be installed in the focus tube.

2. Select additional SM1 tubes (remove and store any retaining rings in the tubes) of sufficient length to allow you to focus the lens onto the camera face. Install them on the camera, using the adapter ring that comes with the camera (note: the adapter ring may already be on the camera).

   a. **Remember, try not to use three 1" tubes instead of one 3" tube, or whatever tube length is appropriate for that lens. You do not want to waste parts. You may still need multiple tubes, however.**

      i. You need to decide how long that tube should be. If you get it wrong, you will just have to swap it out for a different one after you figure it out. That is OK.

3. Start the ThorCam software, and begin acquiring images from the monochrome camera.

   a. Go to the Camera Settings panel, select the "Camera" tab, and under "Exposure time" and check "Auto". Camera Settings can be accessed via the gears icon on the toolbar.

      i. This will make it easier to get images as you pan the camera back and forth over objects of different brightness – you will not have to constantly adjust the exposure.

   b. Set the Pixel Clock to its maximum value.

      i. Alternatively, click "Optimize Pixel Clock" and wait for the system to pick the highest workable pixel clock setting for your system (depends on your USB quality).

   c. Set the Frame Rate to its maximum value.

      i. Depending on your computer's USB port, your computer may have trouble running the camera at high frame rates due to data transfer limitations. If either:

         1. The camera image is not live (not responding to light when you wave your hand over the camera), or

         2. "Dropped Frames" is any number higher than 0.0% (Dropped Frames are listed right under the Pixel Clock slider),

      Slide the Pixel Clock slider to lower and lower values until the Dropped Frames reaches 0.0%.

      This will automatically adjust the frame rate down as well. You should end up with a Frame Rate higher than 12 fps or it will be hard to focus and image things. If it is lower, try connecting the camera to a different USB port on your computer.

REMEMBER!
If you are ever changing a lens, keep the lens tube facing UP while screwing in the retaining ring – despite the dirt that may drift down onto the lens, it is better than having the lens accidently fall on the floor!

4. Screw the focus mechanism into the end of the tube. Adjust the lens position to focus the camera on something in the room. (Note: If you chose the wrong SM1 tube, you may have to change it and/or add or remove a segment.)

    a. **Make sure you have some adjustment room left to screw the lens further in or out. It should not be all the way at the end or tight up against the tube.**

5. **Move the camera back and forth a bit so the image moves. Then set the Frame Rate to 3 fps. Move the camera back and forth again, while looking at the screen. Is it clear how a higher frame rate can be useful?**

    a. **Set the frame rate back to its largest value.**

II. Focusing at Infinity

6. Focus the camera at "infinity." *(Question: how should you do this?)*

    a. If your building permits, go to a window where you can aim the camera at something sufficiently far away.

    b. **From the assigned problem due at the start of this lab, what object (building, etc.) will you choose to focus on?**

    c. This setting will be used for the rest of the course; take the time to get it right. It helps to:

        i. Focus on fine features of something quite far away – at UC Berkeley, we often use the suspension cables of the Golden Gate Bridge (when weather permits) because they are very fine in the image and it is quickly obvious when they are out of focus.

        ii. It can help during this process to use your mouse thumbwheel to zoom in on the screen – even though this does not affect actual resolution, it makes it easier for *you* to see things in the image.

        iii. It is also useful to either mount the camera on something, or brace the lens tube against something to help stabilize the camera and reduce image motion.

        iv. A good way to determine best focus is to adjust the focus back and forth, from blurry on one side (lens too close) to blurry on the other side (lens too far) and back again, then once you have a feel for it, go to the middle of that range.

        v. Tightening down the locking ring on the focus mechanism usually causes the lens tube to turn a bit, spoiling your perfect focus. A trick is to get the locking ring almost snug, then rotate the lens tube roughly 1/8 turn back, then tighten the locking ring all the way (which rotates the lens tube back into correct position).

        vi. **When tightening the lens tubes, hold the tubes, NOT the camera – the camera body is plastic and can be damaged. Hold on to the metal lens tubes.**

    d. **Always put a lens cap over your lens when not in use for a long time (e.g. at the end of the day), or when carrying it (e.g. upstairs or downstairs).**

For this step you will need to be able to aim your camera out a window at an object far away. Where you go to do this will depend on local options; your instructor will tell you what to do.

III. Setting Up Your Optical Rail

> Your instructor will give a number of instructions about how to set this up properly, and without damaging anything. Please do not start this part before the instructor explains some of the different techniques.
>
> **Before starting this, your camera should have been focused "at infinity".**

CAREFUL!
DO NOT screw rail carriers down too hard – you can easily damage the rail by tightening too much and denting the dovetail (it is easy to generate immense pressures with the leverage a screw provides). Finger tight is OK – do not torque down hard. The smaller the screw, the more true this becomes.

7. Your optical rail should already be set up and bolted down to the optical breadboard.

   a. If they are not already there, attach your rail carriers to the dovetail on the top of the rail.

8. Using a post, slip ring, and a rail carrier, set up the camera with its tube lens (the lens you just adjusted) at the left end of the long rail, facing right (down the rail).

   a. **DO NOT change the focus of your <u>camera</u>** when putting the slip ring on the lens tubes. Think about how to do this without adjusting the focus.

   b. **The center of the camera tube should be about 9.5 cm above the top of the optical rail.**

      i. **The best (and easiest) easiest way to verify the camera is at a good height is to check that only ~ 1 mm of post sticks out from the bottom of the rail carrier. If that is true, the camera is as high as it can reasonably go, which is perfect for this setup.**

      ii. **A more time-consuming (and not any better) way to do this would be to measure from the top of the rail to the middle of the lens cap on the camera (you can use the SM1CP2 screw-on target that comes with the QTH10 lamp) to get this exact if you want.**

   c. The camera slip ring should be midway down the lens tubes so the camera is mounted in a balanced way; the camera body itself should be very close to the end of the rail, leaving just an inch (2.5 cm) or so between the end of the camera and the edge of the rail.

   d. **It is very important to get the camera set up parallel to the rail; it is easiest to do this by standing over it and looking down on the tube to see if it is parallel to the rail.**

      i. **Use a post clamp to keep the camera height correct while adjusting the camera angle to get it parallel to the rail.**

9. Your instructor will provide you with a sample stage. **Very gently and after listening to instructions** put this stage into a rail carrier <u>with the left edge of the rail carrier between 43 and 44 cm from the camera (left) end of the rail.</u>

   a. You will never move the sample stage along the rail, but many other distances depend on it. **Important: get the position correct now, to avoid confusion later!**

   b. **Note: The micrometer on stage should face <u>*away*</u> from the camera.**

   c. **Look down onto the stage and try to make sure it is aligned so the sample stage is perpendicular to the optical rail.**

10. Mount the f = 25 mm Plano-Convex (PCX) lens into a lens mount on a 3" (75 mm) post.

    a. This may already be set up (mounted) for you.

11. Put the post into a rail carrier near the end of the camera lens tube.

12. Adjust the lens rail carrier and lens post height so the lens mount is flush up against the end of the camera lens tube (it helps to take the lens cap off before you do this). It is easiest to tell if the lens tube and lens mount are flush by running your fingers around the edge – your fingers are very sensitive to misaligned parts.

    a. **Obviously, be sure to wear gloves and do not touch the lenses!**

    b. Use a post clamp to fix this post height, then – with the post still firmly locked in the rail carrier – remove the entire rail carrier and move both it and the PCX lens assembly down to a point just to the left (camera side) of the sample stage.

        i. **VERY IMPORTANT: have the curved face of the PCX lens facing the sample stage.**

        ii. **Moving the rail carriers like this is a good way to maintain the alignment (height, angle) of the optics when you move them to their new location on the rail.**

        iii. In order to properly focus on the sample, roughly how far will the lens need to be from the sample surface?

IV. Imaging & Measuring Resolution

13. Place the glass Thorlabs resolution test target in the sample holder.

    a. **DO NOT FORCE IT. It is VERY expensive and could break! Ask your instructor for help if you are uncertain!**

    b. **Handle the target with gloves on, and only by the edges.**

        i. **Please be careful with it – they are extremely expensive. ONLY put it back in its special protective box.**

    c. **The chromed side of the target (the side you can read) should face toward the camera** (if it faces the other way, all the extra glass will cause additional aberrations in the imaging system).

    d. Place a diffuser in the post-mounted rail carrier to the right of the stage.

        i. You can make a diffuser by taking an SM1RC (SM1RC/M) slip ring and covering the opening with a single layer of Scotch "Magic Tape" tape to diffuse the light.

        ii. Exact position does not matter that much – you just want it out of focus when you look at the sample. A fine starting point is about 10 cm to the right of the sample (far side of the sample from the camera).

    e. Place your halogen lamp in a sliding rail carrier just to the right of the diffuser.

        i. You will use the lamp in a non-standard configuration:

            1. If it is not already set up for you (as per section 5.2.1 of the User Guide/Manual document, on "Halogen Lamp Assembly"), then:

When adjusting the post height, use a post clamp to keep the lens at the right height.

Remember to wear gloves when handling the target (or any sample)!

2. Unscrew the plate on the back side of the lamp from the lens, and install the Thorlabs SM2A6 adapter plate (with external SM2 threads and internal SM1 threads) into its place.

3. Put the HSLT2 passive-heat sink facing the lens and cover it with an SM1CP2 end cap.

4. Place the lamp on a post on the rail with the hole in the adapter plate facing the sample stage (and thus with the heat sink covering the large lens facing the far end of the rail).

ii. You want the lamp filament at the height of the middle of the camera lens tube.

1. This height does not need to be exact for now – just roughly position the hole in the lamp SM2A6 adapter plate to be about the same height as the rest of the lenses (i.e., about 9.5 cm above the rail. Again, this only needs to be rough – you will adjust it more accurately later).

iii. Similarly, the position of the rail carrier holding the lamp need not be precise – the exact position does not matter that much for now. About 28 cm to the right of the diffuser (on the far side of the diffuser from the sample) is a good starting point. Try moving it closer or farther once you are imaging. What effect does it have?

iv. **Turn on the lamp. CAUTION: It can get hot!**

Figure 1: Rough layout for initial imaging.

14. **Adjust the sample stage micrometer (on the translation stage) so it is in the middle of its travel BEFORE adjusting the lens position.**

15. While watching a live image from your camera, slide the rail carrier of the PCX lens toward the resolution target until you can get a decent image.

   a. Once you are in a decent position, tighten down the rail carrier and use the micrometer on the stage to do fine focusing.

16. You may need to adjust the XY position of the sample so the small markings are in the center of the field of view (note: traditionally, Z is the optical axis, i.e., along the rail, so X and Y will be horizontal and vertical motions, respectively).

   a. **Turn OFF** the autoexposure (and be sure that in Camera Settings/Image tab the Gain is set to 1 ("0" on the slider, with "Auto" and "Gain Boost" unchecked)).

i. On the "Camera Settings/Camera" tab adjust the exposure time to get a nice image.

   1. Alternatively, you can use the Histogram feature on the main ThorCam menu bar; depending on what you are doing, a line profile or a histogram may be most useful. A line profile is handy when you only need one part of your image well-exposed (and may want to under- or over-expose other parts of the image as long as the region you are interested in is good). The histogram is helpful for assessing the overall exposure level of the image (if you are interested in the entire image).

17. Once you have the lens positioned to image the sample on your camera, use the focus knob on your stage to do fine focusing and get the resolution target in focus.

   a. You may need to adjust the exposure setting as you do this.

   b. Look for the small resolution bars on the USAF target **(see the target image and notes at the end of this document; Figure 2 and the page after it).**

18. Explore the camera functions for Exposure time (in Settings/Camera) and Gain (in Settings/Image).

   a. In the Settings, click on the "gears" icon on the ThorCam toolbar to get the Camera Settings panel.

   b. Click on the "Camera" tab and play with the "Exposure" slider.

      i. If you cannot adjust this slider, you may need to uncheck the autoexposure box.

      ii. If changing the slider makes no difference, check that the auto gain box is unchecked (under Gain, in Settings/Image). Ask your instructor if you are unsure how to check this.

   c. Click on 'Image' and play with the 'Gain' slider.

      i. **You should always turn up the exposure first, before the gain – gain introduces extra noise ("graininess" in the image), whereas longer Exposure does not.**

         1. Try this to see – turn the gain to max, then turn down the exposure until you see the noise/graininess in the image.

19. With Gain = 1 (slider at "0") and exposure properly set,

   a. Adjust your stage for best focus.

   b. Save an image (e.g. using the "QuickSave" icon. Be sure you know where that image is saved – go there and name it something descriptive (like "Lab 2 USAF target image exposure 25 ms gain 0 <date>").

   c. **Warning: If you do not use QuickSave, ThorCam *does not* save the image when you click the Save button, but only grabs the image after you enter the filename into the dialogue box and hit enter to save the image. If you save the image in this way, click save, type the name, and then make sure you have the correct image on-screen when you hit enter to save.**

"Nice image" means with a line-profile through your image showing that no pixels are saturating (maxed out) but that the highest pixel values are near maximum (70%-90% of max.)

Settings Icon

Quick-Save Icon

d.   Open ImageJ and then open the image you saved to make sure it worked.

  i.   You can open the image either from the ImageJ file menu, or by simply dragging the file onto the ImageJ toolbar.

20.   In ImageJ, examine the smallest sets of bars in the picture of your resolution target.

a.   What are the smallest bars you can resolve (lines per mm)? Write it down.

b.   Set a line profile (using the ImageJ line tool) through the whole column of the 3-bar resolution elements, including the smallest row of bars on the USAF target (i.e., through the whole column of Group 7, including Element 6, the smallest 3-bar set). Can you see the modulation of the intensity (this is called "contrast" from the little lines)?

  i.   **Hint**: Holding down "Shift" while dragging your line allows you to draw a perfectly horizontal or vertical line. Ctrl+K does a line profile of your line (you can also do this from the Analyze/Plot Profile menu.)

  ii.   **Watch out!** The line profile does not match the image 1:1, unfortunately, so the line profile features underneath the image lines may not be the intensities from those specific lines. Try to figure out what is what. We will return to this issue of contrast later in the course – you are seeing a variant of something called the "Modulation Transfer Function".

c.   **Find the resolution your system is capable of right now:**

  i.   **Using ImageJ, try to figure out which set of bars matches this (approximately).**

    1.   **Use the ImageJ line tool and Ctrl+K to get a quantitative line profile. Note that you can use "copy" to copy the actual numeric data to a spreadsheet where you can look at it in detail.**

      a.   **ImageJ should give you a reading in "pixels" along the horizontal axis – that is all you need right now, since the USAF Group and Element tell you the line spacing for each 3-bar set. If ImageJ is reporting a distance, then someone has set the scale (and not necessarily correctly). To fix this, go to ImageJ Analyze/Set Scale and click "Click to Remove Scale," check "Global," and click "OK."**

    2.   **Given the number of lines per mm for each 3-bar group, what line spacing ("resolution") gives you about 10% contrast? [1]**

---

[1] Advanced note: the dip in intensity between two incoherent point-sources of light separated by exactly the Rayleigh resolution distance (0.61 λ / NA) is 26.5%. In the present case, ~10% is a better number to use for two reasons. The first is that 26.5% is actually a large dip, and (as you can readily verify during this lab) the human eye is often able to distinguish much smaller contrast changes, which underscores the point that the Rayleigh criterion is a guideline, not an exact limit. The second, and more theoretical, reason is that here we are not looking at two point sources, but rather a set of parallel lines on the USAF target. The contrast for a set of lines (known as the CTF; see the Lab 7 notes) spaced by the Rayleigh distance is 11.4%; for a purely sinusoidal sample the contrast (known as the MTF; again see the Lab 7 notes) would be 8.9%. A nice compromise, and closer to what the human eye can readily distinguish at reasonable magnification, is ~10%.

ii. **Does this set of bars match what you think you can resolve, just looking by eye at the image?**

iii. **How does this match what you expect for the Rayleigh resolution for your lens? (Figure out your NA, choose an appropriate λ, and calculate the expected resolution and compare to your measured result.)**

V. Introduction to Lens Limitations & Properties

21. **Turn your PCX lens around so the flat side faces the resolution target.**

22. Adjust the rail carrier for best focus.

23. **Refocus (by sliding the rail carrier and adjusting the** stage **micrometer) and take another image of the target. Save the image as a TIFF.**

24. Repeat the previous steps to find the resolution (take an image, look at the line-scan in ImageJ, etc.)

   a. **You are looking to see whether the orientation of the lens (which way the curved side faces) has an effect on resolution. Does it?**

VI. Clean Up

25. Turn off your lamp.

26. Carefully put away your resolution target in its special box.

27. Turn off the LED flashlight, if you used it.

28. Screw a lens cap onto your camera tube lens.

33. **Carefully disconnect the USB cable from your camera(s) by gently pulling the connector straight out.**

29. Put your optical breadboard system away (if the room has a storage place for it.)

30. Copy your files off the computer onto your USB drive or cloud storage (remember, it is your responsibility to keep copies of your files!).

31. **Shut down** (not just "sleep") the computer.

Lab 2 Write-Up:

1. Due date, etc. will be determined by your instructor.

2. See Appendix A for an example / template of a good lab write-up. Read it over, then do your own write-up for this lab. Include the following material:

3. Paste in your image(s) of the resolution target.

   a. Label any images 'Figure 1', 'Figure 2', etc., and in the caption for each (all figures must have captions) list the relevant information. In this case, the relevant information will include:

      i. Color and wavelength of light (was it white? If so, what is a rough approximation for the wavelength?)

      ii. Exposure and gain settings used for the image.

      iii. Orientation of the PCX lens used for the image.

      iv. Whether or not there was a diffuser being used.

4. Paste in line-scans from ImageJ through the smallest sets of bars you can resolve for each image.

5. Make a table listing the resolution for each image (based on which resolution element/group you could resolve for each):

Table 1:

Lamp Wavelength, nm	Lens NA	Resolution, PCX Lens Round Side Toward Resolution Target	Resolution, PCX Lens Flat Side Toward Resolution Target

   • Note: You may need to estimate the lamp wavelength if you did not measure it. What is a good guess? List it in the table.

6. Calculate the:

   a. NA of the PCX lens you used (25 mm diameter, f = 25 mm).

   b. Expected "Rayleigh" resolution given the wavelength of the light you used.

7. Comment (briefly, 2 - 3 sentences) on whether the resolution you observed matched what you calculated it should be; if not hypothesize (briefly) why not.

   a. Be sure to state what resolution you *measured*, and also show the calculation for what you expected the resolution to be and why.

8. If you got this far: Comment on whether the resolution changed when you turned the PCX lens around, and if so why that might be.

   a. The required reading for Lab 3 (also optional reading for Lab 2) from the IDEX / CVI / Melles Griot Optics Guide may be useful when discussing this; if so, be clear as to what issue may have mattered and why.

Resolution Test Target

The resolution target for this course is chrome on glass. The USAF 1951 pattern[2] in the middle has a series of progressively smaller 3-bar test patterns, where the spacing between the bars is known. Roughly speaking (we will speak more precisely later in the course), the smallest set of bars you can resolve by eye give you the resolution of your system.

Look over the picture below- it will help you when you are trying to figure out where you are on an out of focus target, and how to find the small sets of bars. We use the USAF 1951 target the most because the sets of bars spiral in to the center as they get smaller, allowing you to see them all at the same time without having to reposition the sample. The price we pay for that convenience is that the line spacing is not listed next to the bars as it is for the NBS 1963A lines on the left.

Resolution bar targets that you will use a lot during the first few labs.
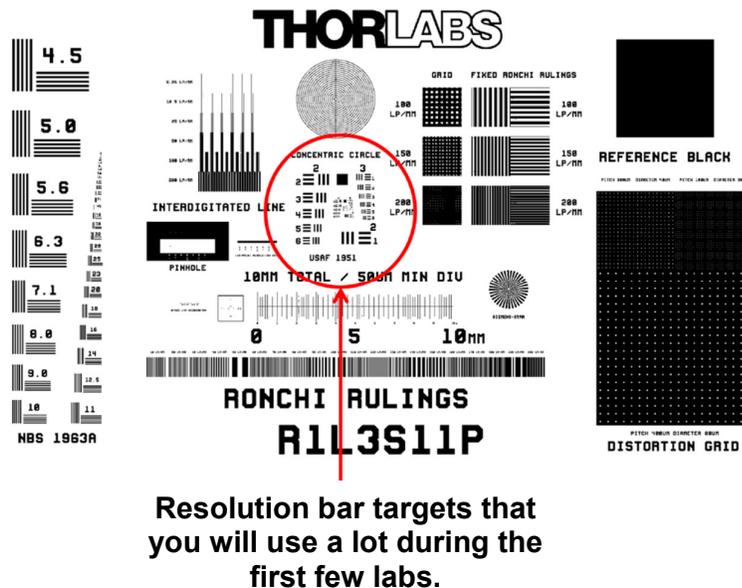
Figure 2: Thorlabs custom resolution target R1L3S11P, showing the USAF 1951 3-bar resolution target area. On the left are the 5-bar resolution elements (NBS1963A standard; NBS stands for 'National Bureau of Standards', the predecessor of NIST, the National Institute of Standards and Technology). The USAF target is more convenient since the elements spiral in to the center as the spacing goes down, so you can see more sizes at once.

---

[2] Did the USAF ever use these targets? Take a look in Google Maps at this giant one in the desert at Edwards Air Force Base in California, Latitude 34.8339167, Longitude -117.7602605. See *Edwards Air Force Base* under the *Reference Links* tab at www.thorlabs.com/OMC.

Reading the USAF 1951 Resolution Target
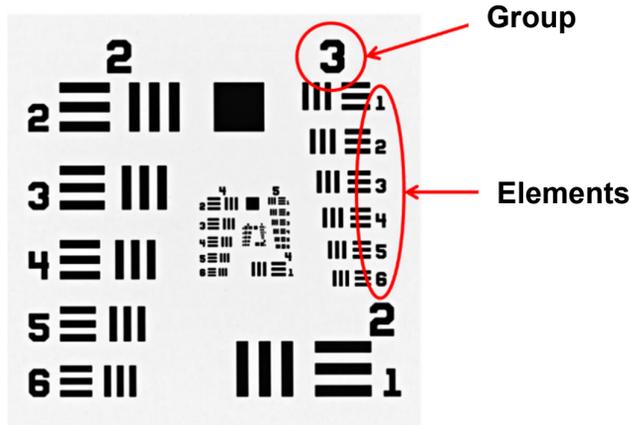
Element	Group Number					
	2	3	4	5	6	7
1	4.00	8.00	16.00	32.00	64.00	128.00
2	4.49	8.98	17.95	36.0	71.8	144.0
3	5.04	10.10	20.16	40.3	80.6	161.0
4	5.66	11.30	22.62	45.3	90.5	181.0
5	6.35	12.70	25.39	50.8	102.0	203.0
6	7.13	14.30	28.50	57.0	114.0	228.0

Resolution: Your resolution will be roughly 1 / (line spacing), so if there are 8 "line pairs / mm" as given by the table for the set of bars you are looking at (i.e., the spacing between the smallest set of lines you can distinguish is 1 mm / 8 bars = 0.125 mm from bar center to bar center), then the resolution would be ~0.125 mm.

Formally, the USAF 1951 target line spacing is:

Line pairs / mm = $2^{\text{Group} + \frac{(\text{Element} - 1)}{6}}$

And so the smallest spacing you can resolve is

Spacing in mm ~ "resolution" = $2^{-\text{Group} - \frac{(\text{Element} - 1)}{6}}$

Example: In the target above, say the smallest set of bars you can make out is roughly Group 4, Element 5. The table above indicates this corresponds to 25.39 line pairs / mm, so the spacing between the lines is = 1 / [25.39 lines / mm] = 0.039 mm (= 39 μm), and so your resolution would be ~ 39 μm.

NBS 1963 Target (left-hand side of the target slide): Here the 5-bar patterns have the line pairs/mm listed next to them; the resolution is then simply 1/(line spacing). Be sure to convert from mm to microns.

Lab 3 Notes: Aberrations and Illumination

Optical Microscopy Course

Overview

Using the optical rail, camera, and tube lens you built in Lab 2, you will now examine the imaging quality of different lens orientations and types, the effects of different colors of light and types of illumination, and familiarize yourselves with a resolution test target and use it to calibrate your magnification.

Lab 3 Reading & Preparation:

1. Lab 3 Notes (this document).

2. "Equations to Memorize," Appendix A in the Course Notes.

   a. Read the entire document.

   b. For the Lab 3 (and a possible quiz), you are responsible only for knowing (memorizing) the following equations on the first page:

      - All equations required for Lab 2 (NA, M, thin lens equation, Depth of Field).

      - Rayleigh Resolution Criterion (do not worry about the "NA$_{condenser}$" part).

      - Colors (at very end of the 'Equations to Memorize' document – know the table of colors and their wavelength ranges).

3. **_Skim through_** the first 36 pages of _IDEX / CVI / Melles Griot Fundamental Optics Guide_ (See the _Reference Links_ tab at www.thorlabs.com/OMC) Note: Though the content is identical, the older version of the "CVI/Melles Griot Fundamental Optics Guide" is better laid-out; if you can find a copy, it is easier to read.

   a. **Read carefully the section on chromatic aberrations, lens shape, and lens combinations, pp. 1.15 – 1.19 (A109 - A113 in the new version).**

   b. **Be sure to read – but do not worry if you get confused by – pages 1.20-1.21 (A114 - 115) and 1.27-1.28 (A122 - 123).**

4. Review the Lab 2 Course Notes.

5. Bring a USB drive to the lab class (unless you have cloud access from the lab computers) so you can copy your saved images for use in your lab report.

Lab 3 Tasks

I. Setup

1. Check that your breadboard is set up as at the end of the Lab 2.

    a. The camera with its tube lens should be on the left end of the rails; the back of the camera should be close (about 2.5 cm) to the end of the breadboard.

    b. The sample stage should be in a rail carrier centered at about 43 cm from the left end (camera side) of the rail.

    c. The f = 25 mm PCX (plano-convex) lens should be in a rail carrier between the camera and stage, with the lens at the same height as the camera.

        i. If you did not set the height of the PCX lens during Lab 2, the procedure is:

            1. Put the post into a rail carrier near the end of the camera lens tube.

            2. Adjust the lens rail carrier and lens post height so the lens mount is flush up against the end of the camera lens tube (it helps to take the lens cap off before you do this). It is easiest to tell if the lens tube and lens mount are flush by running your fingers around the edge – your fingers are very sensitive to misaligned parts.

                a. **Be sure to wear gloves and do not touch the lenses!**

            3. Use a post clamp to fix this post height, then move the PCX lens and mount down to a rail carrier just to the left (camera side) of the sample stage. **Important: by using the post clamp, you will keep the right height for the lens.**

                a. If the thumbscrew on the post clamp interferes with (bumps into) something else, then i) tighten down the main thumbscrew on the rail carrier, ii) loosen the post clamp and rotate it so the thumbscrew is out of the way, and iii) retighten the post clamp so it is secure and maintains the height of the optic.

        ii. In order to properly focus on the sample, roughly how far will the lens need to be from the sample surface?

        iii. **VERY IMPORTANT: start with the curved face of the PCX lens facing the resolution target.**

2. Place the resolution target in the sample holder.

    a. **Handle this target with gloves on, and only by the edges. Always place it back in its special box when not using it. Please be careful with it – they are very expensive.**

    b. The chrome side of the target (the side you can read) should face toward the camera (if it faces the other way, all the extra glass will cause additional aberrations in the imaging system).

3. Set up your lamp and diffuser:

    a. Put the diffuser slip ring SM1RC (SM1RC/M) with the opaque Scotch "Magic" tape over the opening (same as in Lab 2), in the rail carrier roughly 10 cm from the sample, to the right of the sample stage (the side opposite the camera).

The camera and lens should be high enough so they can face the center of the test target or a bit higher; ~9.5 cm from the rail to the center of the lens tube works well.

DO NOT force it! It will break and it is expensive! Ask if you are uncertain how to mount it (or open its box)!

b. Lamp goes in a rail carrier roughly 28 cm from the sample.

c. Turn on the lamp (by plugging it in).

4. Set up your system for proper imaging.

    a. That means open a line-profile, and adjust your camera exposure setting so that the brightest part of the image is almost (but not quite) at the maximum (i.e., not "saturating").

Figure 1: Rough optical layout for initial imaging.

II. Aberrations: Lens Orientation

5. (If you did not do this in Lab 2):

    a. Take an image of the central features of the resolution target; save this as a TIFF.

    b. Reverse the PCX lens (so the flat side faces the resolution target).

    c. **Refocus (by sliding the rail carrier and adjusting the micrometer) and take another image of the target. Save the image as a TIFF.**

    d. **Answer the questions at the end of Part IV of Lab 2 (starting with "Find the resolution your system is capable of right now.")**

    e. **Fill out the information in Table 1 in the "Write-Up" section at the end of this document.**

III. Illumination: Coherence

6. Adjust your camera for proper imaging.

7. Focus using the stage micrometer.

    a. Make sure the sample translation stage micrometer is roughly in the middle of its travel (not all the way at one end).

8. Take an image of the center of the USAF target and save it as a TIFF (or bitmap).

9. Remove the diffuser.

10. Readjust your camera for proper imaging.

    a. **Did you need to change your camera exposure? What does the diffuser do to the amount of light that gets to the camera? Why do you think that is?**

11. Refocus using the stage micrometer.

12. Take an image of the center of the USAF target and save it as a TIFF.

To achieve this, you may have to set the micrometer to the middle of its travel, then re-adjust the rail carrier position, then do fine focus again using the micrometer.

13. **Try putting the diffuser in and taking it out; notice how the image changes when you do this.** What you are seeing is the difference between spatially coherent and spatially incoherent illumination; we will return to this later in the course. **The little fringes around the features on the target are from diffraction – a clear demonstration of the wave nature of light.** This is most noticeable when the light is all coming from the same direction; the diffuser spreads out the directions from which the light hits the sample, and makes the fringes harder to see.

IV. Aberrations: Color

14. Obtain from your lab instructor a set of LEDs (blue, green, red, and infrared) in Thorlabs mounts on 4" (100 mm) posts.

15. Turn off the lamp, and move it to the farthest right end of the rail, maybe 2.5 cm away from the edge.

16. Leave the diffuser in the post-mounted rail carrier to the right of the sample stage (the side opposite the camera).

17. Mount an LED in a rail carrier, about 25 cm to the right of the sample stage (the side opposite the camera).

Figure 2: Optical layout with LED and diffuser.

18. **Repeat the following steps for each LED; it will be easiest to notice the effects if you go in the sequence blue/green/red/IR, or IR/red/green/blue.**

    a. **DO NOT pull or twist the USB cables when they are connected to the camera – be gentle so the connections don't get strained or broken.**

    b. Mount the LED in the rail carrier, so the LED is facing the USAF target.

    c. Connect the LED mount to the micro-USB cable so the LED has power.

    d. Set up your camera for good imaging, and focus on the resolution target.

    e. Adjust the LED angle and height to get the brightest, most uniform illumination.

        1. Click on the horizontal or vertical line profile icon, and position the line across the middle of the sample.

        2. Adjust the LED so the sample looks best. You want the line profile trace to be as flat as possible (except, of course, where it crosses chromed parts of the resolution target).

WATCH OUT! The lamp can get HOT! Either turn it off and wait for it to cool, or hold it by the (cooler!) post.

DO NOT stare at / into the IR (infrared) LED! It will not look like it is on, but it will be working. Turn it off (disconnect it) when you are not using it to image.

NOTE: You may need to adjust the LED height and/or angle each time you change LEDs.

NOTE: You may also need to turn down the room lights if your image is "washed out," and does not go completely black if you block the LED with your hand.

    f. With the image in good focus and the camera properly adjusted:

        i. Write down the value on the stage micrometer.

            1. Get your lab instructor to explain how to read the micrometer. The difference between "10" and "30" on the barrel is 200 μm – it is 10 μm per division; also, the barrel only goes from 0-49, since the major divisions on the barrel are 500 μm (0.5 mm) apart.

        ii. Record the number of lines/mm for the smallest 3-bar element and group you can resolve on the USAF 1951 portion of the resolution target.

            1. Using your mouse thumbwheel to zoom in on the display in the ThorCam image can help you assess this more easily.

        iii. Take an image and save it.

        iv. **Record the relevant information in Table 2 at the end of this document.**

19. Switch to using the next LED and repeat the steps above.

    a. **Which way did you move the sample – is it closer to or farther from the lens?**

    b. **How far did you move it? Record your micrometer setting.**

20. Repeat the steps again for you next two LEDs

    a. **You should now have data for 4 different color LEDs.**

21. Try removing the diffuser – do the fringes come back? They may be even clearer, since the LED emitting surface is even smaller than the lamp filament, so the light is more "spatially coherent."

22. Get out a spectrometer and use it to measure the wavelengths of the red, green, blue, and IR LEDs you used. [If you do not have a spectrometer, get the wavelengths of the LEDs from your instructor.]

    a. Paste images of each spectrum into your Word (or other word processing program) document.

        i. **It is a good habit to save all your raw data** (either in Excel, or as a spectrometer file), though for time efficiency we won't do that now.

    b. Record the main wavelength for each LED in Table 2 at the end of this document.

        i. **What is the most appropriate wavelength to write down?**

V. Aberrations: Achromat Objective

Assembly

23. **[Only if your instructor has not already assembled this for you:]** Construct an objective using your 1/2" (12.7 mm) achromat lens, 1/2" adjustable lens tube (focus), 1/2" lens tube, SM1 - SM2 lens tube adapter, calibrated iris, and a 4" tall post TR4 (TR100/M, 100 mm). See diagram in Figure 3. A description can also be found in the manual.

    a. **BE CAREFUL:**

        i. The socket on the 8-32 (M4) setscrew should face OUT of the post.

        ii. **BE SURE** the setscrew engages most of the threads in the slip ring before tightening the post onto the ring (otherwise you will strip the threads and ruin the slip ring).

           1. It is best to put the setscrew into the slip ring first, then screw the post onto the setscrew and use the driver to thread the screw far enough back down into the slip ring that it will not scratch the lens tube.

        iii. **BE SURE** the setscrew is ~1 mm down into the slip ring so it cannot scratch the lens tube once the lens tube is inserted in the ring.

        iv. <u>Gently</u> tighten the post onto the slip ring using a driver through the post-hole to get leverage.

        v. When screwing on the iris, **DO NOT** tighten it by twisting on the iris adjuster – you will ruin the iris.

           1. **Instead, be sure you are grabbing the fixed /non-turning outer edge of the iris (<u>not</u> the knurled (rough) adjusting ring). If in doubt, ask your lab instructor.**

        vi. Orient the iris such that the white mark indicating its position is facing <u>up</u> before tightening the slip ring (be gentle when tightening things – finger tight is plenty).

           1. Make sure you leave room to get your fingers on the focus locking ring before you tighten the slip ring; otherwise the objective will be hard to focus.
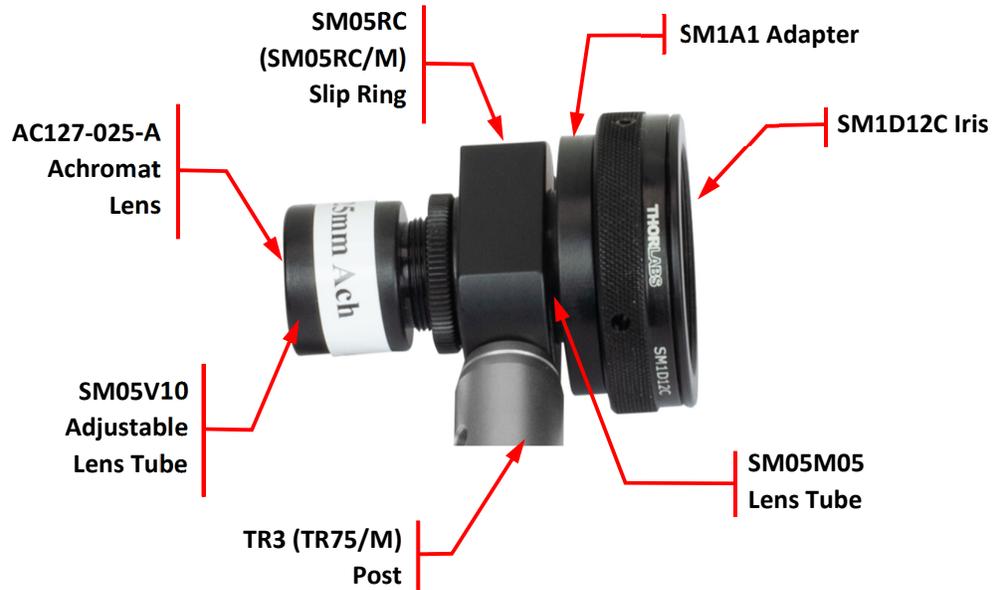
SM05RC
(SM05RC/M)
Slip Ring

SM1A1 Adapter

AC127-025-A
Achromat
Lens

SM1D12C Iris

SM05V10
Adjustable
Lens Tube

SM05M05
Lens Tube

TR3 (TR75/M)
Post

Figure 3: Achromat Objective

This is easiest if you put the post in a rail carrier and <u>slide the iris side up to the camera lens tube</u>, then use your fingers on the edges to decide when it is properly centered.

24. Adjust your objective so it is exactly the same height as your camera lens tube.

Focusing

25. Focus your achromat such that the iris is exactly one focal length away from it. *What easy way do you have of knowing when this is properly done? Hint: consider whether you can* **use your camera to** *image anything useful…*

26. Once you have set the objective lens iris position, make sure the objective is aligned with the rail by sliding the iris up against the camera tube, then locking down the post (and putting on a post collar for good measure), and finally moving the entire rail carrier to the current position near the sample.

Setup

27. Make sure the sample translation stage micrometer is in the middle of its travel (not all the way at one end).

28. Remove your PCX lens and replace it with your objective, lens side facing the sample.

> **NOTE:** You will still be using the LED and diffuser for illumination in these steps.

29. Set the iris on your objective to 7.0 mm diameter, and focus on the resolution target by sliding the objective rail carrier back and forth. Lock down the rail carrier setscrew once you have a decent focus.

30. Use the micrometer (fine focus) on your stage for final adjustments.

31. Make sure the image is in good focus and the camera properly adjusted.

    a. Write down the value on the stage micrometer.

b. Record the smallest 3-bar resolution target (USAF 1951) element and group you can resolve.

    i. **Is this better or worse than the value you got with the PCX lens?**

    ii. **Write down the information in Table 3 at the end of this document (in the "Write-Up" section).**

c. Take an image of the center of the USAF target and save it.

    i. **You will use this image later; make it a good one!**

32. Reduce the iris aperture to 2.5 mm.

33. For each LED (again):

a. Refocus using the stage micrometer and readjust the camera settings for good imaging.

    i. Write down the value on the stage micrometer.

    ii. Record the smallest 3-bar resolution target (USAF 1951) element and group you can resolve.

        1. **Write down the information in Table 3 at the end of this document (in the "Write-Up" section).**

    iii. Take an image and save it.

b. Switch to using the next LED and repeat Step (a).

    i. **Did you move the sample as far as you had to move the PCX lens?**

c. Switch to using the next LED and repeat Step (a).

    i. **Did you move the sample as far as you had to move the PCX lens?**

d. Switch to using the next LED and repeat Step (a).

    i. **Did you move the sample as far as you had to move the PCX lens?**

34. **What is different about the achromat compared to the Plano-convex (PCX) lens? What does 'achromat' mean?**

VI. Calibrating Magnification (and "Pixel Size")

35. Go to the bottom edge of the resolution target, where there are a series of line-targets ("Ronchi rulings") with the line spacings (in line-pairs/mm, or lp/mm, listed above them).

a. Choose a set of lines with reasonably wide spacing – say, 20, 30, or 40 lp/mm.

b. Focus on the lines, set your camera for proper exposure, and take an image.

    i. **Record the lp/mm for this set of lines!**

VII. Clean up

36. Turn off your halogen light so it has time to cool before you put everything away.

37. **Carefully put away your resolution target in its special box.**

38. **Carefully disconnect the LED power cable, and then take the LED off your setup.**

39. **Carefully disconnect the USB cable from your camera(s) by gently pulling it straight out.**

40. Screw a lens cap on over your camera tube lens.

41. Close the iris on the back of the objective (not super tight – just close it to keep dust out.).

42. Put your optical breadboard system away.

43. Copy your files off the computer onto your USB drive or cloud storage (remember – it is your responsibility to keep copies of your files!).

44. **Shut down** (not just "sleep") the computer.
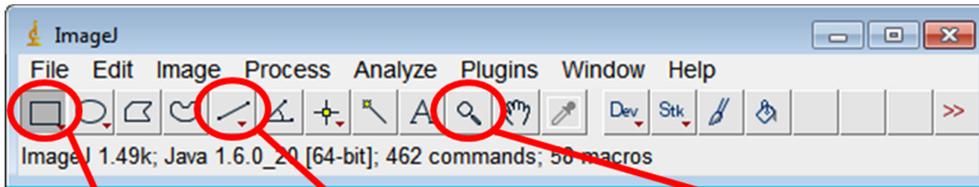
ImageJ

Select Region Tool

Select Line Tool

Zoom Tool:
Cursor over the image
and click " - " or " = "
to zoom out or in.
Ctrl+minus and
Ctrl+plus also work.

Measure   Ctrl+M
Analyze Particles...
Summarize
Distribution...
Label
Clear Results
Set Measurements...

Set Scale...
Calibrate...
Histogram   Ctrl+H
Plot Profile   Ctrl+K
Surface Plot...
Gels
Tools

Select a region and choose
"Histogram" to get a
histogram and statistics on
the pixel values in the region.
Ctrl+h also works for this.

Draw a line and choose "Plot
Profile" to get a plot of the
pixel values along the line.
Ctrl+k also works for this.

x=956, y=812, value=145,118,051 (#917633)

Move your cursor over the image and notice that the pixel x,y
position and RGB (or monochrome, depending on the camera)
pixel intensity at the cursor position are listed here.

Lab 3 Write-Up:

Basic Details:

o Turn in your write-up as a Word document, due date, etc. as specified by your instructor.

o **Number your pages "page X of Y" and have your name in the footer so it is clear if a page is missing if it gets printed out.**

In Microsoft Word (or whatever program you use) be sure to compress all your images before turning in the file; otherwise the files will be too large.

o After appropriately cropping and sizing images – not covering the whole page, not so small one cannot see the features discussed – reduce the image size so the document is not huge. In MS Office as of this writing, you do this by clicking on a picture, choosing the "Format" tab in the top menu bar, and then choosing "compress pictures" on the format menu toolbar. For submitting your assignment, click save and save-as-PDF. Be sure to check that the compression and the PDF conversion have not spoiled some feature in an image you were trying to point out in your lab write-up – this should not be an issue if you cropped and sized your images appropriately. If there is a problem, send the larger document so the image information is preserved.

Write-Up:

1. Paste in your images of the resolution target lines taken using the PCX lens and with the diffuser both in and out.

   a. Comment on the effects of diffraction – is it noticeable? What is the best resolution with and without the diffuser?

   b. Label your images 'Figure 1', 'Figure 2', etc., and in the caption for each list the relevant information. In this case, the relevant information will include:

      • Color and wavelength of LED.

      • Camera exposure and gain settings used.

      • Orientation of the lens (which way is the flat side facing?).

      • Whether or not there was a diffuser being used, and where it was.

2. Paste in line profiles from ImageJ taken through the smallest sets of bars you can resolve for each image.

3. **For Tables 1 and 2:**

   a. Include both tables in your document.

   b. Calculate the NA of the PCX lens (25 mm diameter, f = 25 mm) and state it in the document.

      i. Also state the resolution (in μm) you could measure with the PCX lens using the resolution target. Refer to your line-scans from the last step to back this up.

   c. Do the same for the data from your Achromat objective (f = 25 mm, dia. = 12.5 mm).

      i. **Be careful**; what is the correct NA value to use for your objective?

Important: In all lab reports, you are expected to present *both* measurements *and* theoretical expectations, discuss whether they match, and, if they do not, explain why not.

   d. **Comment on how the resolution you obtained matches (or does not match) the calculation; if not, hypothesize as to why that is.**

e. **It is typical to use a color (usually green) filter in the illumination path for a microscope to limit the white lamp light to a fairly narrow wavelength band. Explain the benefit of doing this, based on your results for this part of the lab (i.e., based on your data in Table 2).**

f. **Compare this to your data from Table 3, and discuss briefly why the data are different.**

4. Using the change in focal distance you measured for the f = 25 mm PCX lens for red and blue light (Table 2), calculate the change in the index of refraction for the f = 25 mm PCX lens assuming that at the red wavelength the focal length of the lens is exactly 25 mm and its index is n = 1.51527.

   a. **Did this match what you might expect from the wavelengths you measured for the LEDs and the index of the BK7 type glass at those wavelengths?**

      i. We expect a quantitative discussion here – calculate values and use the link below (or other data on the index of BK7 glass) to get values to compare with.

   b. For reference, either search the web or see the *BK7 Index Calculator* under the *Reference Links* tab at www.thorlabs.com/OMC for BK7 glass index information (Note: for this link you may have to choose settings, e.g. "Glasses," and then the glass types, "BK7". Use the data for N-BK7 glass.)

5. Magnification and pixel "size" (or, more correctly, pixel ***spacing***).

   a. Open the image you took of the Ronchi ruling (in Part VI, above) using ImageJ.

   b. Draw a line profile horizontally through about 10 lines.

      i. Holding down Shift while dragging the line will force it to be horizontal.

      ii. Ctrl+K is the keyboard shortcut to getting a line profile.

   c. Measure the distance (in pixels) between the start of the first line and the start of the tenth line.

      i. How do you decide where the "start" is? There are different ways to choose. The simplest is to define it as the point where the line has risen by 50% of the distance from its lowest to highest value (this lies behind the definition of curve width called the FWHM, or <u>F</u>ull <u>W</u>idth at <u>H</u>alf <u>M</u>aximum). More advanced (and time consuming) approaches involve using the halfway point between when the line is at 10% and 90% of its range. In our case the difference between these is not critical.

   d. Knowing the distance (in lp/mm) for each line-pair (i.e., one black and one white line), what is the distance in mm between your two points?

   e. Calculate the "width" of a pixel.

      i. Note: this is the "sample-referenced pixel spacing" – the size of one camera pixel as measured at the sample. Since there is magnification in your optics, that is not the same as the actual pixel spacing in the camera!

f. Given that your monochrome camera has pixels spaced by 5.2 µm (as can be verified from the camera manual), what is the magnification of your system?

    i. **Does this match what you expect given the 200 mm focal-length tube lens and 25 mm focal length objective?**

g. You can set this scale in ImageJ, so it reports distances in µm: go to Analyze/Set Scale to do so.

    i. In general it is not good to rely on this unless you have just done it yourself – someone else may have changed an optic, or changed the scale in ImageJ. We prefer to work in pixels and convert ourselves later.

Tables (So You Can Replicate Them in Your Word Document):

From Lab 2 (if you did not complete it then): Make a table listing the resolution for each image (based on the largest number of lines/mm you could resolve for each):

Table 1: PCX Lens Focus vs. Wavelength

Lamp Wavelength, nm	Lens NA	Resolution, PCX Lens Round Side Toward Resolution Target (Element, Group)	Equivalent Resolution, μm	Resolution, PCX Lens Flat Side Toward Resolution Target (Element, Group)	Equivalent Resolution, μm

Note: you may need to estimate the lamp wavelength if you did not measure it. What is a good guess? List it in the table.

Table 2: PCX Lens Focus vs. Wavelength

Be sure to note whether increasing micrometer value corresponds to the sample being closer or farther from the objective.

Wavelength of LED, nm	Stage Micrometer Setting for PCX Lens Best Focus

Table 3: Achromat Focus and Resolution vs. Wavelength ith Iris Set = 2.5 mm

Be sure to note whether increasing micrometer value corresponds to the sample being closer or farther from the objective.

Wavelength of LED, nm	Stage Micrometer Setting, Achromat Objective	Resolution Target Element Group, Achromat Objective	Resolution Based on Resolution Target, Achromat Objective, μm	Expected (Rayleigh) Resolution for Achromat Objective, μm

Lab 4 Notes:
Köhler Illumination

Optical Microscopy Course

Overview

In this lab you will set up "proper" illumination for your microscope; after doing this, your microscopes will have all of the important features of a major research microscope, and the stage will be set for exploring the Abbe theory of image formation as well as darkfield, phase contrast, and other techniques. The purpose of Köhler illumination is to provide bright, uniform illumination to just the area of the sample you wish to examine, and with complete control over the angles at which the illumination light hits the sample. Time permitting, you will also explore some of the effects of changing the objective NA.

Lab 4 Reading & Preparation:

1. Lab 4 Lab Notes (this document).

2. Lab 4 Course Notes.

3. *IDEX / CVI / Melles Griot Fundamental Optics Guide*; see the *Reference Links* tab at www.thorlabs.com/OMC.

   a. **READ CAREFULLY:** Lens Performance Factors, in the IDEX/Melles Griot Optics Guide, pp. 1.11– 1.20 (A105-A113 in the new version) (pages 1.15-1.20 (A109-A113) should be review from previous weeks). The new material includes four pages on aberrations (pp. 1.11– 1.14 (A105-A107). **This is important material!**

4. Tutorial on *Objectives and Numerical Aperture* under the *Reference Links* tab at www.thorlabs.com/OMC.

5. Some information about August Köhler: along with Otto Schott (a glass manufacturer) Ernst Abbe (whose theory we will spend several labs investigating), and Carl Zeiss. Köhler is one of the four people most responsible for the modern microscope. Wikipedia article on Köhler: See *August Köhler* under the *Reference Links* tab at www.thorlabs.com/OMC.

Supplementary Reading (Not Required):

6. Ernst Keller, "Proper alignment of the microscope," Meth Cell Bio 2007. Ernst Keller is/was one of the main optical engineers and designers at Zeiss – it is notable that the thing he thinks is most important for microscopists to know is how to set up proper illumination.

    a. **Note: In this paper, in Figure 2, plane C is the plane at which you would put your camera; ignore the eyepiece and eyeball.**

    b. Do not get bogged down in this article, but it is worth reading through.

        - **Focus on PART II of the article (all sections starting with Köhler Illumination).**

        - DO NOT get bogged down in Part I; consider reading Part I **after** you read Part II; if it is confusing, skip ahead / skim until you get to Part II.

    c. Many terms will be unfamiliar, and the article covers a full research microscope (which has many more parts and features than ours will). However, much of this will make more sense over the course of the next two labs, and it is good background.

7. The article by Peter Evennett (a noted British microscopist), "Köhler Illumination: a simple interpretation," in the "Köhler Illumination Centenary: A collection of papers detailing Köhler illumination," published by the Royal Microscopical Society, 1994, pp. 10-13.

    a. In this paper, in Figure 1, the "Field Diaphragm of Eyepiece" and the "Primary Image" are where your camera goes; ignore the eyepiece lens and eye above that. Note: "diaphragm" is another word for "iris".

8. **ONLY** the section on "Uniformity" (about 3/4 of the way down the page) in the Zeiss/Davidson article on *Illumination Source Fundamentals* under the *Reference Links* tab at www.thorlabs.com/OMC.

9. "Equations to Memorize," Appendix A in the Course Notes: Memorize the entire first page.

    a. This will be required for Lab 5; you can get a head start if you want.

10. **ONLY** the section on Köhler illumination in Davidson, Abramowitz "Optical Microscopy," Microscopy Review 2009.

    a. We encourage you to read around in the rest of the document, but that is **not** required.

11. The sections on aberrations in Hecht, Optics (§6.3, p. 258 in the 5th ed.) Read but do not get bogged down; in the discussion of thin achromatic doublets, stop at the point when it starts to get complicated.

Lab 4 Tasks

I. Collection Optics Setup

1. Set up your system; use the instructor demo/teaching unit (if there is one) as an example, and/or refer to the diagram later in these notes. This will involve a bit of rebuilding of your system.

    a. Make sure that all lenses are at the same height; otherwise getting an image will be complicated, and images themselves will be poor.

        i. You will do best to start with the camera at the right height, then carefully match the height of all your lenses (objective, condenser, and collector) to that.

            1. If the camera tube lens center is ~9.5 cm above the center of the top rail (so ~ 1 mm of post is projecting below the rail carrier), you will have enough leeway to adjust the resolution target height as needed.

                a. Remember that you can always move the resolution target **up**, but there is a limit to how far **down** you can push it.

        ii. **Alignment Trick:**

            1. Place an optic in a rail carrier in front of the camera, so the optic is up against the end of the camera lens tube.

            2. Get the height correct by looking to see if the camera tube and the new optic are flush (or feeling around the edge with your fingers to verify – be sure to wear gloves if doing this!)

            3. Lock down the rail carrier set screw to fix the height.

            4. Use a post collar to fix the height of the post against the rail carrier.

            5. Remove the rail carrier from the rail, and reattach it wherever you want the optic to be. This maintains both the correct height and correct angle for the optic.

            6. Alternatively, loosen the rail carrier thumbscrew, remove the optic, and put it in a different rail carrier. This maintains height but not angle.

2. Adjust the lamp to roughly the correct height (need not put it up against the camera); you will fine tune it later. It is the ONLY element whose height you will change later.

3. **It is important to have the optics aligned – this means both at the same height and at the same angle. You want your camera tube, for instance, to be parallel to the optical rail you have set up, and you want any other lenses (e.g. your objective, etc.) to be similarly aligned so the optical axis goes straight through the axis of the lens.**

    a. **The best way to check that things are parallel to the rail is to slide them up against another lens tube in a separate rail mount (e.g. slide the condenser lens tube up against the camera lens tube so that the edges are flush with each other) – the two separate connections to the rail force alignment of the optics along the rail axis.**

    b. **The next-best approach is to look down on the optic to check that its mount looks perpendicular to the rail when viewed from above.**

c. **Note: It is possible to correct for a lens incorrectly angled in one direction by angling some subsequent lens the opposite direction, but this results in increasingly bad aberrations and performance.** The closer everything is to being aligned along the rail axis – and optics are actually quite sensitive to misalignment – the better your imaging will be.

4. The time to adjust your camera height – if you want to – is now, before adjusting all the optic heights. Camera position is flexible, but the camera body should NOT extend beyond the end of the rail; keep it at least ~2.5 cm from the rail end.

5. With your camera placed at one end of the rail, adjust your home-built achromat objective to be ~ 200 mm from it (essentially, space the objective and tube lens by roughly $f_{obj} + f_{tubelens}$).

6. Remove your diffuser.

II. Illumination Optics Setup

7. Place your lamp at the far opposite end of the rail from the camera (lamp body should not extend off the rail; keep it ~2.5 cm inside the end of the rail.)

8. Place the f = 35 mm PCX lens (as your collector lens) close to the lamp (~1" or 2.5 cm away.)

   a. Remember: this lens needs to be the same height as the lens on the camera.

   b. **Which direction should the flat side of the collector lens face?[3]**

   c. **Place the condenser** in the sliding rail carrier on the lamp side of the sample; see Fig. 4b later in the notes for this lab.

      i. If necessary, assemble your condenser lens system; see Fig. 1 below (the condenser system is very similar to the objective from Lab 3).

   d. Remember, the lens must be the same height as the lens on the camera.

   e. **Roughly how far from the resolution target should the condenser lens be? Place the assembly on the rail such that the lens is in about the right place.**

   f. Separately, think through how you will adjust things to get your field stop in the correct position.

---

[3] If you cannot figure this out, consider reviewing the CVI/IDEX/Melles Griot reading, particularly Figure 1.25 showing a symmetric bi-convex lens (which is equivalent to two plan-convex lenses with their flat sides together) and the system below that (with two identical plano-convex lenses with their curved sides together). Fig. 1.23 showing aberrations as a function of lens shape is also relevant.
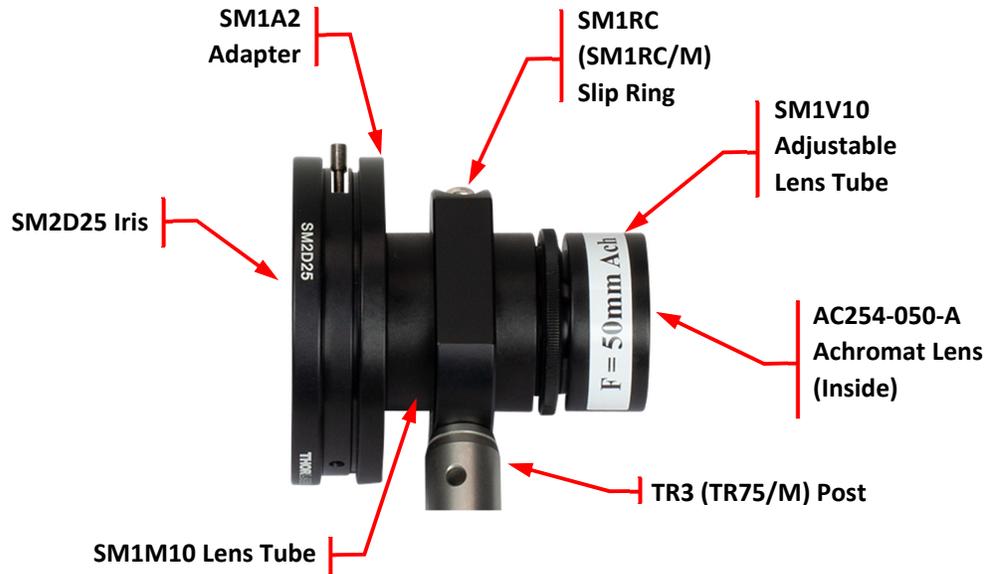
Figure 1: Condenser lens assembly.

9. Put a 1" diameter iris (for your field stop; item # is SM1D25) on a post in a rail carrier ~ 8.5 to 9.0 cm away from the front of the lamp housing.

   a. Open this iris fully.

10. Place your green glass color filter (FGV9) in a flip mount (TRF90) in a rail carrier on the far side of the field stop from the lamp.

    a. **If the flip mount is already assembled, make sure the arrow drawn on it (if there is one) faces away from the lamp (arrow should face the direction the light is propagating.)**

11. Position your neutral density filter wheel at some convenient place between the lamp and condenser – the exact placement is not critical – so that you can use them to control the intensity of the illumination.

III. Köhler Illumination (& What the Light Does in the Optical Path)

You may find it helpful here to use a more transparent (lower ND) ND filter, or no filter at all, so that the light is very bright.

12. Turn on your lamp.

13. Set up Köhler illumination as described in the Course Notes for this lab.

    a. **This means you need to be familiar (VERY familiar – we often put it on the midterm exam) with the steps on "Setting up Köhler Illumination" near the end of this chapter. You need to be ready to do this without trying to read the notes during the lab.**

    b. Leave the green filter flipped OUT (not in the light path) for now.

WATCH OUT!
The halogen lamp gets HOT after it is been on for a while!

c. When adjusting the height of the filament image at the aperture stop, adjust **only** the height of the lamp – everything else should already be indexed to the height of the camera lens. The lamp is the last thing left to adjust, so set the height of the lamp so the image of the filament at the aperture stop is at the correct height (centered on the condenser iris aperture).

    i. General check: The above procedure should work, but if your alignment is bad (or your collector height was set incorrectly) then you may have difficulty. A good rough check is as follows: use a piece of paper to look to see if the light stays parallel to the rail (the same height) after the lamp and collector lens. Take other parts off the rail to check this, so you can move the paper back and forth. If the light path is going up or down, adjust the lamp height (assuming the collector is at same height as the camera; otherwise fix that first).

14. Using a scrap of notebook paper, examine the image of the filament between the collector lens and the aperture stop. **For your write-up, note briefly what you see.**

15. Using the same scrap of paper, and with your microscope set up for Köhler illumination, examine the light between the condenser lens and the sample. **For your write-up, note briefly what you see.**

16. With the iris in the back of your objective wide open, use a scrap of paper to examine what you can see in the objective back focal plane. **For your write-up, note briefly what you see; this is easiest if you take the sample out so you can move your piece of paper through the sample position.**

    a. Put the resolution target back in, and focus on it properly.

17. Set the aperture of the iris on the back of your objective to 3 mm in diameter (This is the objective "BFP" iris, for <u>B</u>ack <u>F</u>ocal <u>P</u>lane.)[4]

18. Open the aperture stop on the condenser just barely **as wide as the height** of the filament image (a few millimeters in diameter) **(be sure you know what the aperture stop is and what to adjust)**.

    a. Adjust your camera gain and exposure to their minimum values, adjust your ND filters so that the camera is not saturating; readjust exposure so that the camera dynamic range is well used (i.e. so that the camera line scan values are nearly at not quite 255).

19. Adjust the diameter of the field stop so that the edges are just visible in your image.

    a. What is the smallest USAF bar pattern (Group and Element) on the resolution target that you can resolve?

    b. Take an image and save it.

---

[4] Advanced note: it may seem that reducing the BFP diameter will reduce resolution, and in fact it will. The reason for choosing this value right now is that the filament image in the BFP is only ~ 3 mm tall, and by choosing this value for the BFP aperture we assure that we can fill the entire objective NA with illumination. If you open the BFP aperture wider, then the illumination NA will improve on the horizontal axis (which has a wider filament image) but not on the vertical axis (with a narrower filament image), and so the expected resolutions and behaviors will be different on the two axes. Normally larger is better, up to ~ 7.5 mm diameter for this objective, above which the lens barrel restricts the aperture and induces aberrations.

20. Use the light blue IR filter (item # FGB37M) in a short SM103 lens tube and screw it gently onto your lamp housing.

    a. If this filter was already present, remove it for the following steps, so that you have images both with and without the filter. Reinstall it afterwards.

21. Adjust your ND filters and exposure again, and then take an image.

    a. What is the smallest 3-bar pattern on the resolution target you can resolve now?

    b. Take an image and save it.

    c. **Is your resolution improving with the filter? If so, why is that?**

22. Flip the green filter into the light path, and optimize your ND filters and exposure again. Then adjust your aperture stop to give the best image contrast, and take an image.

    a. What is the smallest 3-bar pattern on the resolution target you can resolve, and how does this compare to your results in the previous two parts?

    b. (If you have a spectrometer) Measure the spectrum of the green filter. Save this (you can paste it into your notes document).

    c. **Why might you use this filter, given what you saw in Lab 3 with the PCX lens?**

23. Focus on the smallest 3-bar pattern you can resolve easily.

24. Take an image, optimized for best exposure, etc., that has the USAF target bars well-centered in the image (use 1:1 imaging for this) so that you can see both large bar-sets and the very small ones in the center. **This image is for use in the 'Contrast and Resolution' part of the write-up.**

    Only start Part IV if you have at least 30 minutes to complete it.

IV. Numerical Aperture

NA (I): Resolution

25. Adjust gain and exposure for good imaging.

26. Close the objective iris to 1 mm diameter, and adjust the aperture stop iris size to match the **height** of the filament image on the aperture stop.

    a. **Note: Over time, people sometimes twist the objective iris knobs too hard and the alignment gets messed up**– so "1" no longer corresponds to 1 mm iris opening. You can tell if this has happened by twisting the knob gently and seeing where it stops – if it stops at "1," it is correct.

        i. If it does not, but stops at something above "1," then you can use that as your "1" value and still increase in steps of 0.5 mm, keeping track of the offset so you can adjust for it in your calculations.

        ii. If it stops below "1," the only choice is to consult your instructor.

27. Focus the objective and set the camera for best imaging.

   a. As you close the objective aperture down, the image will get dimmer; you will need to turn up the exposure (and possibly the gain) in order to see your image well.

      i. If your image remains too dim, you may need to <u>turn the frame rate down</u> in order <u>to be able to turn the exposure up</u> further.

         1. In that case you <u>may also</u> have to turn down the pixel clock in order to be able to turn the frame rate down enough to allow longer exposures. These features are available from your camera and image menus.

28. For every value for the iris diameter from 1 to 3.5 mm (i.e., 1.0, 1.5, 2.0, 2.5, 3.0, 3.5):

   a. Adjust your exposure for proper imaging.

   b. Find the smallest 3-bar resolution target you can resolve, and focus carefully.

   c. Write down the diameter and 3-bar pattern number (lines/mm) in Table I, below.

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29. Note: Backlash

   a. Your iris setting will be inaccurate unless you always move in the same direction (this is due to slop in the mechanical system, known as "backlash"). The way to work with this is to open it back up to (e.g.) 7 mm and then close the iris down to whatever setting you want. You do not need to open back up if you continue closing the iris down; you only need to do this if you want to move to a larger aperture while preserving the proper calibration – in other words, you always need to approach your setting from the same direction.

NA (II): Image Brightness

30. Flip OUT the green filter glass and take the IR filter OFF the lamp. **Be careful – the lamp is hot; you may need to turn it off and let it cool a bit first.**

   a. This is just to get more light; adding the diffuser will make things dim.

31. Put your diffuser between the lamp and the condenser (about 4 cm from the condenser) using a rail carrier.

   a. The diffuser serves to make a bigger blob of light at the aperture stop, so the whole condenser NA is filled with light.

32. Close the objective BFP iris to 1 mm diameter, and adjust the aperture stop to ~1.0 cm diameter.

   a. See previous 'Notes' boxes under NA (I), above, on iris size calibration and backlash.

33. Open the line-scan profile so that you can see how intense the illumination is in your image; adjust the exposure (and gain, if need be) to keep the line profile <u>**at about the same level**</u>, near the top of the range, for all images.

Histogram Window Icon

34. You want to know how bright the image is for different diameters of the objective iris (which sets the objective NA). The most accurate way to do this is to change the exposure to keep the pixels seeing a similar amount of light, then later divide out the effect of using longer exposures (more time) to collect that light. The brightness of the image will be proportional to: (Average pixel counts – Average background counts) / (Gain * Exposure). To do this:

35. Open a Histogram Window (see page with diagram at end of these notes).

    a. Adjust your exposure such that the pixels are using the camera range without saturating too much (average level ~ 200 or so).

        i. **Record this exposure level in Table II, below.**

    b. Record the average pixel intensity from the histogram in Table II, below.

    c. Block the lamp light close to the lamp.

    d. **Record the average *background* pixel intensity from the histogram in Table II, below.**

        i. This is the "background" intensity value from room lights, etc.

36. Repeat the last set of steps for every objective iris dia. from 1.0 to 3.5 mm in 0.5 mm steps.

> For purposes of your lab report, the relative image intensity = (Average pixel intensity – Average background) / (Gain * Exposure).
>
> Note: it is critical to subtract the background off the image before figuring the pixel intensities – i.e., if the intensities of some pixels in a bright area you are using for reference are ~ 245 counts, and (with the same objective BFP setting) you measure 40 counts when you block the lamp light (or in a large black area nearby in the image), then the number to use for your pixel intensity is 245 – 40 = 205 counts.

37. Remove the diffuser, and screw the IR filter back onto the lamp.

V. Clean up

38. Turn off your halogen light so it has time to cool before you put everything away.

39. **Carefully put away your resolution target in its special box.**

40. **Carefully disconnect the USB cable from your camera(s) by gently pulling the connector straight out.**

41. Screw a lens cap on over your camera tube lens and condenser.

42. Close the irises on the back of the objective and condenser (not super tight – just close it to keep dust out).

43. Put your optical breadboard system away.

44. Copy your files off the computer onto your USB drive or cloud storage (remember – it is your responsibility to keep copies of your files!).

45. **Shut down** (not just "sleep") the computer.

### Setting Up Köhler Illumination: When Building a Microscope

46. With your camera focused at infinity, put the camera approximately one tube lens focal length behind the objective (the best distance depends on lens design; if in doubt, closer than a focal length is better than farther, to avoid clipping rays on the edge of the lens, known as "vignetting").

47. Turn on your lamp so that there is some illumination on the sample.

48. Position a sample in front of the objective such that it is in focus on your camera (a marker drawn on a slide works fine; even a fingerprint on a slide can work well).

49. With the aperture stop and field stops wide open, position the condenser roughly one focal length from the sample.

50. Position the lamp, collector lens, and field stop at the far end of the optical rail.

    a. Get all lenses at the same height as the camera tube lens; this is hardest to do with the lamp, so adjust lamp height as best you can.

        i. One way to check is to put optics next to each other and adjust the heights so they match. In this way, index all of them off the heights to one specific optic (such as the camera). Remember: moving the entire rail-mount can allow you to maintain both height *and* angle of the optic, which helps with alignment.

        ii. The lamp height only needs to be approximately right; do it as best you can.

51. Adjust the collector so that the filament is in focus at the aperture stop.

    a. Adjust the lamp height to get the filament vertically centered on the aperture stop. Holding a piece of paper or business card at the aperture stop can make it easier to see the filament image.

52. Position the field stop conveniently close to the collector.

53. Close down the field stop and adjust the condenser position along the optical rail axis in order to bring the field stop into focus at the sample (and hence on the camera).

    a. If the field stop is not centered, adjust its height and/or horizontal position to get it centered on the camera image. (Some microscopes have adjustments for this; others are designed so they are unnecessary.)

    b. Note: in this kit, horizontal position of the field stop cannot be adjusted; if the field stop position in the image appears incorrect, the issue is the angular alignment of the intervening lenses.

54. Reposition the collector (if necessary) to bring the filament back into focus at the aperture stop.

55. Open the field stop as much as desired – usually so no larger area is illuminated than what is being viewed on the camera.

56. **Close down the aperture stop until contrast is optimized. This is usually ~ 70% of the objective back aperture (after any magnification effects); we will return to this in a later lab.** Note: filters (e.g. ND filters, color filters) or diffusers should be positioned away from the field stop so that they are not in focus at the detector – that way the diffuser texture and/or scratches or dust on the filters will not be visible in the image.

Camera Histogram Function
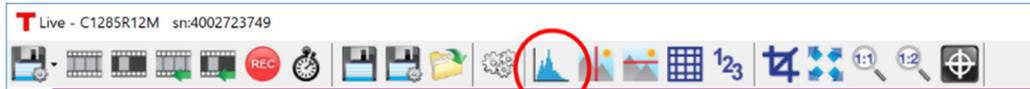
Histogram Window Icon
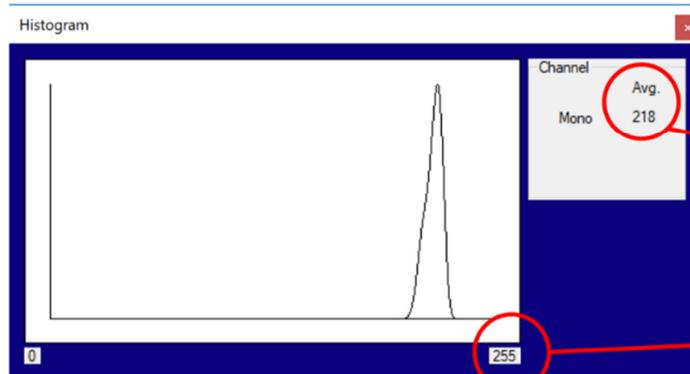
Average Pixel Value

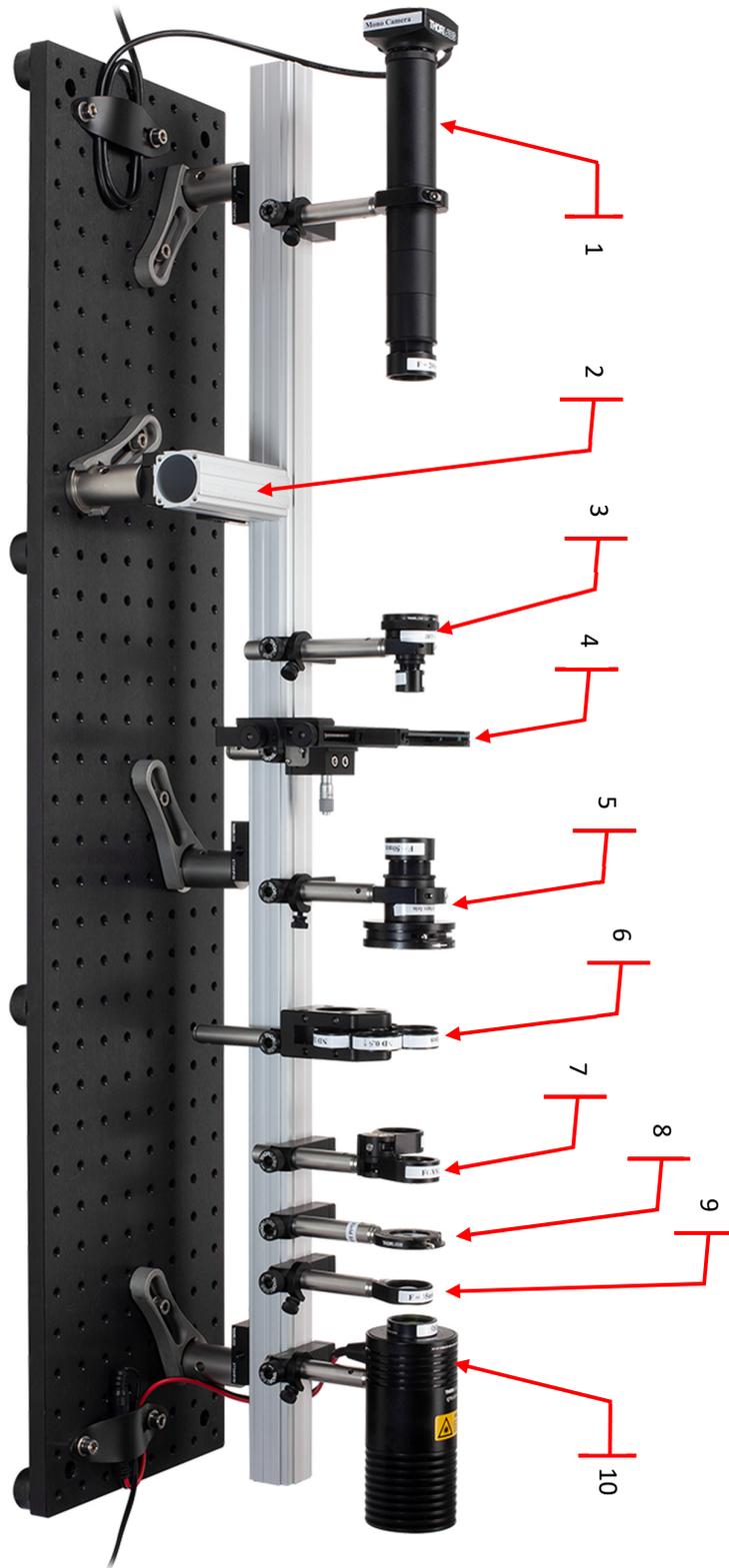**Adjust the exposure so a few pixels have values near 255.**

Be careful – lower the exposure until you see the histogram is all shifting down; the placement of the "255" is not exact.

Note how I have lowered the exposure to place the histogram values to the left of 255 here.

Details for Optical Rail Layout (Figure 2 on Following Page)

# in Fig	Details
0	Left side of rail
1	Camera. Exact position is non-critical, and will vary depending on where the slip ring is secured. Important thing is to keep camera body from extending too far beyond edge of rail.
2	**Distance from end of main rail to left side of perpendicular stub rail. Helpful if this is the same for all rigs, ideally 26.5 cm from left edge.**
3	Homebuilt objective. Exact position will vary with device, and where the slip ring is attached.
4	**Sample stage. Helpful if this is the same for all rigs, ~ 43 - 44 cm from left edge of rail to left edge of stage rail carrier.**
5	Condenser. Exact position will vary with field stop location and slip ring positioning.
6	ND filter wheel. Exact positioning not critical.
7	Flip mount filters. Exact positioning not critical.
8	Field stop (iris). Exact position will vary with lamp and collector lens position.
9	Collector lens. Exact position will vary with lamp and condenser lens position.
10	Lamp. Exact position not critical. Important thing is to keep lamp from extending off edge of rail.

Figure 2: Photo of optical rail layout for Köhler illumination.

Lab 4 Write-Up:

Basic Details:

o Turn in your write-up as a Word document, due date, etc. as specified by your instructor.

o **Number your pages "page X of Y" and have your name in the footer so it is clear if a page is missing if it gets printed out.**

o After appropriately cropping and sizing images – not covering the whole page, not so small one cannot see the features discussed – reduce the image size so the document is not huge. In MS Office as of this writing, you do this by clicking on a picture, choosing the "Format" tab in the top menu bar, and then choosing "compress pictures" on the format menu toolbar. For submitting your assignment, click save and save-as-PDF. Be sure to check that the compression and the PDF conversion have not spoiled some feature in an image you were trying to point out in your lab write-up – this should not be an issue if you cropped and sized your images appropriately. If there is a problem, send the larger document so the image information is preserved.

Write-Up:

1. (Re: Part III, Köhler Illumination steps 14 - 16) Describe what you see when you examine the light in the optical path in the following places. Does it makes sense based on the course notes? Explain why or why not.

   a. Between the collector lens and the aperture stop.

   b. Between the condenser lens and the sample.

   c. In the objective back focal plane.

2. (Re: Part III, Köhler Illumination steps 17 - 22) Make a table showing:

   a. Your resolution in white light with the aperture stop open to the same height as the filament image.

   b. Your resolution with the IR filter in place.

   c. Your resolution with the green filter in place (as well as the IR filter).

   d. How do these compare to the Rayleigh resolution you expect?

      i. **What wavelength seems to make sense to choose for white light if you want to match the Rayleigh resolution? Note: this is a subtle question.**

      ii. What wavelength should you use for the green filter? If you have one, paste your spectrum for it into your write-up.

      iii. The image of the filament at the aperture stop is ~ 3 mm across, roughly, in case that information is useful.

   e. Comment on the utility of the green filter and of the IR filter (i.e., why you use them).

3. (Re: Part III , Köhler Illumination steps 23 – 24):

   a. Open ImageJ

      i. **Look at the helpful ImageJ description and guide in the Lab 3 Lab Notes.**

b. **For each image you took of your resolution target in Step 24** (i.e., for the images of USAF 3-bar patterns):

    i. Open the image.

        1. You can open this from the 'file' menu or by dragging and dropping the image onto the ImageJ window.

c. Change to the line tool. Draw a line through the three bars of one of the Elements (plus a little on each edge).

    i. The line should be perpendicular to the bars.

    ii. Use analyze/plot profile, or "Ctrl+K," to plot the pixel values along the line you drew.

    iii. Drag your cursor over the line plot, and record the pixel value at the darkest point on one of the bars and at the brightest point in between it and an adjacent bar.

        1. **Write these in Table III, below.**

d. Use Excel to calculate the **_contrast_** of the bars [(max – min) / (max + min – 2 * background)] versus the resolution (line spacing for each Group, Element).

    i. **If any of your values end up being negative, then your background value was too high (either due to not having a good place to measure it in your image, or due to some non-uniformity in your illumination). In this case, use a value of background = 0 and re-plot the data.**

    ii. Fill all this information into Table III, below.

    iii. Paste the table into your write-up.

e. In Excel, plot the contrast vs. the spacing between the lines (in lp/mm).

    i. Paste this plot into your write-up; be sure to note the 'background' value used.

f. Paste your line-profile plots for the 3-bar patterns from USAF Group, Element: 6,5; 6,6; 7,1 into the your write-up. Note in the caption what the line spacing is for these in microns.

    i. **Comment on how much contrast (difference between black and white) there is for the bars as a function of resolution – is the contrast the same right up until you hit the resolution limit, or not? Does this make sense to you?**

        1. This plot is related to the "Modulation Transfer Function," which we will discuss in a lot of detail more later in the course.

g. If your result seems obvious, it is not – some systems can give fairly constant contrast right up to the resolution limit, while others do not.

4. (Re: Part 4: NA (I) resolution): For Table I (below):

a. Calculate the NA for each objective BFP iris size. Use the BFP diameter formula from the 'Equations to Memorize' (Course Notes Appendix A) sheet for this.

b. Using Excel, or a similar program, plot, on the same graph, as two separate curves:

    i. Measured resolution vs NA

    ii. Calculated Rayleigh resolution vs NA

       1. Assume that your condenser NA = objective NA.

    iii. **Comment on whether these calculated values match, and whether the lines scale the way you expect.**

5. (Re: Part 4: NA (II): image brightness): For Table II (below):

a. **Use a spreadsheet to plot the relative image intensity vs. the (objective NA). Comment on <u>why</u> this graph looks as it does. Be as quantitative as you can.**

>   i. NOTE: Be sure to take into account which ND filters you used, if any. If you used ND 0 for one measurement and ND 1.0 for the next, the ND 1.0 measurement is 10X *brighter*, since you reduced the intensity 10X from what it would have been by using the filter.
>
>   ii. Note: For purposes of your lab report, the relative image intensity = (Average pixel intensity – Average background) / (Gain * Exposure).
>
>   1. It is critical to subtract the background off the image before figuring the pixel intensities – i.e., if the intensities of some pixels in a bright area you are using for reference is ~ 245 counts, and (with the same objective BFP setting) you measure 40 counts when you block the lamp light (or in a large black area nearby in the image), then the number to use for your pixel intensity is 245 – 40 = 205 counts.

Table I: Resolution vs. Achromat Aperture

Iris Setting, mm	Calculated NA	Rayleigh Resolution	USAF Target Smallest Resolvable Group, Element	USAF Target Max Resolvable, lines/mm	Resolution Calculated from Max Resolvable, lines/mm
1.0					
1.5					
2.0					
2.5					
3.0					
3.5					

Table II: Brightness vs. Achromat Aperture

Iris Setting, mm	Calculated NA	Pixel Avg.	Background Avg.	Exposure	Brightness
1.0					
1.5					
2.0					
2.5					
3.0					
3.5					

Table III: Contrast vs Resolution

USAF Target Group, Element	USAF 1951 3-Bar Target, lines/mm	Min Pixel Value	Max Pixel Value	Resolution (Line Spacing)	Contrast
G5, E4					
G5, E5					
G5, E6					
G6, E1					
G6, E2					
G6, E3					
G6, E4					
G6, E5					
G6, E6					

Background Value Used:

Lab 5 Notes:

Köhler, Conjugate Planes, and Darkfield Imaging

Optical Microscopy Course

Overview

In this lab we will continue exploring conjugate planes and Köhler illumination, and use them to explore new contrast mechanisms: darkfield and oblique illumination.

Darkfield microscopy is historically quite important; until the 1950s it was the only way to image transparent objects, and even now it is the cheapest way to do so. The utility of this cannot be overstated: cells are usually transparent, and staining them to make them visible usually kills them. Hence darkfield and similar techniques (e.g. phase contrast and differential interference contrast, DIC) offer a way to observe cells while they are alive and behaving normally, something of obvious scientific importance. From the perspective of this class, darkfield also presents a nice way to explore the use of conjugate planes to manipulate image contrast, consider resolution in slightly more detail, and to begin to develop intuition about how image information is distributed in the objective back focal plane. That will lead us in the next lab on the Abbe theory of image formation, one of the most important topics of this course.

This lab should not be enormously time-consuming during class; this is partly intentional, with the idea that you can take advantage of the additional time to make sure you thoroughly understand Köhler illumination and how to set it up. **Remember: Köhler illumination is very important. We often make setting it up properly a part of the practical portion of the midterm exam.**

Lab 5 Reading & Preparation:

1. Lab 4 Course Notes (on Köhler illumination), if you have any doubt or confusion, re-read these. **This material underlies everything we will do for the next 4 labs. Be sure to review it.**

2. Lab 5 Lab Notes (this document).

3. Lab 5 Course Notes.

4. Memorize all formulas on the first page of "Equations to Memorize" (Course Notes Appendix A).

5. Zernike's description of phase contrast, from Course Notes Appendix B:

    a. Read the first 5 pages, stopping just prior to Fig. 4. Skim this material; do not get bogged down if it is confusing. It represents another way of thinking about darkfield microscopy, one which we will explore more in coming labs. We will return to phase contrast in Lab 8.

Supplementary Reading (Not Required):

6. Look at the diagrams of the 3D point-spread function in either or both of:

    a. Figure 8.41, p. 490, in "Principles of Optics, 7th expanded ed.", by Born and Wolf. ISBN 0521642221

    b. Discussion and images of the 3-D PSF with spherical aberration, from Wikipedia. See the links on *Spherical Aberration* under the *Reference Links* tab at www.thorlabs/OMC.
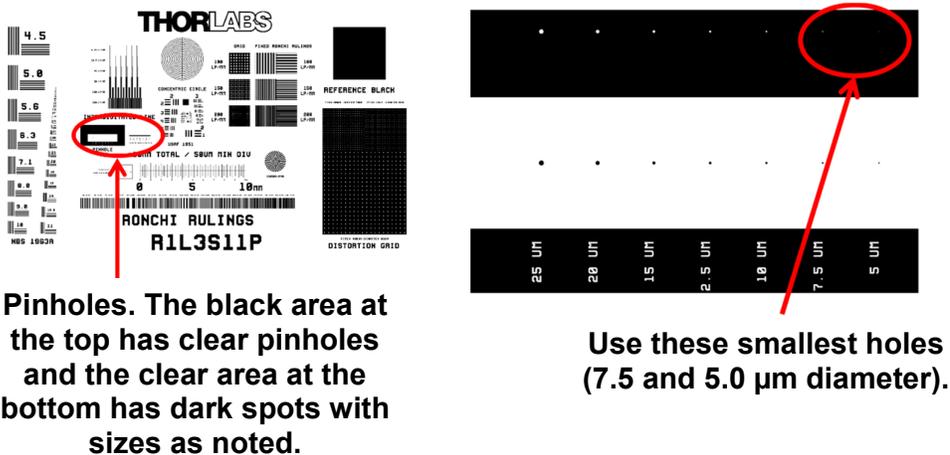
Lab 5 Tasks

I. Alignment Check

Review your Köhler alignment, and make any changes/improvements you want before moving on. There will be (some) time at the beginning of this lab to help/answer questions. This is a valuable opportunity to get your systems (and your understanding) squared away before moving on.

II. Building Intuition: The PSF

We have discussed the fact that an image requires taking light from any individual point in your object (sample) and putting that light at a corresponding place (ideally another individual point) in your image. When the system does not get all of that light into one point in the image (and it never does), the image is worse – not perfectly representative of the original sample.

The distribution of light at the image from a single spot in the object is called the Point Spread Function (PSF); one obvious way to measure this is to have a sample that is a tiny point, smaller than anything we could resolve, and to see what that point actually looks like in the final image; hence the name "Point Spread Function".

Let's examine the imaging of a sub-resolution spot by your system: insert the Thorlabs test target and image the clear pinholes with the black background:

Pinholes. The black area at the top has clear pinholes and the clear area at the bottom has dark spots with sizes as noted.

Use these smallest holes (7.5 and 5.0 µm diameter).

Figure 1: Pinholes on the Thorlabs target. Left: location on target slide; Right: details of pinhole feature, with diameters on bottom, opaque spots in middle, and clear apertures on top.

With your rig set up for well-aligned Köhler illumination:

1. Find the pinholes on the Thorlabs target (see Figure 1).

    a. This is easiest if you find the diameter information, then move up to the pinholes.

2. **With your objective BFP iris diameter set to 3.0 mm**, image the black 7.5 µm diameter pinhole in the center of your field of view.

    a. Have your field stop and aperture stop wide open, and your green filter in place.

3. Move the sample so you are imaging the clear 7.5 µm pinhole in the middle of the field of view.

4. To see if they are exposed properly, examine the pixel values at the pinhole image. To do this:

    a. Use the mouse thumb-wheel to zoom in on the image.

    b. If you cannot see the image of the pinhole when you do this, try turning up the exposure.

    c. Play with the amount of zoom, but then make the spot fairly small.

    d. Click the "Pixel Peek" icon on the ThorCam toolbar.

        i. The numbers you see are the pixel values for the pixels around the place where the cursor is. You can see more pixel values by dragging the Pixel Peek Window to a larger size.

    e. Move your cursor to the center of the pinhole image, and look at the values of the pixels in that vicinity.

5. If any values are 255, then you are saturating those pixels; if so, turn down the exposure.

6. Turn up your exposure so you can see (in the main camera window) the smaller 5 μm diameter pinhole to right of the 7.5 μm one. Move it (the 5 μm hole) to the center of your field of view.

7. Focus carefully, and maximize the exposure (while using the Pixel Peek window to make sure no pixels in the image of the pinhole are saturating, i.e. at 255 counts).

    a. Is the background completely black? What does the Pixel Peek window say the background (in a black area slightly away from where the bright spot) is? Is it zero counts?

    b. Close down your field stop; the 5 μm pinhole should still be visible at the center of your field of view (if not, move the stage so that you can see it).

        i. You will probably see a large gray circle around the pinhole defined by the field stop diameter; this is the lit area of the sample, and clearly the chrome of the sample does not completely block the illumination light.

            1. Advanced question: how much of the light does the chrome block? What OD (Optical Density) is it? Do not worry about this unless you have lots of extra time.

    c. **What happened to the background level when you closed the field stop? Did it drop everywhere, even in the illuminated area of the sample?**

        i. The reason for this is that the light on other parts of the sample and optics scatters off of dust, etc., and gets into other parts of the image. This is exactly why you use a field stop – by reducing light on things you are not interested in you also reduce the scattered light in the system and improve the image of what you care about seeing.

    d. Turn off the room lights, keeping a desk light or two (aimed away from the rigs) so everyone can see what they are doing. What happens to the background level now?

8. Re-optimize your exposure, so the spot is close, but not quite 255 counts at the brightest point.

Pixel Peek Icon

Lab 5 Notes: Köhler, Conjugate Planes, and Darkfield

9. Close the "Pixel Peek' box so you can see the pinhole image in the camera window.

   a. **Can you see any rings around the central spot? This should remind you of the "Airy disk" from the Optics Guide readings and other Course materials.**

   b. **Focus back and forth across the pinhole – does it stay centered? Do the rings open up symmetrically around the central spot as you change focus? This is a good test of how well your system is aligned.**

   c. **If you focus far enough, you should see a bright ring with a *dark center* and if you keep going, you should get a (less) bright spot in the center again, etc… we never cease to find this amazing (it is a diffraction effect, due to the wave nature of light).**

   d. As you turn the focus back and forth, you are exploring the 3-D PSF, i.e., the 3-D distribution of light at the focus of the system. Try to imagine what this looks like in 3-D (we will return to this in a later lab). You can also compare what you see to figures such as those **for the PSF and spherical aberration** listed in the Reference**s** / Optional reading links for this lab. (If you have web access, try comparing right now).

10. With everything exposed properly, take an image of the 5 μm pinhole with the BFP at 3.0 mm.

   BE SURE to name this image properly and SAVE as a TIF for later.

   a. **It is important in the later analysis to have low background – try taking your image, properly exposed (spot not saturating) with the room lights off to reduce stray light.**

11. Close the BFP iris from 3.0 mm down slowly to 1.0 mm while <u>carefully</u> watching your zoomed-in image of the 5 μm pinhole.

   a. **What happens to the diameter of the rings around the spot as you make the BFP diameter smaller?**

      i. **You may want to open and close the BFP iris a few times to watch this.**

   b. **What happens to the diameter of the central spot?**

      i. **Why does it get dimmer? What formula on the formula sheet might help explain this?**

      ii. You will need a long exposure to see the spot properly with a 1.0 mm BFP – possibly close to 1000 ms (one second!). To do this you may have to turn down the pixel clock and the frame rate for the camera on the "Settings" menu.

         1. Be sure to be at the best focus first; you need a frame rate of at least 5+ fps to be able to focus very well. Once you turn up the exposure, the frame rate will be too slow to focus easily.

         2. You may find the green interference filter (if it is already in the TRF90 flip mount) allows more light through than the green VG9 glass filter. Either is OK to use.

         3. The room lights will definitely have to be off at such long exposures, or else the background will be so high the image will saturate.

         4. You can also explore using the Gain slider and Gain Boost checkbox on the ThorCam Settings / Image tab (be sure to

leave Gamma = 1 on that tab). First set the exposure to be long, however.

12. With the spot properly exposed (near, but not quite 255 counts max, per your Pixel Peek window), **save an image as a TIF for later.**

    a. **Warning: ThorCam *does not* save the image when you click the save button, but only grabs the image after you enter the filename into the dialogue box and hit enter to save the image; so click save, type the name, and then make sure you have the correct image on-screen when you hit enter to save.**

13. Turn the pixel clock and frame rate back up, to at least 10 fps, and set the gain back to zero (and uncheck the Gain Boost, if you used that).

III. Darkfield & Oblique Illumination

Darkfield

14. Set up your microscope for Köhler illumination, with the USAF 3-bar resolution targets as a sample. Readjust exposure, etc., as necessary.

    a. If you adjusted the Gain previously, make sure to set it back to zero.

    b. Open the field stop so the whole camera image is illuminated.

15. Close down the aperture stop as small as you can make it.

    a. If you find too little light getting through, you may need to slightly reposition the image of your filament on the aperture stop such that one of the bright lines in the filament coil passes over the tiny hole in the iris.

16. **IMPORTANT: In the following, DO NOT move your sample between the next few steps. You can change the BFP diameter, etc., but do not move the sample back and forth with the XY adjustments or change the focus or you will not be able to do the lab write-up later.**

    a. **You want the sample positioned so the image is centered on the smallest USAF 3-bar set.**

17. Set the iris in your objective back focal plane to 7.5 mm.

    a. Optimize your exposure, focus, and ND filters for good imaging.

    b. **Take an image and save it as a TIF.**

18. Set the iris in your objective back focal plane to 1.0 mm

    a. Optimize your exposure and ND filters for good imaging.

        i. If your image intensity drops a lot when you close the objective BFP iris down, the image of the aperture stop in the objective BFP is probably not properly centered (and hence the BFP iris is blocking the light before it is fully closed).

            1. To fix this, slightly adjust the alignment of the condenser by tapping on the side of it with a balldriver handle (this allows very small adjustments) until the light comes through OK / alignment is good.

            2. If you tap the objective, you will move the image on the camera and will have to go back and repeat Step 17 above.

    b. **Take an image and save it as a TIF.**

CAREFUL!
Do not force the aperture stop or it will break.

CAREFUL!
Do not force the iris; be gentle!

19. Open the iris in your objective back focal plane up to 7.5 mm again.

   a. Block the middle of the BFP with something; the tip of one of the smaller balldrivers works well.

      i. It helps to brace your hand on the breadboard / optical rail, and the driver on the edge of the objective iris housing so the driver tip is more stable.

      ii. Ideally, you want the tip of the driver at the same plane as the iris diaphragm (i.e., in the objective BFP).

      iii. Imaging will be better if you bring the driver in at an angle (say 45°) to the horizontal or vertical. (The reason for this will become clear next lab; the issue is the rectilinear nature of the sample you are examining).

        ➔ **Try holding the driver in different orientations!**

      iv. Aluminum foil can be molded around the objective post to make a stable "finger" that reaches in to block the middle portion of the BFP. This is quite handy, as it leaves your hands free. If you do this, be sure the aluminum foil blocks only as much of the BFP as necessary to get darkfield, and not too much more.

   b. Optimize your exposure and ND filters for good imaging.

   c. **Take an image and save it; the image should be centered on the USAF target.**

20. **Stop and think carefully about why your image looks the way it does. What happened to the background light? What did placing the driver in the center of the objective BFP do, and why did it have that effect?**

Oblique Illumination

21. Open the iris in the objective back focal plane to 7.5 mm.

22. Set both your field stop and aperture stop wide open.

23. Using some aluminum foil, mask off the right or left **_half_** of the aperture stop so that you have oblique illumination on your sample (i.e. the light is hitting your sample at an angle).

   a. The edge of the mask should be **vertical**.

24. Using aluminum foil in a similar way, mask half of the objective BFP to get a dark field image.

   a. You may need to adjust your exposure.

   b. **Is this image different from the previous darkfield image? Why might that be?**

   c. **Which side of the BFP do you have to mask to get darkfield? Why that side and not the other?**

   d. **Take an image and save it; the image should be centered on the USAF target.**

MAKE SURE you have a sharp, straight edge on the foil running vertically through the aperture stop. This is easiest to get by folding the foil over first to make a clean, straight edge.

25. Rotate your foil masks 90° (so the aluminum foil edge runs horizontally through the aperture stop and BFP).

    a. **Does the image looked different than in the previous step? Why might that be?**

        i. Note: the filament is narrower vertically than it is horizontally, which makes it harder to set up darkfield this way. It can be easier to do this by molding some tinfoil over the objective and aperture stop than by trying to hold a driver in the right place by hand. However, this issue is not what the question above is alluding to – something else is different about the images taken this way and with the apertures blocked at 90° to this. What is it?

    b. **Take an image and save it; the image should be centered on the USAF target.**

26. Try spacing your aluminum foil masks apart just a little bit so that a sliver of light can get through; does that change the image in an interesting way?

    a. This is easiest with the foil edges oriented vertically

    b. If so, **take an image and save it; the image should be centered on the USAF target.**

IV. Building intuition about the BFP: Image subtraction

27. **Image Preparation: The point of this step is simply to get the images to roughly the same brightness, to account for slight differences in exposure time, etc. when you took them.**

    a. In ImageJ open both your 7.5 mm and smallest (1 mm) objective BFP iris diameter images of the resolution target (from the beginning of Part III, above).

    b. Use the box tool in ImageJ to outline a large box in a bright area of the 7.5 mm BFP image.

        i. Use Ctrl+H to get a histogram; read the mean pixel value for the bright area off of that.

    c. Repeat step (b) for the smallest iris (1 mm) BFP image, using roughly the same box location and size.

        i. **If the pixel value for either of steps (b) or (c) is below 40, you need to retake these images.**

    d. In ImageJ, use process/math/multiply to multiply the 7.5 mm BFP image by the ratio of (7.5 mm BFP mean bright pixel value) / 250.

        i. This scales the image so that the bright areas are close to the display maximum of 255 counts.

    e. In ImageJ, use process/math/multiply to multiply the 1.0 mm BFP image by the ratio of (1.0 mm BFP mean bright pixel value) / 250.

28. Use process/image calculator to subtract the two images.

    a. Use the 32-bit mode and 'create new window'.

29. Use process/math/abs to take the absolute value of the image pixel values.

    a. This step is important because you cannot see negative electric field values – the camera (and your eye) detect intensity, which is the magnitude-squared of the electric field. We are trying to duplicate that effect in a rather hand-waving way.

30. Save this image as a bitmap for use in your lab write-up.

31. **Think carefully about why subtracting these two images would give something that looks like your darkfield image. What did you do in Part III (Darkfield) that might have had an effect similar to subtracting an image?**

V. Building Intuition About Imaging: Convolution & the PSF

What is the radius of your PSF?

32. Using ImageJ, open your image of the 5 μm pinhole taken using the 1.0 mm BFP iris diameter (this should be the last image you took in Part II).

33. **What is the magnification, M, of your system? (You should be able to calculate this easily.)**

34. **You are looking at a 5 μm dia. pinhole; the pixels on the DCC1545M camera are 5.2 μm apart. How many pixels would you expect to be illuminated by the pinhole image?**

35. **Open your image in ImageJ, and take a line-profile through the spot.**

    a. **If you hold "Shift" while dragging the line across the spot, it forces the line to be horizontal and straight)**

    b. **If you hit "Ctrl+K" after that, ImageJ will plot the line-profile. The x-axis will be measured in pixels.**

    c. **What is the diameter from the minimum on one side of the spot to the minimum on the other?**

>   i. Note: Even if there is no background, or you subtract it off, because the pixels are not infinitely small you will probably not see the profile go all the way to zero – the pixel averages over a bit of the nearby bright rings and so the pixel value just dips near where the (ideal, theoretical) zero would be.

36. **Calculate the Rayleigh resolution for your system and compare that to what you just found. Does it match appropriately? If not, why not?**

Implementing ("Faking") that PSF using Convolution

> "Convolution" simply means that for every pixel in your image, software places a blob function centered at that pixel (but extending to other nearby pixels) and scaled to the intensity of the original pixel value at that point. It then does that for every pixel in the image. This is exactly what would have happened if your optical system has a point spread function (PSF) shaped like the blob function you convolve with.

Theoretically, for a perfect optical system, the "blob function" is actually an Airy function, with form $f(r) = [2 \cdot J_1(2\pi \cdot NA \cdot r / \lambda) / (2\pi \cdot NA \cdot r \cdot / \lambda)]^2$, where J_1 is a Bessel function of the first kind. It is computationally (and generally) a hassle to work with Airy / Bessel functions, so most people (and ImageJ) use a Gaussian (Bell-curve) function to approximate it instead. The Gaussian that looks most like the central bump of the Airy function has a standard deviation $\sigma = 0.35 *$ Airy disk <u>radius</u> (not diameter).

37. Using ImageJ, open your images of the resolution target you took with the BFP iris set at 7.5 mm and at 1.0 mm.

    a. Put the two images side by side.

    b. We will assume the 7.5 mm image is "perfect."

        i. Click on the 7.5 mm image, and use process/filters/Gaussian Blur.

        ii. Choose radius = the value you decided on in the last step.

        iii. Click the "preview" box.

38. Compare your <u>actual</u> 1.0 mm BFP image with the 7.5 mm BFP image that you just made by convolving (smearing out or blurring) with the PSF you calculated to be the same size a spot would get using a 1.0 mm BFP iris.

    a. Are they the same – i.e., can you resolve the same smallest 3-bar element in both images?

    b. If so, click OK and save your convolved image using a different name (so it does not overwrite the old image).

    c. If not, uncheck the preview button, adjust the radius value you chose, and click 'preview' again. Repeat this until the images match.

        i. Record the radius you found, and save the image (with a different name).

        ii. Explain why your calculation was wrong.

Darkfield Comparison (Again)

39. Close all images, then reopen the 7.5 mm BFP image and the Gaussian blurred (convolved with the larger PSF) version of it you just made.

    a. Closing the images is necessary to reset the image names in ImageJ.

40. Use process/image calculator to subtract the two images.

    a. Use the 32-bit mode.

41. Use process/math/abs to take the magnitude of the pixel values in the image, like before.

42. **Save this image as a TIF for use in your lab write-up.**

43. **Does this image look like the one you got in Part IV? Think carefully about why blurring an image, then subtracting it from the original generates a similar result to blocking part of the objective back focal plane.**

VI. Clean up

44. Turn off your halogen light so it has time to cool before you put everything away.

45. **Carefully put away your resolution target in its special box.**

46. Screw a lens cap on over your camera tube lens.

47. **Carefully disconnect the USB cable from your camera(s) by gently pulling the connector straight out.**

48. Close the iris on the back of the objective (not super tight – just close it to keep dust out).

49. Put your optical breadboard system away.

50. Copy your files off the computer onto your USB drive or cloud storage (remember – it is your responsibility to keep copies of your files!).

51. **Shut down** (not just "sleep") the computer.

CAUTION!
The light will be hot! Let it cool down.

Lab 5 Write-Up:

Basic Details:

- Turn in your write-up as a Word document; due date, etc. will be specified by your instructor.

- **Number your pages "page X of Y" and have your name in the footer so it is clear if a page is missing if it gets printed out.**

- After appropriately cropping and sizing images – not covering the whole page, not so small one cannot see the features discussed – reduce the image size so the document is not huge. In MS Office as of this writing, you do this by clicking on a picture, choosing the "Format" tab in the top menu bar, and then choosing "compress pictures" on the format menu toolbar. For submitting your assignment, click save and save-as-PDF. Be sure to check that the compression and the PDF conversion have not spoiled some feature in an image you were trying to point out in your lab write-up – this should not be an issue if you cropped and sized your images appropriately. If there is a problem, send the larger document so the image information is preserved.

Write-Up:

Darkfield Illumination (Part III)

1. Compare your darkfield image from Part III, step 19 with the images you generated in Part IV and Part V "Darkfield Comparison (Again)".

    a. Paste in and caption all three images.

    b. Explain why the images you made using ImageJ and your darkfield image are similar (they will not be exactly the same – do not worry about that).

        i. For the image you made in Part IV, explain, based on the Lab 5 Notes and your understanding of resolution and NA, what the relationship is between the way you made this image and the way you took your darkfield image in lab (using the balldriver).

        ii. Explain, in detail:

            1. Why blocking the middle of the objective BFP generated the same image you got by subtracting a low-resolution image from a high resolution image.

            2. Why convolving the high resolution image with a larger PSF, then subtracting the two images, produced a similar result. Please explain this in terms of formulae and concepts; "the image looked the same so the result was the same" is *not* an acceptable answer.

2. Compare the results above to what happens when you use the process/find edges command in ImageJ on your 7.5 mm diameter BFP iris image from Part III.

    a. Paste in your "find edges" image.

    b. Note: "Find edges" also makes the image binary (that is why the images look so white) – if you want (**_not required_**), you can make your subtracted image binary like that using process/binary/make binary, then inverting it using image/lookup tables/invert LUT.

c. Discuss (briefly!) what this might imply about where the information about edges in an image is distributed in the objective BFP.

d. Any ideas **why** that might be?

Oblique Illumination (Part III)

3. Comment briefly on how your images taken with oblique darkfield illumination look different from your central darkfield image (the one taken using the balldriver). What is the most noticeable difference? What does this imply about where the information from the various elements of the image is distributed in the BFP of the objective?

Point Spread Function (Part I)

4. Paste in the (cropped out and blown up so easy to see!) images of the pinhole with BFP diameter = 3.0 mm and 1.0 mm.

    a. Include figure captions listing λ, exposure, gain, BFP diameter, etc.

5. For the 3.0 mm and 1.0 mm BFP PSF images, explain briefly:

    a. Why they were so dim (especially the 1.0 mm one).

    b. Why using the gain still did not allow you to see the Airy disk (if it did not).

        i. What **did** you see a lot more of when you turned up the gain?

6. For the 3.0 mm BFP PSF image,

    a. Paste in the (cropped out and blown up so easy to see!) image of the PSF.

    b. Paste in the line profile through the PSF (use Ctrl+K in ImageJ to get a line profile.)

    c. In ImageJ, crop the image (you can crop in ImageJ by drawing a box around the spot and using Ctrl+Shift+X)

        i. Use ImageJ Analyze/Surface Plot, and check (only) Draw Wireframe and Draw Axis. Leave the Polygon Multiplier at 100%.

            1. If you get an error, you probably have your image open as RGB (color). Use Image/Type/8-bit (or 16-bit) to change it to grayscale; then the previous step should work to create the surface plot.

        ii. Paste this image into your write-up, with an appropriate caption.

    d. Explain clearly what the surface plot represents – what do the numbers, etc. mean in that plot? What does that plot tell you about (and about what part of) the PSF?

7. Explain what you measured (based on your image or line profile) for your Airy disk diameter (or radius), in pixels.

    a. Given that camera pixels are spaced 5.2 μm apart, also list your Airy disk diameter (or radius) in microns.

8. What radius do you expect for an objective with BFP diameter 3.0 mm and f = 25 mm? How many pixels is that? How does that correspond to your answer above?

9. Assume the tube lens is one focal length from the objective BFP.

    a. What NA does the f = 200 mm tube lens have as it focuses light from the sample, through the BFP, and down onto the camera? (A point source sample will fill the BFP uniformly with light).

    b. What is the diameter (or radius), in pixels and microns, of the Airy disk you expect to see based on that?

    c.   How does that correspond to the BFP diameter you measured from your image?

10. What would the expected Airy disk diameter (or radius) on the camera be for a 1.0 mm BFP iris diameter?

    a.   How did that correspond to the value you used in your convolution (Gaussian blur) of the 7.5 mm BFP image that produced an image similar to your 3.0 mm BFP image?

11. Calculate the ratio of the Airy disk expected for a 3.0 mm diameter BFP iris using the objective NA on the sample side, and the tube lens NA on the camera side (these are the values you calculated above).

12. Calculate the magnification of your system using the focal lengths of the lenses.

    a.   Discuss the link between the NA ratio you calculated and the magnification.

Lab 6 Notes:

The Abbe Theory of Image Formation (I)

Optical Microscopy Course

Overview

From the Lab 6 Course Notes you will have the basics of the Abbe theory of image formation; your instructor will likely spend several lectures on it as well. This lab will allow you to explore some of how Abbe theory overlaps with what you have already done in terms of darkfield illumination, and to investigate phenomena that might help you understand the oblique/graded-field illumination you did last lab. We will also revisit conjugate planes to help cement your knowledge of these, and look toward Labs 7 and 8 by exploring contrast in a way related to Abbe theory.

In order to do all this you will set up a second camera, which images the back focal plane of the objective. In typical microscopes you have access to this view using by either of two methods: first, on many research microscopes there is a lever which allows you to insert a lens (called the Bertrand lens) which shifts your view through the eyepieces to the back focal plane of the objective; if this lens is absent, then you can do the same thing, roughly, by removing the eyepiece from the microscope (the eyepiece can usually just be pulled out of the tube it fits in) and looking into the eyepiece barrel with your eye (this if fine with a normal microscope; do NOT do it if using a laser-based miroscope!). In our case, the use of two cameras will allow you to watch what is happening in both the sample plane and the objective back focal plane at the same time.

Lab 6 Reading & Preparation:

1. Lab 6 Lab Notes (this document).

2. Lab 1 and Lab 6 Course Notes. **VERY IMPORTANT, particularly the Lab 6 Course Notes**

3. The amazingly well-done videos of a lecture on Abbe theory by Prof. Peter Evennett. Although we learned about these videos long after we had started planning this course, we could not hope to do better in exploring Abbe theory, and he has kindly made them public for you to watch. You will notice that we borrowed the idea of having Abbe's image in a conjugate plane directly from this lecture. Do not worry about the discussion of phase contrast, or other things which we have not covered yet. See the *Reference Links* tab at www.thorlabs.com/OMC for the links to the videos.

Lab 6 Tasks

I. Preliminary

1. Insert your resolution target and set your microscope up for Köhler illumination. Make sure your alignment is good.

II. Setting Up the Back-Focal Plane Imaging Camera

2. Get out your color camera, which should be mounted on a 3" (75 mm) post as shown in the OMC1 manual. The manual, which includes assembly instructions, can be found at www.thorlabs.com/OMC.

3. The SM1V10 adjustable lens tube is screwed into a 1" SM1 lens tube; a 1" dia., f = 35 mm plano-convex lens is mounted in the adjustable lens tube. You will be imaging the objective back focal plane onto the camera, with the shortest distance being between the color camera and the lens. **Which way should the flat side of the lens face?**

   a. **NOTE**: We have done everything so far with one thing at infinity and the other at exactly one focal length from the lens. This time, for convenience, we will not. The thin lens equation (see the 'Equations to Memorize') would allow you to figure out exactly where the object should go to image on the camera if the lens is a bit more than 35 mm from the camera face, as it is here. However, we will do this in a simpler way: screw the focus all the way in so that the lens ends up as close to the camera as possible (this is still ≥35 mm). You will then move the entire camera along the rail, thus adjusting S_o, in order to focus on the back focal plane.

4. If this has not already been done, screw the 1" SM1 lens tube onto your color camera. Keep the lens tube horizontal while doing this to keep any debris off the camera and optic.

5. Adjust the camera so that it is at the same height as the monochrome sample camera.

   a. Use the usual trick of putting it in a rail carrier in front of the monochrome camera and adjusting its height until the SM1 tubes fit together face to face at the same height. Then put a post clamp on the color camera to keep it at the right height.

6. Mount the color camera with the special long rail carrier (the only one longer than the others), labeled XT34TR2 (XT34TR2/M), on the "dogleg" optical rail as shown:

**Figure 1**: Color Camera and Beamsplitter Positioning

7. Connect the color camera to a USB port on the side of your computer opposite the monochrome camera (this is so that you will have maximum bandwidth USB connections for both cameras).

8. Open both cameras using the ThorCam software – initialize one window for the monochrome camera and one for the color camera. Make sure that both cameras are detecting light.

9. Click on the "Fit to Screen" icon at the top of the ThorCam window for both camera displays.

10. Place a rail carrier on the main rail at the base of the short rail (see Figure 1), and mount the beamsplitter angled at 45°, so that light will come from the objective and reflect off it to the color camera.

   a. The beamsplitter is the EBP2, a large transparent 2" (5 cm) diameter circular plate mounted in an LMR2 lens mount on a post. It reflects 30% of the incident light off one surface and allows 70% to pass through – hence its "70/30" designation.

   b. The exact height is not critical, but try to get the plate centered on the optic axis.

11. Open the BFP iris, aperture stop, and field stop wide, so that lots of light is getting through the back of the objective.

12. Adjust the color camera rail carrier to be close to the end of the short rail.

13. Make sure the color camera is facing along the rail (i.e., facing perpendicular to the main optical rail. It is easiest to see whether this is correct by standing up and looking straight down on the camera, the same perspective as used to take the picture in Figure 1 above).

14. Make sure the color camera is imaging (sensitive to light if you put your hand in front of it). Open up a line profile (notice how there are three colors in the line profile, one each for the red, green, and blue pixels) and have the camera properties open.

15. Adjust the position and angle of the beamsplitter until lots of light hits the color camera (this should be very obvious, since it will be a lot of light – the camera will saturate).

   a. Turn down the color camera exposure until there is little (or no) saturation in the image.

Fit to Screen Icon

i. If everything worked unusually well, you will see a somewhat-focused image of the BFP. What would you expect to see in the BFP?

16. Close down the BFP iris to 1.0 mm. The light on the color camera image should be reduced to a small area, though probably out of focus and looking strange.

  a. If this is not the case, open the BFP back up, adjust the beamsplitter, and close the BFP down again until you keep some light (e.g. a lighted spot of some sort; see Figs. 2 and 3 below) on the screen when it is closed down.

17. Adjust the Beamsplitter:

  a. Use BOTH HANDS.

    i. Loosen the beamsplitter rail carrier and use one hand to slide it back and forth.

    ii. Loosen the set-screw and use one hand to rotate the beamsplitter post (use a post clamp so the post stays at the same height while being rotated).

    iii. If the light is flaring away from the spot on the screen (see Figure 2), then <u>twist</u> the beamsplitter / adjust its angle to reduce the flaring / get the flare centered.

      1. You cannot adjust the vertical rotation of the beamsplitter, so flaring up or down from the spot cannot be fixed; ignore it, if there is any.

      2. When you turn the beamsplitter, the spot will move across the screen. <u>Slide</u> the rail carrier to bring it back to the center.

        a. The sliding will make the light flare again, so now twist to fix that. This will move the spot, so slide to fix that… etc.

        b. Mess with this until you get the hang of it.

  b. End Result: You should have the spot in the center of the screen, with the flare going out evenly on either side (see Figure 3).
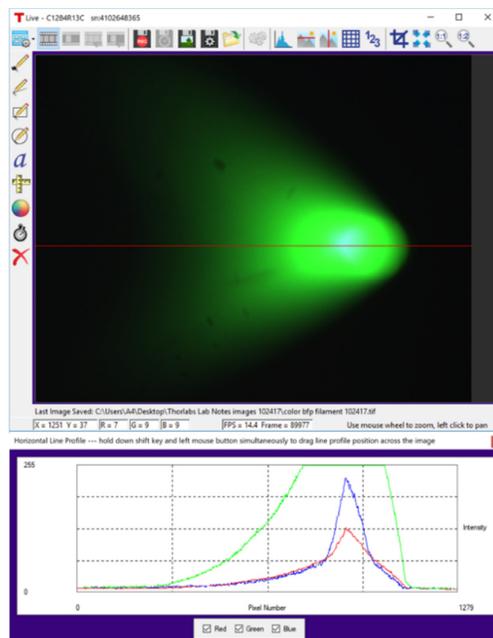
Figure 2: Example of undesirable flaring. The image is overexposed to make the flare more apparent.
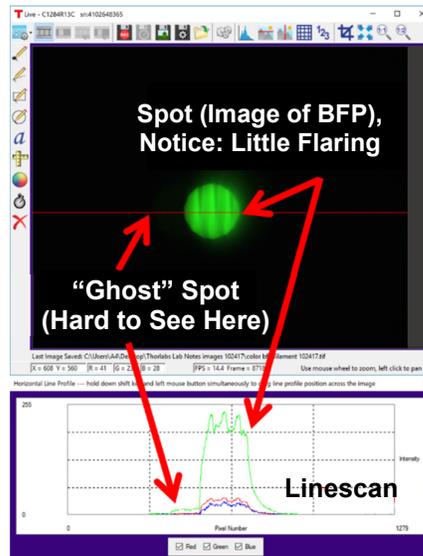
Figure 3: BFP spot appearance on the color camera when properly aligned.

18. Adjust the position of the color camera along its rail, by sliding the rail carrier back and forth. This should allow you to bring the edges of the BFP iris into sharp focus on the camera.

19. When you think you have it right, make sure the line profile goes through the spot, and look to see how large the secondary reflection (off the back side of the beamsplitter) is. This reflection will be slightly displaced from the main spot.

   a. There is a right and a wrong orientation for the beamsplitter; try reversing the beamsplitter (rotating it 180°). Is the "ghost" spot a smaller fraction of the intensity of the main spot (you can tell by looking at the line profile, which should go through both)? Choose the orientation with the dimmer ghost spot. See Figure 3.

   b. **Advanced question (only if you have extra time): can you explain where this "ghost" spot comes from?[5]**

20. Verify that the aperture stop and objective BFP iris are concentric (i.e. properly aligned). To check this:

   a. Alternately close down the aperture stop and the objective BFP iris to make sure that in the image they start to clip each other equally on both sides (left and right; there is nothing you can do about the vertical on this system).

   b. If they are not concentric (e.g. if the aperture stop starts cutting off the BFP iris on one side before the other, or vice-versa for the BFP iris and the aperture stop) then either the condenser or objective is at the wrong angle. If they need adjustment, either:

      i. Put post collars on the condenser and objective, and then tap the sides of either or both of lens tubes (start with the one that looks least parallel to the optical rails, when you look down on them) with a balldriver to turn them a bit and superpose the images of the aperture stop and BFP.

---

[5] Note: One can buy beamsplitters (e.g. Thorlabs item # BSS16) that are designed to minimize these "ghosts," but they are usually thicker, and always more expensive. It is good to see this effect here – we purposefully chose a beamsplitter that shows "ghosts" so that you understand where they come from.

ii. A better/more thorough (but also more time consuming) approach is to:

1. Remove the objective rail carrier, with the objective still mounted in it, and check its height and alignment against the monochrome camera. Slide the objective up against the camera lens tube so that the edges are flush with each other; this will force both to be aligned along the rail. Lock down the objective height and tighten it in the rail carrier, then remove the entire rail carrier and put it back near the sample stage.

2. Repeat this for the condenser (and afterwards adjust its position so the field stop is in focus).

3. This approach will usually get you very close to perfect alignment, such that only small adjustments are necessary to get the aperture stop and BFP iris images to be concentric.

c. **Remember**: The field stop (FS) should also be centered on the monochrome camera after you have done this. If the objective and condenser are parallel to the optical rails, the aperture stop will superpose on/in the BFP <u>and</u> the field stop will image to the center of the monochrome camera all at the same time. Note: use "Fit to Screen" on the camera window to see if the field stop is in the center.

III. Reminding Yourself About Conjugate Planes

21. With the BFP iris open, close the condenser aperture (aperture stop) until you just start to see the edges on the color camera. Then close down your objective aperture until you just start to see it with the camera, inside the area of the condenser aperture (both should be in focus on the camera.)

22. Close your field stop down until it is just visible on your monochrome sample camera – you should be able to look at the BFP on the color camera and the sample on the monochrome camera at the same time.

23. Insert your green filter, and adjust the camera exposures for good imaging on both cameras.

24. Get two of the slides with the clear plastic images of Abbe and the images of his name. If not already done, ask your instructor to remove the hard plastic frames. With some part of the resolution target in focus on the monochrome camera, hold Abbe's name in the field stop, and Abbe's picture in the aperture stop, and take images from both cameras and save them.

a. **Pause and think about this: you have both images in the optical path at the same time, yet one images to one camera and one to the other camera.**

b. **Important Note**: (Not important if you are saving your images as TIFs.) If you save images from the color camera as bitmaps (.bmp), ImageJ cannot open them. Save the color images as .TIF or .PNG format, which ImageJ recognizes.

Aside: If you hold Abbe's name at the field stop, and his face at the Aperture Stop, then the image of his name can be blurry – the reason for this has to do with how much of the aperture stop his face blocks… you are blocking many of the spatial frequencies in his name. It is actually quite impressive that the imaging is not worse.

IV. Investigating the Abbe Theory of Image Formation

Line Spacing I

25. Find the "Ronchi rulings," i.e. sets of parallel lines, on the resolution target. There are a number of these sets, with decreasing spacing between the lines:
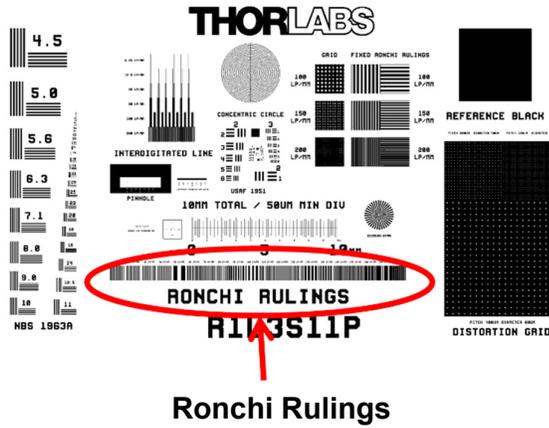
Ronchi Rulings

Figure 4: Ronchi ruling placement. The line spacings are printed on the target just above each set of lines.

26. Focus on the most closely-spaced set of lines (200 lp/mm, on the right in Fig. 4). Set up for proper imaging of the sample; make sure while you are focusing that you have a line profile window open for the sample (mono) camera, and that you are using all of the camera dynamic range (i.e. the line profile is near the maximum value – but not saturating! – when it hits a light area).

27. Adjust the focus. Notice how the lines come in and out of focus several times as you move the micrometer. This is not an error: what you are seeing is due to the "Talbot effect," an odd (and in this case annoying) result of imaging a periodic structure.

    a. If you are curious about the Talbot effect, some references are: Wikipedia; Inoué, Video Microscopy 1st ed., p. 39 footnote; H. F. Talbot "Facts relating to optical science" No. IV, Philos. Mag. 9, 1836; Lord Rayleigh "On copying diffraction gratings and on some phenomenon connected therewith" Philos. Mag. 11, 1881.

    b. In order to get good images, you will need to make sure that you are in the best plane of focus. This is where using the ThorCam line profile is especially important: it will appear to have lots of little fringes, and **the amplitude of these fringes will be the largest at the plane of best focus**. You may have to scan the focus back and forth once or twice to make sure you are actually at the best spot.

    c. If the fringe of peaks in your line profile window goes up and down to zero and back up in a weird way, instead of staying fairly flat, then your stage is not perpendicular to the optic axis (rail); try adjusting the angle of your sample stage. When it is truly perpendicular to the optical path the fringe of lines should be constant height all the way across the image, though this can be hard to achieve. If it is close, that is fine for this lab.

28. Now shift the sample to image the most widely-spaced set of lines (on the left in Fig. 4). If your focus was good before, it is probably best to leave it alone. Set up for proper exposure so you are using all of the camera dynamic range (i.e. the line profile is near the top when it hits a light area).

   a. Click on the "Fit to Screen" icon at the top of the ThorCam window in order to fit the whole image into your window, so you are seeing the whole camera image. Close down the field stop until the edge is visible in the image, and adjust the sample so that only one size (spacing) of Ronchi lines is visible on the camera (you may have to center the set of Ronchi lines and adjust the field stop diameter to achieve this).

      i. Note: If your system is well aligned, the field stop should be centered in your image when you close it down. If it is not, adjust your alignment so that it is – the reason we are using it is to block light to nearby sets of lines with different spacings than the one on your screen; if your field stop is halfway off-screen, then you may be illuminating parts of the sample that you do not expect (and that make your results confusing).

29. Prepare for imaging:

   a. Close down the aperture stop as small as it will go. **(Without forcing it!)**

   b. Flip <u>out</u> the green filter (if you had it in).

   c. Set the BFP iris to 7.5 mm diameter.

   d. For the color camera, click "Fit to Screen" on the ThorCam menu bar.

   e. Set the monochrome camera to 1:1 imaging, "Display Image in Normal Size" mode.

      i. If you stay in "Fit to Screen," then for closely-spaced sets of lines you may see weird patterns in the image. These are due to "aliasing" of the image when it is resampled to fit the window, a topic beyond what we will cover today. 1:1 imaging makes each pixel on the camera display as one pixel on the monitor, instead of scaling (resampling) the image to fit the size of the window you have open.

   f. Optimize exposure for the sample (mono) and the BFP (color) cameras, so they are not saturating.

   g. Make sure both camera images are visible on your monitor, side by side.

30. Without changing the exposure, start sliding the sample left, so that you are sliding more and more closely spaced sets of vertical lines through the field of view.

   a. **While you are doing this, WATCH THE IMAGES. What do you notice?**

      i. **What is the relationship between the Ronchi line spacing and the image in the BFP camera?**

Wavelength

31. While focused on the 200 lp/mm Ronchi ruling,

   a. Set the BFP iris to 7.5 mm diameter.

   b. Close your condenser aperture (aperture stop) down all the way.

c. Remove your green filter, if it is in (but keep the IR filter in place).

d. Set up your cameras for good imaging.

e. Use "Fit to Screen" and your field stop to make sure you are imaging only the 200 lp/mm set of lines and not parts of any other sets. Then switch back to 1:1 imaging.

f. You should see the main (white) spot in the color camera, the "ghost" spot (ignore this one), and some rainbow smears to either side of the main spot.

32. **Take three images with your color camera (remember to save in TIF format):**

   a. One with a deep red Roscolux filter in the light path. (Light Red #26 or Storaro Red #2001 works well if the filter book has it, but any filter with a transmission peak in the red – and **only** the red – will do).

   b. One with a green Roscolux filter in the light path. (Moss Green #89 or Storaro green #2004 works well, but again any filter with a peak **only** in the green will do.).

   c. One with a blue Roscolux filter in the light path. (Baldassari Blue #381 or Midnight Blue #384 works well, and again what you are looking for is any filter with a peak **only** in the blue – no peaks in the red or green).

   d. **Consider why the spots for each of these colors are in the locations they are relative to each other.**

33. Use the printed spectrum for each filter (in the Roscolux filter book), and write down the main/center wavelength that the filter transmits. Ignore peaks in the far red, since your IR filter blocks that light.

   a. If you have a spectrometer, instead use it to measure the spectrum of the red, green, and blue filters above; save screenshots of these spectra with a note about the main/center wavelength for each.

34. While holding the red filter paper in the illumination path, optimize your cameras for viewing.

   a. **Take pictures from both cameras (remember: save in TIF format).**

   b. Close down your objective back aperture until you have **just** blocked out the red diffracted beams in the BFP. **Take pictures with both cameras.**

      i. **What do you notice about the image of the sample (monochrome camera)?**

      ii. If your system is not well aligned, your BFP iris will not block both of the spots symmetrically – it will cut one off first (you can see this on the BFP camera). If that happens, you need to adjust your alignment.

   c. Without changing the BFP iris, remove the piece of red filter paper, flip in your green filter, and optimize settings for both cameras if necessary.

      i. **Take pictures with both cameras.**

      ii. **Why is it that the image changed the way it did between steps (b) and (c)? This is a *very* important thing to understand.**

To adjust alignment, try tapping the condenser or objective to get the BFP iris set till it closes down evenly on the spots on both sides.
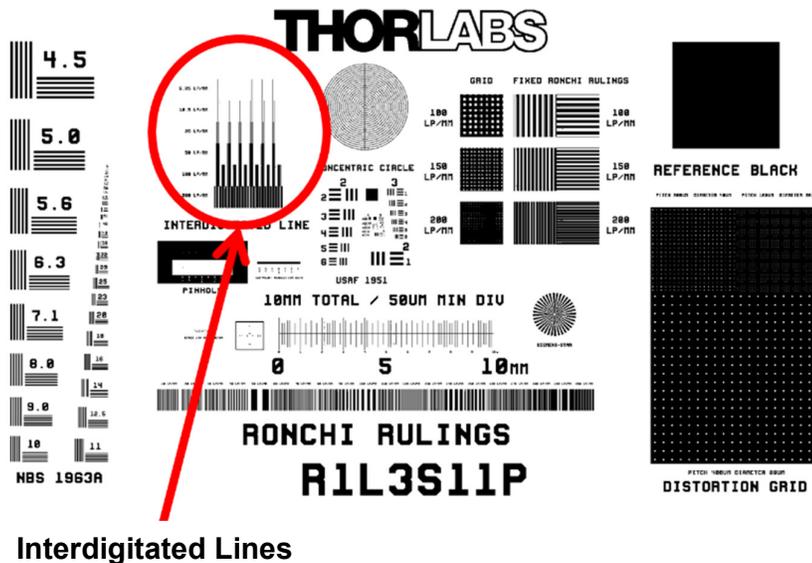
Interdigitated Lines

Figure 5: Interdigitated line sample location.

Line Spacing II

35. Move your sample so you are imaging the "Interdigitated Lines" sample.

    a. Have your aperture stop closed down.

    b. Set your BFP iris to 7.5 mm

    c. Flip your green filter in (though you can also try it with the filter flipped out).

    d. Check (using "Fit to Screen") that you have only the lines on the camera, and no other samples.

36. Move to the bottom (200 lp/mm) set of lines, then move up until you have **both** the 200 lp/mm and 100 lp/mm sets of lines on the screen at the same time.

37. Set up both cameras for proper imaging.

    a. **Save images from both cameras (remember: use TIF format for the color camera).**

38. Watching your BFP camera image, close down your BFP iris until:

    a. **You notice a change in the sample image. What happened?**

    b. **What change in the BFP camera image happened at the same time the image of the lines changed?**

    c. **Save images from both cameras (remember: use TIF format for the color camera).**
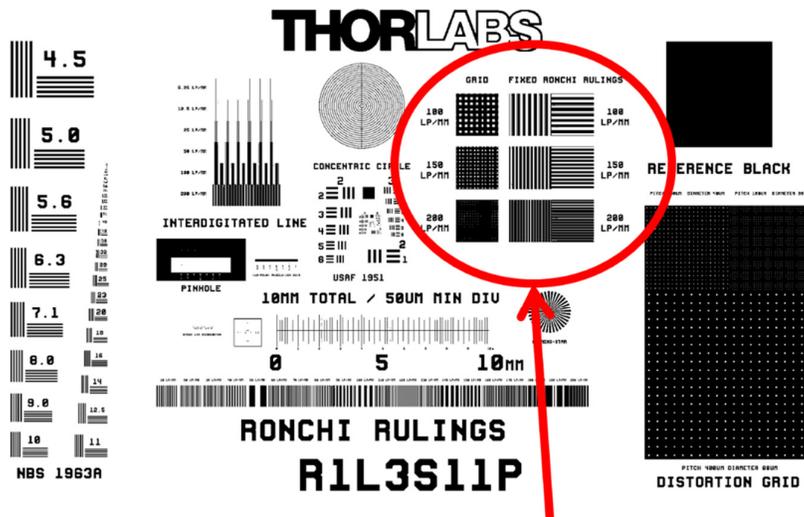
**Grid / Vertical Ronchi / Horizontal Ronchi,
3 Different Spacings: 100, 150, 200 lp/mm**

Figure 6: Grid and Ronchi ruling location. Grids are on left; vertical line Ronchis are in middle, horizontal line Ronchis are on right. Top row has 100 lp/mm; middle row is 150 lp/mm; bottom row is 200 lp/mm.

V. Spatial Filtering in the BFP

39. Open your objective BFP up to 12 mm; make sure your aperture stop is all the way closed. Remove your green filter.

> a. Note: For parts of the imaging below, it can be helpful to have the room lights low or off, to reduce the background on the color camera (which makes it harder to see the dimmer spots, especially for the grid samples). It can also be helpful to turn up the exposure so that the central spot is somewhat overexposed, which again makes the dimmer spots more visible.

40. Using Figure 6 as an aid, find and image the horizontal 100 lp/mm Ronchi ruling (upper right of the sets on the resolution target).

    a. Close down your field stop so you are only imaging the <u>horizontal</u> lines.

    b. **Does what you see in the BFP camera make sense to you?**

41. Move back to the middle, so you are imaging the <u>vertical</u> 100 lp/mm Ronchi.

    a. Check to make sure you are only imaging vertical lines.

    b. **Does what you see in the BFP camera make sense to you?**

42. Move slightly further left to the 100 lp/mm grid target (this is essentially just a vertical and horizontal 100 lp/mm Ronchi on top of each other).

    a. Check to make sure you are only imaging the grid (field stop blocking anything else), but go back to 1:1 imaging to look at the grid on the sample camera.

    b. **Does what you see in the BFP camera make sense to you?**

You may want to check in "Fit to Screen" to see the whole camera image and make sure nothing but horizontal lines are present.

Lab 6 Notes: Abbe Theory of Image Formation (I)   © Switz, Fletcher; 2019

c. **Optimize your cameras and take pictures with both.**

   i. Remember: Save in TIF format from the color camera.

43. Get two pieces of aluminum foil, and fold each so you have two straight edges.

44. Using the foil, block all the diffracted spots in the objective BFP except for the <u>vertical</u> row in the center.

   a. **Take pictures with both cameras.**

   b. **Does what you are seeing make sense to you? Why do you see what you see on the sample camera?**

45. Repeat this, except that this time block all except the <u>horizontal</u> central row of diffracted spots.

   a. **Take pictures with both cameras.**

   b. **Does what you are seeing make sense to you? Why do you see what you see on the sample camera?**

46. Repeat this again, but now block all except a **_diagonal_** line of diffracted beams.

   a. **Take pictures with both cameras.**

   b. **There is something subtle going on here. See if you can explain/understand why the effect is different than in steps 40 and 41.**

      i. **Note: Are the diagonal lines oriented the way you would expect?**

VI. Darkfield Revisited: the Zero-Order Mask

47. We now have the ability to directly observe the objective BFP; let's use that ability to improve darkfield imaging by using a mask that blocks only the very center of the BFP:

   a. Check that your aperture stop and BFP iris images are concentric by closing them down alternately as you did before (Step 20).

      i. If they are not concentric, fix your alignment before continuing.

   b. Open the iris in your objective BFP up to a 7.5 mm diameter again.

   c. Open the aperture stop so you can see plenty of filament on your BFP camera; adjust exposure so nothing is saturating.

   d. Screw on your zero-order mask, which is a 1.25 mm diameter black dot in the middle of a 25 mm diameter glass plate. This should be mounted in a short SM1 lens tube; just screw that onto the back of your objective BFP aperture, which is threaded to accept it.
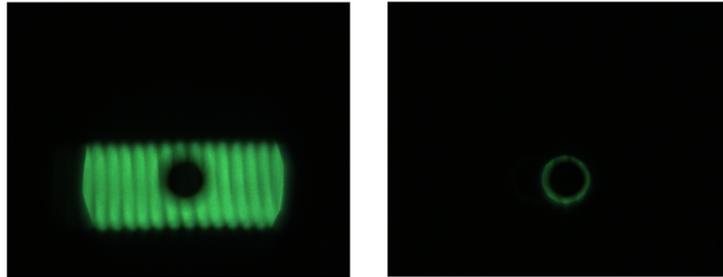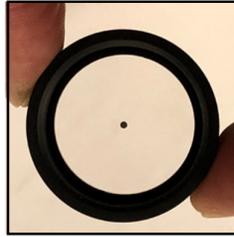
Figure 7: Top: Zero-Order Mask in SM1 Tube. Bottom Left: Image of Dot on BFP Camera; slightly out of focus because dot is ~2 mm behind the exact BFP iris plane. Bottom Right: Image of Aperture Stop Closed Around Dot Showing Good Alignment; closing aperture stop slightly farther will result in darkfield imaging.

  i. As you screw the mask on, you should see the black dot superposed on the filament image on your BFP camera (Fig. 7). If the mask is well centered in its SM1 tube, then the dot image will not rotate much as you screw it on, but stay in the same spot in the image.

 ii. If it moves around, shifting more than about 1/5 of its radius as you turn it, then:

   1. Use the SM1 spanner to slightly loosen the retaining ring holding the mask in the SM1 tube.

   2. Hold the tube so that the glass plate is horizontal, and then gently shake the tube to help center the optic.

   3. Retighten the SM1 retaining ring using the spanner

   4. Screw the mask on again and see if it is better centered.

   5. If not, repeat these steps. This may take a couple of tries to get right.

 e. Once the mask is reasonably centered and screwed all the way on, close down the aperture stop to see that it closes down evenly on the dot (Fig. 7, above).

  i. If it is still (only slightly) off, then:

   1. Rotate the SM1 tube to center the dot *vertically* in the aperture stop (see Fig. 8).

   2. Adjust the angle of the condenser or objective to center the dot *horizontally* in the aperture stop.

Rotate Tube 90°

Slightly Adjust Condenser or Objective Angle to Center Dot in Aperture Stop
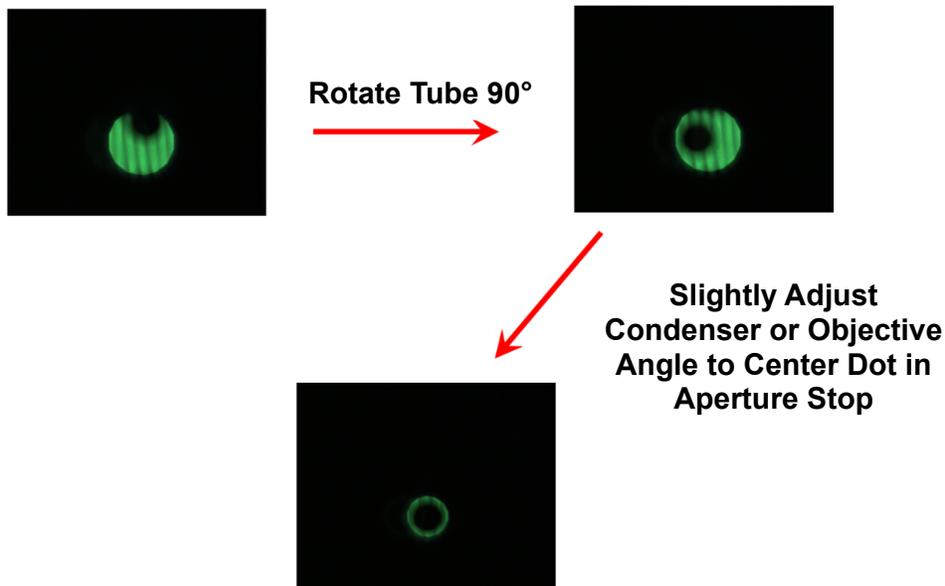
Figure 8: Rotate SM1 mounting tube to center dot vertically in aperture stop; then (slightly) adjust condenser or objective (try the condenser first) angle to center dot horizontally. Aperture stop should close evenly down on dot once alignment is complete.

48. Darkfield imaging: once your zero-order mask is aligned,

   a. Optimize your exposure and ND filters for good imaging.

   b. **Take an image and save it; the image should be centered on the USAF target.**

   c. **Why does this image look better than the one you did in the Lab 5 using the balldriver? What is different?**

VII.   Clean Up

49. Turn off your halogen light so it has time to cool before you put everything away.

50. **Carefully put away your resolution target in its special box.**

51. **Carefully disconnect the USB cable from your camera(s) by gently pulling the connector straight out.**

52. Screw a lens cap on over your camera tube lens (and onto the color camera too).

53. Remove the zero-order mask from the back of the objective (if you have not already), and store it properly.

54. Close the iris on the back of the objective (not super tight – just close it to keep dust out).

55. Put your optical breadboard system away.

56. Copy your files off the computer onto your USB drive or cloud storage (remember – it is your responsibility to keep copies of your files!).

57. **Shut down** (not just "sleep") the computer.

Lab 6 Write-Up:

Basic Details:

o   Turn in your write-up as a Word document, due date, etc. as specified by your instructor.

o   **Number your pages "page X of Y" and have your name in the footer so it is clear if a page is missing if it gets printed out.**

In Microsoft Word (or whatever program you use) be sure to compress all your images before turning in the file; otherwise the files will be too large.

o   After appropriately cropping and sizing images – not covering the whole page, not so small one cannot see the features discussed – reduce the image size so the document is not huge. In MS Office as of this writing, you do this by clicking on a picture, choosing the "Format" tab in the top menu bar, and then choosing "compress pictures" on the format menu toolbar. For submitting your assignment, click save and save-as-PDF. Be sure to check that the compression and the PDF conversion have not spoiled some feature in an image you were trying to point out in your lab write-up – this should not be an issue if you cropped and sized your images appropriately. If there is a problem, send the larger document so the image information is preserved.

Write-Up:

Conjugate Planes (Part III)

1.   Show your pictures of the BFP and sample plane, with Abbe, the filament, and the aperture stop shown in the BFP image and Abbe's name, a resolution target feature, and the field stop in the other image.

    a.   Comment on why you can image both Abbe and his name without one badly interfering with /overlapping the other.

Abbe Theory (Part IV)

2.   Show one image of the 200 lp/mm Ronchi grating sample, and all three images of the corresponding BFP (one in red, blue, and green light using the Roscolux filters).

    a.   Measure the distance from the 0th-order spot to the 1st-order spots for each color. (With a ruler on the screen is OK, or you could measure in pixels.)

    b.   Include your spectra and note the center/main wavelengths for the red, green, and blue filters. Explain how these wavelengths relate to the distances you measured.

    c.   Plot the relative change in wavelengths vs. the relative change in spot spacing. Does this match up as you would expect? Be quantitative about this.

3.   The lab instructions (Part IV, Step 34) asked you to do the following things. Please include your images from this section, along with explanation of what was happening and why the images looked as they did. **Also explain, in terms of what you saw here, why (for a fixed objective NA) one gets better resolution in green light than red light.**

    a.   *With the red filter paper in the illumination path, optimize your cameras for viewing. Take pictures with both cameras.*

    b.   *Close down your objective back aperture until you have just blocked out the red diffracted beams in the BFP. Take pictures with both cameras.*

    c.   *Remove the red filter, flip in your green filter, and optimize settings for both cameras if necessary. Take pictures with both cameras.*

4. Paste in your images of the interdigitated lines from Part 4, steps 35 - 38 ("Line Spacing II").

    a. Explain what happened to the sample image, and why, when you closed down the BFP iris.

Spatial Filtering in the BFP (Part V)

5. Show your images of the grid sample, both the sample itself and the BFP.

6. Show your images (of both the sample and BFP) when you have used aluminum foil to block parts of the back aperture, leaving only a horizontal or vertical line of spots.

    a. Comment, briefly but accurately, as to why you see what you do in these images of the sample, despite the fact that the sample itself remains the same throughout.

7. Explain why what you saw when blocking all but a diagonal row of spots is different than what happens in Prof. Evennett's demonstration in the video "Abbe's Experiments Part 5 – Interference and Coherence of Beams" (See www.thorlabs.com/OMC for the links to the videos.) Explain (possibly using a diagram) why he got one result and you got a different one, noting especially what is different in what you did vs. what he did, and what is different about the spots in your back focal plane (if anything).

Lab 7 Notes:

The Abbe Theory of Image Formation (II)

Optical Microscopy Course

Overview

So far we have focused on issues of ***resolution***; during Lab 7 we will move beyond that to look at how resolution and **_contrast_** play together in terms of image quality and what features can be seen (and how effectively). To do this, we will examine what happens to the contrast of samples containing different spatial frequencies (i.e., different line spacings); the resulting function is known as the Modulation Transfer Function (MTF) and is often used to characterize optical systems in a more informative way than simply stating the one-number resolution. The MTF is strongly affected by how the illumination of the sample is done, so we will also look at that. This falls under the name of "coherence;" you have already seen it at work in one of the early labs, where decreasing the coherence of the illumination reduced the number of fringes visible at the edges of the features of the resolution target.

Lab 7 Reading & Preparation:

1. Lab 7 Lab Notes (this document).

2. Lab 7 Course Notes.

3. (Optional): Volkmann, "Ernst Abbe and His Work," Applied Optics, v. 5, n. 11, Nov. 1966, pp. 1720-1731.

Lab 7 Tasks

I. Coherent and Incoherent MTF

It is important to understand where the MTF (Modulation Transfer Function) really comes from: it is just the contrast you see in your image of a sine wave sample at a given spatial frequency (spacing between the lines). Since it is hard (and hence very expensive) to deposit chrome lines with a sine-wave transmission profile onto glass, we will do this with lined-gratings (Ronchi rulings) as you have used before.

> **NOTE**: Of course, because the Ronchi rulings are essentially a square-wave transmission function, they will produce multiple spots rather than just one in the center and two on either side that a true sine-wave sample would, but for simplicity we will ignore that (it can be accounted for formally; see the note on "CTF vs. MTF" at the end of this lab's notes, after the "Write-Up" section).

We will start by looking at contrast using Ronchi rulings with the aperture stop open and closed, both to get see how it can be done and to see how changing the aperture stop size affects the MTF. After that we will examine another method (faster, but slightly less intuitive) for getting the same information.

Ronchi Rulings

Reference Black; Edge
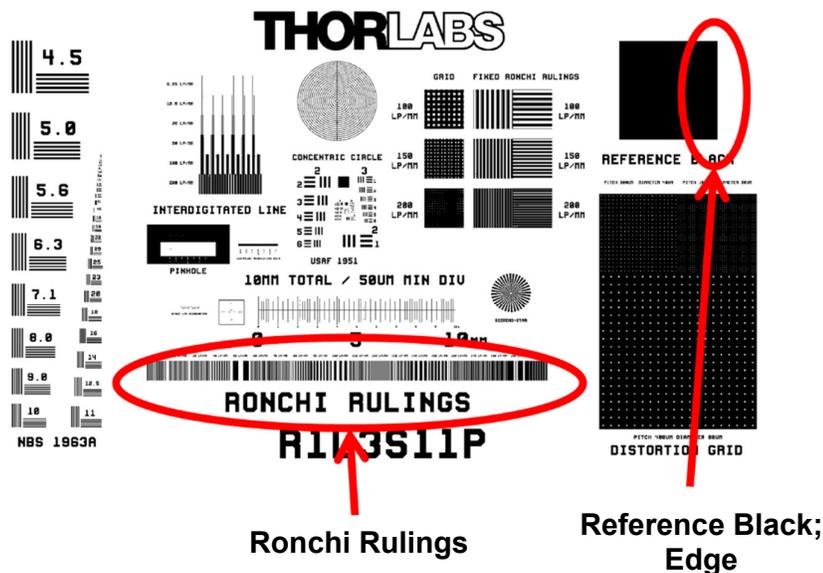
Figure 1: Location of Ronchi rulings on the resolution target. Largest line spacing (10 lp/mm) is on the far left; smallest (200 lp/mm) is on the far right. The 'Reference Black' area is also shown; use the edge indicated for finding the MTF using the edge-spread function.

Incoherent MTF

1. Set up Köhler imaging using the resolution target.

    a. Flip your green filter into the light path.

    b. Remove the zero-order mask from the back of your objective, if it is present.

2. Verify that the aperture stop (AS) and objective BFP iris are concentric (i.e. properly aligned):

    a. Alternately close down the AS and the objective BFP to make sure they start to clip each other equally on both sides (left and right; there is nothing you can do about the vertical on this system).

    b. If they are not concentric (e.g. if the AS starts cutting off the BFP iris on one side before the other) then either the condenser or objective is at the wrong angle. If they need adjustment, either:

        i. Put post collars on the condenser and objective, and then tap the sides of either or both of lens tubes (start with the one that looks least parallel to the optical rails, when you look down on them) with a balldriver to turn them a bit and superpose the images of the AS and BFP.

        ii. A better/more thorough (but also more time consuming) approach is to:

        Remove the objective rail carrier, with the objective still mounted in it, and check its height and alignment against the monochrome camera. Slide the objective up against the lens tube of the monochrome camera so that the edges are flush with each other; this will force both to be aligned along the rail. Lock down the objective height and tighten it in the rail carrier, then remove the entire rail carrier and put it back near the sample stage.

        Repeat this for the condenser (and afterwards adjust its position so the field stop is in focus).

        This approach will usually get you very close to perfect alignment, such that only small adjustments are necessary to get the AS and BFP iris images to be concentric.

    c. **Remember**: the field stop (FS) should also be centered on the monochrome camera after you have done this. If the objective and condenser are parallel to the optical rails, the AS will superpose on the BFP <u>and</u> the FS will image to the center of the monochrome camera all at the same time. Note: use "Fit to Screen" in the camera window to see if the FS is in the center.

3. Once that is done, set up Köhler illumination, using (as a sample) the right side of the Ronchi rulings, with the 200 lp/mm line spacing. Open the BFP iris to 7.5 mm and keep the AS closed down small.

    a. Make sure you have a line profile window open, so you can see the modulation in the image.

    b. Make sure your sample stage is perpendicular to the optical axis – if it is not, your line profile will not show steady oscillations in intensity, but instead have additional, large oscillations visible (going from large amplitude to almost zero; see Fig. 2) as the lines come in and out of focus (with secondary foci due to the Talbot effect – these are most noticeable when using coherent illumination). Adjust your sample stage angle until these are

Note that the lp/mm value of the Ronchi rulings is printed on the target right above the lines.

Lab 7 Notes: Abbe Theory of Image Formation (II)

minimized and you get (fairly) steady pixel value oscillations all the way across your line profile window.

Oscillation   **Corresponding Out of Focus Area**
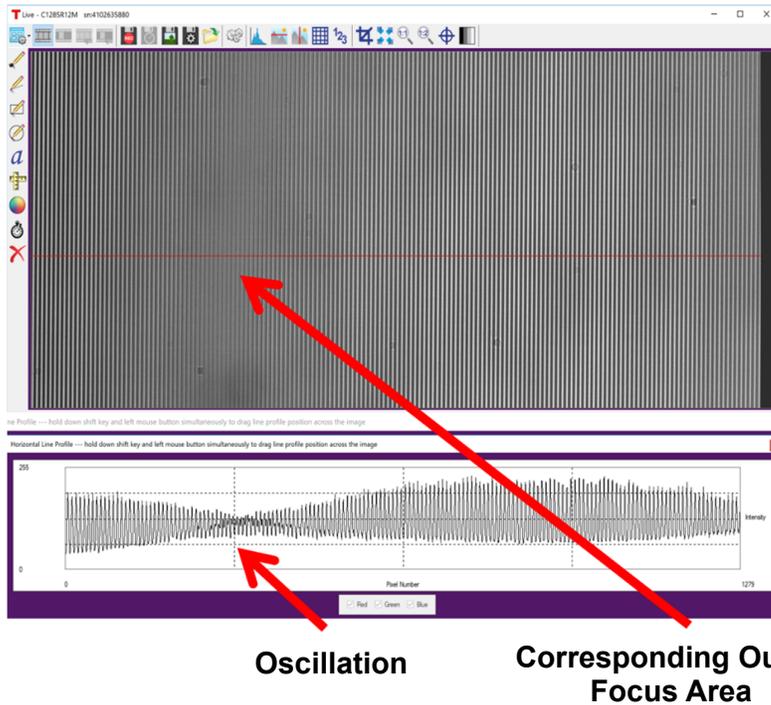
Figure 2: Focus gradient. A 200 lp/mm Ronchi sample with a focus gradient – i.e., the sample is tilted with respect to the optic axis so that the lines go out of focus. Normally they would just get fuzzy, but due to the Talbot effect (an artifact of the very periodic nature of the sample), there are multiple secondary foci, and it looks like the sample comes in and out of focus. Adjust the angle of the stage so this effect is minimized (try to get no minima, or only a slow change over the width of the image). Best focus is when the amplitude (top-to-bottom distance) of the peaks in the line profile is maximized.

> **Note:** There is dirt on the target in the image above. Keep yours cleaner! This dirt is on the back (which is why it is out of focus); probably from putting it down on a surface instead of in its special case. The back could be wiped to clean it; NEVER wipe the front!

4. Open the AS up wide, and then reduce your objective back focal plane (BFP) iris such that the lines are just barely visible / modulation is very slight in the line profile.

    a. This will likely be a BFP diameter of about 3.0 mm.

        i. This may vary a bit depending on whether your iris has gotten out of calibration.

    b. **Record the BFP value you use.**

    c. You will have to turn up the exposure as you reduce the BFP diameter in order to keep your image bright enough to see the contrast.

Remember!
NEVER wipe the front of a grating target – you can severely damage it.

5. Looking on your color BFP camera, adjust your AS size so that it is exactly the same size as the objective BFP diameter.

    a. Reduce the AS diameter until you see the edges of the AS occlude the edges of the image of the BFP iris, then open it back up so it just hits and gets slightly larger than the side edges of the objective aperture (do not worry as much about vertical alignment, which we cannot adjust here anyway).

    b. Now your objective NA and illumination (condenser NA) are the same.

6. Move the target so that you are imaging the 10 lp/mm lines (at the far left of the set).

7. Adjust your ND filters and exposure settings so that your camera dynamic range is used fully, without saturating.

    a. Use your green and IR filters.

    b. Make sure you use the lowest exposure possible – i.e., do not have a large value ND filter in the optical path while you have the exposure up (this is general advice, not specific to this lab).

        i. If you are exposure-limited by the software, you can get shorter exposures by turning up the pixel clock and then the frame rate (in that order) on the camera Settings menu. Note: beware of dropped frames when you exceed the USB bandwidth of your computer; if dropped frames are reported, or the camera is not working (no live image), turn the pixel clock down until it works again.

8. **Focus on the grating and save an image (under file/save) in TIF format.**

9. Repeat this at least 7 times, moving across the gratings, ending with an image of the narrowest line-spacing Ronchi ruling.

    a. **DO NOT** adjust your camera settings after you move on from the 10 lp/mm grating. **DO** refocus using the stage micrometer before taking each image.

    b. You do not need to image every set of lines; only 7+ sets, evenly spaced so you can get a sense of the shape of the MTF. E.g., do at least 10, 40, 70, 100, 130, 160, 190 lp/mm. More data points produces a nicer plot later on, but is more work.

10. **Keeping all your camera settings the same, take an image (well-focused!) with the sample positioned at the right edge of the "Reference Black" target (see Figure 1) so the edge runs through the middle of your image.**

    a. **The point of this image is to get a background reading (measure of the amount of light leaking in everywhere, even in the black areas of your sample – mostly from the room lights), which you can subtract from all your other images later.**

    b. You will also use this for finding the MTF by a different method, so being in good focus is important.

Coherent MTF

11. **BE SURE the aperture stop iris opening is concentric with the (larger) objective BFP iris.**

    a. **Close the AS all the way down; DO NOT adjust the objective BFP (which should still be at about 3.0 mm.)**

b. **Focus on a set of small Ronchi lines (say, the 80 lp/mm set; use the line profile amplitude to tell when you have the best focus).**

c. It is fine to change the camera settings to optimize your exposure.

12. **Repeat steps 6-10, but now keeping the condenser aperture closed all the way down.**

   a. **You may not need to adjust your focus or camera setting during these steps.**

   b. **Be sure to save all your images, titled so you can figure out what they are and use them for your lab report later.**

   c. **During this part of the lab, pay attention to the link between the line spacing and the distance of the diffracted orders from the center of the BFP camera image. What is the relationship?**

13. Using a relatively low line spacing (e.g. 60 or 70 lp/mm), but small enough spacing that you can see several diffracted spots in the BFP (with your green filter in), use a balldriver to block out the zero order diffraction (the spot in the middle of the BFP).

   a. Optimize camera settings and take a picture; does this remind you at all of darkfield?

   b. **Take a picture and save it.**

   c. **Also take and save a picture *without* blocking anything in the BFP, for later comparison.**

II. Plotting Your Values

◀

> For ***both*** sets of images (those for incoherent illumination, with the condenser aperture open, and those for coherent illumination, with the condenser aperture closed), do the following:

14. Open your "Reference Black" image, define a box in the black area, and use a histogram (Ctrl+H) to find the mean pixel value of the background in your images.

15. Open your images of the different line spacings in ImageJ (you can just drag them all onto the ImageJ bar to do this).

16. Take a line profile through a portion of each image, perpendicular to the sample grating lines.

17. From your line profiles, record for each image:

   a. The intensity of a white area near, but not between, the 3-bar targets (will usually be brighter than right amidst the target lines)

   b. For a given peak and valley in the modulation, the minimum and maximum values.

   c. The spatial frequency is just lp/mm; do not forget to convert units to μm^{-1}.

   d. The line spacing, given by 1/ (lp/mm); again, convert to μm.

18. Calculate the spacing between pixels: use the focal lengths of your objective and your tube lens to get the magnification (be careful!) and then use the fact that your (monochrome) camera pixels are 5.2 μm apart to find the distance between pixels at the sample.

   a. Then measure the spacing between the lines in pixels, convert that to microns, and finally calculate the corresponding spatial frequency in μm^{-1}.

   b. Note: the line spacing you are looking for is from one maximum to the next (or one minimum to the next), **not** the width just of the line, or just of the space between lines. It can be helpful to find the spacing of, say, 10 lines, then divide by 10… that averaging will reduce the uncertainty in your results by the same factor.

19. Subtract your background value from your max and min values, and then calculate the modulation M = [(Max – Min) of the 3-bar line sets)] / [(Max + Min) of the brighter white area just away from the lines and the background. Since you have subtracted the background from everything, "Min" in the denominator should now be zero or close to it.]

20. Plot the modulation on the y-axis vs. the spatial frequencies you calculated earlier on the x-axis.

21. Using the BFP diameter you used for your objective, calculate the NA, and then the maximum spatial frequency you expect to see for both the coherent and incoherent illumination cases (note: the formulae for these are in the "Equations to Memorize" section, Appendix A of the Course Notes).

   a. Does this match your plots?

   b. Do your plots look as you would expect from the Lab 7 Course Notes?

III. The Edge and Line-Spread Functions: An Easier Way to Find the MTF

There is an easier, though less intuitive, way to get the MTF information: we could pick a sample that requires sine waves with ALL spatial frequencies to make it up, take an image of it, and then see what amounts of which spatial frequencies we see in the image. If this sounds simple, it actually is!

In fact, a very thin line is such a sample. However, it is hard to make really thin lines, and they do not scatter that much light; so a trick is used: one uses an *edge* instead. These are easy to make (in many cases one can use the edge of a razor blade), and easy to image since the light on one side is quite bright and on the other very dark where it is blocked by the edge.

To get the equivalent of the image of a line, you just take the derivative of the image of the edge. An edge is a step-function – constant on both sides with a step discontinuity at the edge. If you think about the derivative of that, the derivative of a constant is zero, so it will be zero on either side (for a perfectly sharp edge), and infinite right at the edge transition, a lot like a thin line. Of course, the real image will not be perfectly sharp, so you get a "hump" shape just as you would if you imaged a line. The line profile across the image of the edge is called the "ESF," for Edge Spread Function, but remember: it is just a line profile across the edge. The derivative of this is called the "LSF," or Line Spread Function – because it is what you would get if you imaged a very thin line and took the line profile across it.

Note: If the idea that it takes all frequencies to make a thin line bothers you, consider adding up cosines, $\cos(a\text{ x})$. At x = 0, they will all add up, since $\cos(0)$ = 1 always. If you use all values of a, then at other points x ≠ 0, the cosines will vary between positive, negative, zero, etc., at each point for the different values of a. The more values of a you use, the more the cosines will all cancel out at points x ≠ 0, and the closer you will be to an infinitely sharp line at the location x = 0.

The "hump" in the profile across a line image (the LSF) should remind you of something… the PSF. It is the same concept: the PSF is just the profile across the image of a point source instead of a line.

We can use software to figure out (using an algorithm called the Fast Fourier Transform) how much of which sine or cosine waves are needed to re-create the "hump" shape. This is the Modulation Transfer Function, or MTF.

Practicing engineers and scientists use this technique for figuring the MTF, since it is quick, easy, and you can do it for multiple angles of the line and at different places in the image to see if the MTF (and hence resolution) are different at different places due to, e.g., aberrations. Plus you do not need a lot of expensive Ronchi rulings (line targets.)

To avoid problems related to the alignment of the edge and the pixel pattern on the camera, and the finite spacing of the pixels, it is best to have a small angle (about 10°) between the edge that you image and the camera vertical (the pixel column axis on the camera). As a result, the method is called the "Slanted Edge MTF" in the literature.

Edge Spread Function and MTF

22. Return to the edge of the "Reference Black" target (see Figure 1).

23. Set up Köhler illumination with good exposure, green filter, etc.

24. Open your BFP to 7.5 mm, and use your BFP camera to set the aperture stop so it is very small (but open enough that illumination is not too nonuniform, if you get a problem when you close it too far).

25. If possible, have the room lights off, or low.

26. Take an image and save it as a TIF (focus well and adjust exposure first, of course).

27. Set the aperture stop so it is the same size as the objective BFP. If it is not exact, err on the side of being just a tiny bit larger.

28. Using proper exposure, etc., take and save an image as a TIF.

29. Move your sample 50 μm out of focus (one major division on the micrometer knob – if you were, e.g., at "20," now you will be halfway to "30" or "10".)

    a. Take an image and save it as a TIF.

    b. Repeat this again for

        i. 100 μm out of focus

        ii. 150 μm out of focus

Test your system, in advance of the write-up:

30. Using ImageJ, open your **in-focus image** taken with the aperture stop open (i.e., in incoherent illumination), from Part A above.

31. Draw a box across the edge as shown:
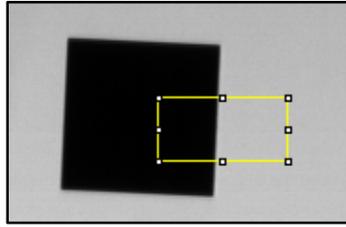
Figure 3: How to draw the box around the edge to use with the SE MTF ImageJ Plugin. Note: the box you draw MUST have the dark side to the left; drawing the box on the other side of the square won't work due to limitations of the software.

32. Run ImageJ and use ImageJ / Plugins / SE MTF 2xNyquist[6] to start the MTF software. Then click on 'Generate MTF' (depending on the software version, you may need to redraw your box (see Fig. 3).

    a. Leave Frequency Units as "Relative (cycles/pixel)".

    b. Ignore the other settings.

    c. Click 'OK".

        i. If nothing happens and the box goes away, click 'Generate MTF' again, draw a bigger box this time, and repeat the steps above.

        ii. Drawing bigger boxes in ImageJ, and using larger sample size (in pixels – say 64 or 128) can make a nicer plot, but you must have a small enough box that does not overlap too much dust, etc.

    d. Close the "SPP Clipboard" window that opens.

33. Examine the next three windows (stacked on top of each other) that have been created:

    a. Make a line profile (using the line tool in ImageJ and Ctrl+K) through the same edge in your image from the resolution target.

        i. Compare this to the ESF Clipboard figure you just created with the MTF software.

            1. Are they similar?

    b. Look at the LSF Clipboard figure.

        i. Do you see how this profile could come from the derivative of the ESF figure?

        ii. How wide is the hump, (from the center to the edge) in pixels?

        iii. Based on that, what is the resolution of your system in microns? Remember: the pixels are spaced 5.2 μm apart horizontally and vertically; also do not forget to factor in the magnification.

        iv. Does this resolution match the Rayleigh resolution you calculate for your system?

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[6] If you cannot find the SE MTF plugin option, ask your instructor. Also see the *Reference Links* tab at www.thorlabs.com/OMC for link.

c. Examine the MTF figure.

    i. Does it look like what you would expect for the incoherent MTF?

    ii. What is the spatial frequency, in μm^{-1}, where it hits zero?

        1. Remember, the pixels are spaced 5.2 μm apart; do not forget to take the magnification into account.

    iii. Calculate the highest spatial frequency you would expect to see from this system (formula is on the 'Equations to Memorize' page).

    iv. Does this match what you get in your image, more or less?

    v. How does the inverse of that number match the resolution you expected (from step 33.b.iii, just a few steps back)?

34. Open your in-focus coherent image, and use ImageJ and SE MTF to take the MTF for it.

    a. Does the MTF look as you would expect? How does it compare to the one you did the hard way, in Part II?

    b. At what spatial frequency does it go to zero? Does that match, roughly, what you would expect based on your Equations to Memorize?

IV. Clean Up

35. Turn off your halogen light so it has time to cool before you put everything away.

36. **Carefully put away your resolution target in its special box.**

37. Screw a lens cap on over your camera tube lens (and onto the color camera too).

38. Close the iris on the back of the objective (not super tight – just close it to keep dust out).

39. Put your optical breadboard system away.

40. Copy your files off the computer onto your USB drive or cloud storage (remember – it is your responsibility to keep copies of your files!).

41. **<u>Shut down</u>** (not just "sleep") the computer.

Lab 7 Write-Up:

Basic Details:

o Turn in your write-up as a Word document, due date, etc. as specified by your instructor.

o **Number your pages "page X of Y" and have your name in the footer so it is clear if a page is missing if it gets printed out.**

o After appropriately cropping and sizing images – not covering the whole page, not so small one cannot see the features discussed – reduce the image size so the document is not huge. In MS Office as of this writing, you do this by clicking on a picture, choosing the "Format" tab in the top menu bar, and then choosing "compress pictures" on the format menu toolbar. For submitting your assignment, click save and save-as-PDF. Be sure to check that the compression and the PDF conversion have not spoiled some feature in an image you were trying to point out in your lab write-up – this should not be an issue if you cropped and sized your images appropriately. If there is a problem, send the larger document so the image information is preserved.

Write-Up:

Modulation Transfer Function (Parts I and II)

For **_both_** sets of Ronchi images (those for incoherent illumination, with the condenser aperture open, and those for coherent illumination, with the condenser aperture closed), do the following:

1. Show your plots for the coherent and incoherent MTFs (these are the plots vs. the spatial frequencies you calculated earlier).

   a. Paste small images from your Ronchi ruling line profiles next to the appropriate data points in your plots (this should help you make the connection between the modulation and how the image looks). Compare your plots to the Figures (9 and 22) in the Lab 7 Course Notes which show the MTF with coherent and incoherent illumination and also the back aperture of the objective. Do you see the connection between the modulation you see in your Ronchi ruling images and the back aperture pictures?

      i. Note: taking small portions of line profile that show the modulation, and cutting and pasting them near the relevant part of your plot is easy to do in PowerPoint or other programs.

2. Using the BFP diameter you used for your objective, calculate the NA, and then the maximum spatial frequency you expect to see. (N.b.: this formula is in the "Equations to Memorize").

   a. Does it match what you expect from your graphs?

Modulation Transfer Function (Part III)

3. For the image you analyzed in steps 33a – c,

   a. Show the ESF (paste it into your write-up) you get from the SE MTF ImageJ plug-in, along with the line profile you did through the edge. Explain why these are similar or different.

      i. Note: make your line profile about the same # of pixels across as the ESF, so the images will be the same size and will be easier to compare

b. Show your LSF, and list the width (in both pixels and μm at the sample) of the "hump".

     i. Show your calculation for the resolution, and discuss how this matches (or does not match) the width of your hump. Comment on how this is linked to the PSF calculations you did in Lab 5.

c. Show your MTFs for the in-focus incoherent and in-focus coherent images

     i. Note in your figure captions what the conversions to cycles / μm (in μm^{-1}) are.

     ii. Comment, with calculations, on how these match what you expect for the system.

4. Plot MTF and LSF images for your incoherent in-focus, and each out-of-focus image.

a. Show these, one under the other, side-by side (MTF on the right, LSF on the left) in order of increasing out-of-focus distance.

b. Make a table listing, vs. out-of-focus distance, the:

     i. Width of the LSF, in pixels and μm

     ii. Cut-off of the MTF (where it goes to zero), in cycles/pixel and cycles / μm.

c. Comment about what happens to the MTF and LSF as your system goes out of focus, and **use this to explain why out-of-focus images look blurry**.

     i. What do you think happens to the PSF?

Building BFP / Spatial-Frequency Intuition

Fast Fourier Transform

1. Using Google Image Search, find an image with several different clear spatial frequencies in it (e.g. a striped shirt or something like that). You will need an image with a decent number of pixels – at least 600 x 800 or bigger.

2. Paste this image into ImageJ.

3. Make the image grayscale by choosing Image/type/8-bit.

4. Use Process / FFT / FFT to get a version of what this sample would look like in the objective BFP.

5. Draw a small-ish circle around the center spots in that "BFP" image, then use Edit / Fill (or use Ctrl+F) to zero them out (compare to Fig. 4, on the next page).

a. If the area does not become black when you use Ctrl+F, you may need to double-click on the "color picker," and on the palette click on the black icon at the lower right. After this, Ctrl+F should fill with black (0's; be careful: a non-zero dark (say "3" value) will not work correctly – if in doubt, double-click on any color in the color picker and then manually set all values to zero). Note: the paintbrush tool will also now paint with black, if you want to get more selective later.

6. Use Process / FFT / Inverse FFT to get a version of what the sample would look like at your imaging camera, if you had blocked out that same area of the BFP.

a. **What does this image remind you of?**

Color Picker Icon

Black Palette Icon

7. Paste these four images into your write-up:

   a. Original image, converted to 8-bit grayscale.

   b. FFT of the image.

   c. FFT with the area you blacked out.

   d. Inverse FFT showing what the resulting image would look like.

   e. **BE SURE to use 'compress images' in your write-up before sending it in!** Otherwise your file will be unreasonably huge.
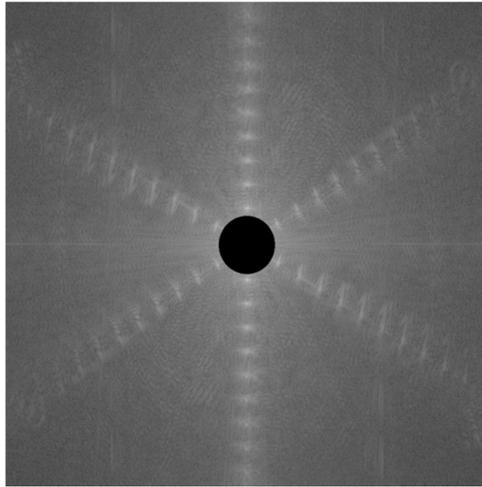
Figure 4: ImageJ FFT of an image, with the center area subsequently blocked out.

Inverse Fast Fourier Transform

8. Close ImageJ, then re-open it to clear any variables.

9. Paste the original image back in and convert to grayscale.

10. Obtain the 'BFP' image using the FFT the same way as in Step 4.

11. Draw a box or circle; use either Shift-Ctrl+X to cut out some sections, or Ctrl+F to blank out some sections. The paintbrush can also work.

12. Obtain the image you would get at your camera using Process/FFT/Inverse FFT.

13. Mess around with the image until you can show some interesting phenomena – certain parts getting emphasized, or some parts (e.g. sleeves, as in a shirt image we worked with) going blank, etc.

14. Paste in a couple of representative examples of your image, what the BFP looked like, what you kept, and what the resulting image would look like.

15. For the image you manipulated (in Step 13, above), explain in basic terms, for things that changed, **why they changed in the way that they did.** I.e., why did blanking out some section of your BFP image result in what you see in the final image?

Note: CTF vs. MTF

You may have wondered whether using a square-wave (Ronchi) grating is acceptable to measure the sine-wave based MTF. The answer is… sort of. As covered in the last two Course Notes, a square wave is made up of a series of sinusoidal frequencies, so we do not expect to get exactly the same results as we would with a sinusoidal grating. The use of a square-wave grating results in something called the "CTF," for Contrast Transfer Function, but do not worry about the name.

One can actually calculate back from the CTF to the MTF (or vice-versa), but few people actually get the MTF this way. The reason anyone does (and why we used Ronchi rulings) is that square wave gratings are *much* cheaper than sine wave gratings.

At higher spatial frequencies, near the limit of the system resolution, the CTF and MTF are essentially the same. Though they are both normalized to one at the origin, at lower spatial frequencies the CTF tends to run a bit higher than the MTF. We will let you think about why that is… consider what happens to diffracted spots in the objective BFP as you get close to the edge of resolution..

Lab 8 Notes:
Contrast Methods and Abbe Theory

Optical Microscopy Course

Overview

In this lab we will explore different contrast mechanisms, building on what we have covered in Labs 6 and 7. In particular, you will set up phase contrast microscopy, and then compare brightfield, darkfield, and phase contrast for different samples. Not surprisingly, different contrast mechanisms are best for different samples; the point of this lab is to introduce you to both the differences and how various samples look using the different techniques.

The samples you will look at include 100 nm polystyrene beads, stained tissue samples, diatoms (which are the glass skeletons of algae), and unstained human cheek epithelial cells. The human cheek epithelial cells are handy samples to know how to make, since you can use them to test your own microscope systems.

This lab is the culmination of the past several labs; after this we will move on to the final contrast mechanism we cover in this course: fluorescence. There are a number of differences in implementation between this lab's techniques and those of fluorescence microscopy, so our discussion will shift a good bit starting with the next lab. Notably, fluorescence is almost never done by itself: it is almost always implemented in combination with one of the techniques covered in this lab. One thing all the techniques share in common, however, is that they are ways of generating contrast from a sample.

Lab 8 Reading & Preparation:

1. Lab 8 Lab Notes (this document).
2. Lab 8 Course Notes.
3. Read **all** of Course Notes Appendix B, "Zernike's phase contrast".

Lab 8 Tasks

I. Darkfield

Brightfield Contrast

1. Set your objective BFP aperture for 7.5 mm.

2. Set up Köhler illumination properly.

   a. This means using your BFP camera to make sure the aperture stop is set to the same size as the objective BFP.

3. Obtain (from the instructor) the bead sample slide.

   a. This is a ThermoFisher Tetraspeck slide, #T14792. Bead sizes in the various positions are:

      i. Position 1: 4.0 μm diameter

      ii. Position 2: 1.0 μm diameter

      iii. Position 3: 0.5 μm diameter

      iv. Position 4: 0.2 μm diameter

      v. Position 5: 0.1 μm diameter

      vi. Position 6: all five sizes mixed together.

4. Focus properly on the slide, and set up proper exposure, etc.

   a. Be sure to have the coverslip side of the slide facing the objective. Imaging through the main (1 mm thick) slide will increase aberrations.

   b. The XY sample stage may not have enough travel to allow you to image at each of the six bead areas; if you cannot reach Position 6 on the slide, then orient the slide such that you can reach Position 1, since the larger beads will be easiest to find.

   c. Start at Position 6, or Position 1. It is easiest to focus first on a printed feature on the slide (e.g. the surrounding edge around the Position 1 area), then shift the stage over to the bead area. The 4 μm diameter beads should be very easy to see in brightfield (though small and point-like in the image), and there should be a large number of them spread over the sample area.

      i. If you have trouble finding beads, check:

         1. Are you imaging the back or front side of the slide, instead of the region under the coverslip where the beads are? To avoid this, focus first on the inked edge surrounding the bead position area – that edge is at approximately the same plane as the beads (under the coverslip).

         2. Are you properly exposed (not over-exposed), and in good Köhler illumination?

         3. Are you accidentally zoomed in? Be sure you are using 1:1 imaging, or "fit to screen" so you can see a large area.

   d. Once you have found some beads, use your mouse-wheel to zoom in on one. A 4 μm bead should actually look round in your image at the right zoom level.

e. Focus back and forth. If the bead stays round, and just goes in and out of focus, your alignment is excellent. If the bead gets elliptical in one direction (say, horizontal) when out of focus on one side, and elliptical in the direction perpendicular to that when focused on the other side, then you are at the very least somewhat misaligned. (This aberration is called *astigmatism*.) This is a sensitive test of your alignment; only a very well-aligned rig will not show some astigmatism at this point.

f. Focus to find the larger beads, which are easier to see. After you have found those, try again in a different region for the smaller beads, which will be harder to see. The small beads – especially the 0.1 or 0.2 µm beads – will be the smallest thing in the image, smaller than most dust particles that may be on the slide.

g. **As you focus back and forth, is the center of the bead always dark?**

5. Once you have the 4 µm beads in good focus,

a. Put a line-profile through your chosen bead. You should be able to roughly estimate the contrast based on the values shown in the line-profile plot.

i. Contrast is defined as $[(I_{max} - I_{min}) / (I_{max} + I_{min})]$

b. Open a Pixel Peek window in the ThorCam software by clicking the icon on the menu bar.

Pixel Peek Icon

i. Position the red cursor over the image point that you want to be the center of the Pixel Peek data table.

1. It is possible to take a screenshot of the Pixel Peek window (if you want one) by clicking the Pixel Peek widow to make it active, followed by positioning (without clicking) your red cursor where you want it in the image. Then use Alt-PrtScn (or Fcn-Alt-PrtScn) to take the screenshot.

ii. If you have your exposure set properly, values around the bead will be at least 225 counts, but less than 255 (the saturating value for this camera), while the (darker) center of the bead will have a lower pixel value. You may have to adjust your exposure to avoid saturation.

1. If you change the zoom while doing this, check the position of your red cursor to make sure the Pixel Peek is still centered where you want it.

2. You can see more pixel values if you drag the Pixel Peek window to be larger.

Pixel Peek				
190	189	173	185	173
178	181	184	180	173
180	169	172	179	179
177	176	175	174	171
175	172	180	179	175
178	179	176	181	175
177	190	180	183	174
180	179	184	178	172
179	181	168	188	186
189	183	186	178	182
182	185	188	173	175

6. **Write down the:**
   a. **Average pixel value <u>around</u> the bead (i.e., the background for the bead image)**
   b. **Lowest pixel value (at the center of the bead)**

7. Save the image as a TIF file so you can refer to it later.

8. Calculate the bead contrast $[(I_{max} - I_{min}) / ((I_{max} + I_{min})]$
   a. This is sometimes known as the "Michelson contrast."
   b. Calculate the signal to background ratio for the bead
   $S/B = [|I_{bead} - I_{background}| / I_{background}]$
   c. Note the absolute value signs – it makes sense to discuss S/B as a positive quantity.
   d. The signal to background ratio is sometimes also known as the "Weber contrast".

9. Look for 1.0 µm beads in Position 2. These may be very hard to see; if you cannot find them, continue to the next step.
   a. If you do find them (or better yet, any even smaller beads at Position 3 or higher), then zoom, focus, open a Pixel Peek window, and record the value at the bead and well away from the bead as before. Save an image as well.

Darkfield Contrast & Signal-to-Background

10. Close the aperture stop to its smallest value (~1 mm)

11. With the room lights off, or very dim:
    a. Focus on the ink edge next to Position 2 of the bead slide, then shift over to the main bead area.
    b. Set up darkfield imaging (without moving the sample.)
       i. Using your zero-order mask will probably give the best results.
       ii. If you prefer, you could use the smallest size driver possible, or use molded aluminum foil to make a "finger" that reaches into the BFP and blocks the appropriate spot.
          1. If you do this, keep the foil "finger" narrow, and just big enough to block the BFP image of the aperture stop (AS).
       iii. If the beads you are seeing are not spherical at all, but elongated and with many fringes, check that you are not blocking more light than necessary with your driver or tinfoil "finger."

12. If you need to, turn up the exposure so features start to be visible, and then focus so you can find beads.

13. Ideally you will do the next step using beads of 0.5 µm or 0.2 µm diameter (Positions 3 or 4). **It is definitely possible to find beads at these positions**, but it becomes increasingly more difficult as the beads get smaller (and somewhat slide-dependent in terms of which regions on a given slide have well-distributed beads). The smallest (and often dimmest) things in the image are likely to be the beads if you are imaging Position 4 (0.2 µm beads) – you will also see random crud, often very non-spherical looking. Ignore this.
    a. Once you have found beads in Position 2, try to find them at Position 3 or Position 4, and then do the next steps. However, if you cannot find beads at those locations, use Position 2.

You will need the pixel values of the bead for your lab report, so you can find it again in ImageJ.

i. **Record the Position (bead size) you are imaging!**

14. Zoom in on a bead, and with it in good focus (as good as you can get in darkfield),

   a. Place your cursor on the bead.

      i. At the bottom left of the ThorCam camera software window you will see the X, Y pixel position of the current cursor location, as well as the pixel Intensity, I (in counts, from 0 to 255).

      ii. **Record the pixel values (x, y position) for this point (so you can find the bead again later).**

15. Open a Pixel Peek window, and right-click on the bead to center the Pixel Peek window on the bead.

   a. Adjust your exposure and gain settings until the maximum signal from the bead is at least ~ 225 counts (but not saturating!)

16. **Write down the:**

   a. **Average pixel value from the dark background in the vicinity of the bead.**

   b. **Highest pixel value (at the center of the bead).**

17. **Save the image as a TIF.**

18. Calculate the bead contrast $[(I_{max} - I_{min}) / (I_{max} + I_{min})]$

19. Calculate the signal to background ratio for the bead $S/B = |I_{bead} - I_{background}| / I_{background}$

20. <u>Without moving the sample</u>, remove your zero-order mask (or driver), so you are imaging in brightfield again, and adjust the exposure so it is not saturating.

   a. Adjust your focus so you can see the bead optimally.

      i. **Was the bead already in best focus, or did you have to shift the focus when you changed from darkfield to brightfield?**

   b. **Save the image as a TIF.**

   c. As above, use the Pixel Peek window to find the intensity at the bead center, and also a little away from the bead.

      i. Calculate the bead contrast and S/B ratio as before.

         1. How does this compare to the contrast and S/B ratio you got using darkfield?

   How much better is your bead contrast in darkfield versus brightfield? How much better is the signal to background ratio? Why was it important to turn out the lights when taking your darkfield image to measure this quantity?

II. Diatoms, MTF, & Contrast

Brightfield & Darkfield

21. Get a diatom sample slide from the instructor (slide included with the kit). Diatoms are small water-dwelling creatures that make intricate glassy skeletons.

22. Set up proper Köhler illumination to image the diatoms.

23. Set your objective BFP aperture for 7.5 mm.

24. Close the AS down to its smallest diameter (~ 1 mm)

25. Use your zero-order mask (or a driver) to block the illumination in the BFP so you have a darkfield image.

Notice the difference in the images; is the darkfield image harder to interpret? If you can, try to separate the effects of fringing (due to having the aperture stop closed down so far, i.e. coherent illumination) and what is due to the darkfield technique. It may be worth looking at more than one diatom type in the sample to get a feel for this.

Aperture Stop and Resolution

Here you will be looking for an example of the increasing resolution which can be achieved by opening the aperture stop (commensurate with the $NA_{condenser}$ term in the resolution formula). You can go about this any way you want; one way to do it is as follows:

26. Set your objective BFP to 7.5 mm, and your aperture stop set to match the objective NA (you will need to use your BFP camera to check this),

27. Find a diatom with a lot of small internal structure.

28. Close your aperture stop down.

    a. It may be helpful to set the exposure to "Auto" in the checkbox under the "Exposure" slider on the Camera Settings menu. That way when you adjust the AS you will not have to keep changing the exposure.

29. If you see a substantial resolution change by opening and closing the aperture stop, then:

    a. **Take images with the aperture stop both open and closed.**

Save the images as TIFs.

30. If you do <u>not</u> see a substantial change in the diatom, either look for a different diatom to try, or:

    a. Close down the aperture stop to its smallest value.

    b. Adjust your objective BFP until the internal structure of your chosen diatoms is no longer easily visible.

    c. Open your aperture stop to the same size as the objective BFP.

    d. If the internal structure of the diatom is not reasonably visible now, you need to find another diatom with different structure and try again. **The effect is very noticeable for the right diatoms.**

31. Uncheck the "Auto" exposure box so it does not cause you trouble later.

Aperture Stop and MTF

Now you will look for examples among the different diatoms of cases where a given set of features look better with the aperture stop closed somewhat (not necessarily all the way), and others where you need the full condenser aperture. Full condenser aperture means with the aperture stop set to the same NA as the objective BFP, i.e. so it appears the same size as the BFP iris on the BFP camera.

Good microscopists usually set the aperture stop to about ~70% of the size of the objective BFP when setting up Köhler illumination. This generally improves contrast at lower spatial frequencies, at the expense of some resolution at the high-end (explained in the Lab 7 Course Notes). While viewing the sample, the microscopist will then adjust the aperture stop setting to achieve optimal contrast – for samples with a lot of super-fine detail, the AS may need to be fully open; for samples with more detail near the middle of the spatial frequency range the system can image, a smaller AS setting may be

better. A good microscopist adjusts the aperture stop fairly often, at least when doing brightfield microscopy on changing samples.

32. Look around in your diatom sample for diatoms which have extremely fine structure along with some medium scale structure.

33. Adjust your AS back and forth to see if the diatom you are looking at shows better contrast on the medium scale structure with the AS slightly closed, even if you do not actually lose the ability to see the fine structure.

    a. What you are looking for is a case where your view of the fine structure is improved with the aperture stop slightly closed.

    b. Do not spend more than 20 minutes on this task; if you are unable to find good examples, move on to the next task.

34. **When you find a good example, save images (as TIFs):**

    a. **With the aperture stop fully open.**

        i. **Note the approximate aperture stop size for your lab report.**

    b. **With the aperture stop adjusted for best contrast.**

        i. **Note the approximate aperture stop size for your lab report.**

III. Absorbing (Stained) Sample

35. Get a stained tissue section sample (e.g. Carolina Biosciences H&E stained Histology slide) from the instructor.

36. Set your objective BFP diameter to 7.5 mm.

    a. Take a properly exposed image.

    b. **Save it as a TIF.**

37. Use your zero-order mask (or a balldriver) to set up darkfield.

    a. Take a properly exposed image.

    b. **Save it as a TIF.**

        i. **Is darkfield a helpful technique for looking at stained samples like this?**

38. Feel free to explore different samples.

IV. Phase Contrast

Here you will set up phase contrast, and investigate how phase imaging compares to brightfield and darkfield, as well as a few traits (e.g. halo effects) characteristic of phase imaging.

Setup

39. Put in your resolution target and image the smallest set of the USAF target bars.

40. Set the BFP to 7.5 mm and the AS open so that it matches the BFP (based on the BFP camera image).

41. Set exposure correctly, then focus slowly. Carefully note how far you can move the focus knob between when the bars are just starting to get fuzzy on one side of focus until they are just starting to get fuzzy again after you go through best focus.

    a. Each tiny division on the micrometer rotating barrel is 10 μm. How many μm is it between times when the sample is starting to be out of focus?

b. This is the "depth of field." Calculate whether it matches the formula you know from the "Equations to Memorize" sheet.

42. Remove your achromat objective.

43. Get the 10X, 0.25 NA phase objective. Note the markings on the objective barrel; it is worth learning how to read these:

   a. 10X / 0.25: gives 10X magnification when used with the lens it was designed to go with / the numerical aperture is 0.25

   b. Ph1 ADL: it is a phase objective, and there is a "#1 phase ring" you should use in the aperture stop of the microscope when you use it. We will fake it with aluminum foil.

      i. ADL is more technical, and you can ignore it. For reference, it stands for "Apodized Dark Low"; this means the objective has additional apodization (the extra lighter rings in the BFP; see Fig 1) to help reduce the typical "halos" seen in phase images, and is designed to produce darker features when there is a phase delay (by choosing a 180° different phase shift for the ring, you could get features to be bright instead of dark; there are two choices in phase contrast, and this one is 'dark'). It also means it is designed for relatively low contrast, which is to say that the rings do not block that much light – they do not block as much of the main illumination, so the background is still fairly bright.

   c. ∞ / 1.2: the objective is designed to form its image at infinity when properly focused. This means we also want our camera focused at infinity, as it already is. 1.2 means the objective is designed to look through 1.2 mm of glass, and will not give the best performance unless it is doing so. This means that for this objective it might be best to image through the thickness of the slide, and have the sample on the condenser side, but it will not matter that much for us. More common is a "0.17," since many microscope coverslips are 0.17 mm thick.

   d. WD 6.2: the so-called "working distance" – the end of the objective housing should be 6.2 mm away from the sample.

44. Try holding the objective up to the room lights, look into the back, and see if you can see the phase rings.

45. Mount the objective onto a lens mount and post (using the M25 thread adapter ring), and set it to be the same height as the sample camera (in the usual way – slide it flush against the camera and set the mount to be the same height, and mark it with a post collar).

46. Mount the objective, and get it aligned.

   a. The rail carrier will need to be farther away from the sample stage, as this objective is longer than the previous one.

   b. This may be easiest to do by closing down the field stop (FS), setting the sample camera to "Fit to Screen" so you can see the entire image, and then adjusting the objective height and angle until the image of the FS is centered. The final height may be slightly different than you had set from matching the camera originally; this is OK.

   c. Slide the rail carrier until the sample (and field stop) are in reasonable focus, then fine-focus with the stage micrometer.

47. Adjust the position of the rail carrier holding the BFP camera, moving the BFP camera back and forth to get the BFP of the 10X objective in best focus (because you changed objectives, the location of the BFP is now different). You will need the image to be properly exposed to get the focus correct.

48. Check (using the BFP camera image) that when you close down the AS it closes evenly on the rings visible in the BFP of the objective (see Fig. 1).
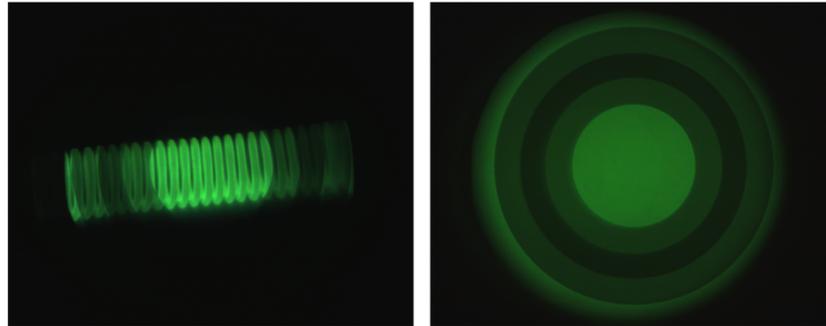
Figure 1: Phase rings in BFP of objective. Left: standard image. Right: with a diffuser (a Kimwipe, held just in front of the field stop) to aid in filling the entire AS with light. The darkest ring is the phase ring; the lighter rings inside and outside the main dark ring are "apodizing" rings, designed to help reduce the "halo" effect typical of phase contrast images. For our purposes, these gray apodizing rings can be ignored.

49. Depth of field (DOF): notice how much shorter the DOF is with this objective than the homebuilt achromat – it will probably be hard to measure well, but rack back and forth slowly through the focus a few times to see how quickly it changes.

    a. **Why is the DOF smaller for this objective?**

Cheek Epithelial Cell Sample

Cheek epithelial cells are a great sample either for demonstrations or for testing your optical setup (they have small features at the limit of the resolution of even the best systems). Knowing how to make these cheek cell samples can be useful to you later.

> **NOTE**: If for some reason you cannot do the cheek epithelial cells, then use the Plankton slide included with the kit (look for plankton in your slide without a lot of dark chlorophyll in it; the samples vary and you want a clear object if you are trying to use phase contrast). Otherwise, the plankton is also fun to look at, after you have done the cheek epithelial cells.

50. Get a clean glass slide.

51. With a permanent marker, make an "X" on the slide.

52. Using a (clean!) 1 mL pipette tip, coffee stirrer, or other plastic stick:

    a. Rub the inside of your cheek a little bit

53. Rub the stick gently on the slide near the "X" you drew (putting the cells on the same side of the slide as the ink "X".)

54. Ideally you want a little bit (size of a small drop) of saliva on the slide to keep the cells hydrated.

55. Quickly place a coverslip on top of the drop of saliva.

56. Seal the edges of the coverslip with clear nail polish.

    a. Never do this with cells you care about; the acetone in the nail polish is not good for them. You would use wax or grease if it mattered.

57. Place the slide flat for a minute or two so the cells can settle out and the nail polish can dry.

Phase Contrast Imaging

58. Set the field stop so it is just outside the field of view of the camera (i.e., open it up until the field stop is just out of sight.)

59. On the BFP camera get a good image of the BFP, properly exposed.

60. Close down the aperture stop until it blocks everything outside the main (darkest) phase ring.

    a. There should be nothing outside the dark ring, but you do not want to block any more of the main ring itself than absolutely necessary.

    b. See Fig. 1 if you are unsure what the rings look like – on your BFP camera you should see something like the left side of Fig. 1, prior to starting to close down the aperture stop.

61. Make a strip of aluminum foil that is 9.0 mm wide.

    a. The easiest way to do this is to cut a wider strip, then fold over one edge to get it very sharp, then hold it over a ruler while folding the other edge to get the strip sharp on both sides and the correct width.

62. While watching your BFP camera, gently tape this strip such that it crosses the aperture stop in a way such that it blocks all the light except for what goes through the main dark phase ring.

    a. Tuck it in so it is close to the aperture stop iris.

    b. **All that should remain visible in the BFP camera is two strips of the main phase ring, one on either side of the blocked area.**

    c. If there is still light coming through inside the main dark ring, you may need to make a slightly wider foil strip.

Of course, without touching the iris with your fingers!

Figure 2: Aluminum foil strip blocking center of the aperture stop.

63. Mount your sample on the stage, find an epithelial cell, and focus properly on it.

    a. Sometimes it is easier to focus first on the ink "X" you drew on the slide. That is why we had you draw it!

    b. Properly adjust your exposure.

        i. You may need to use high Gain (under the Image tab on the Camera Settings menu) as well a long exposure in order to be able to see things properly.

        ii. You will want to use a line-scan through the cell to make sure you are not overexposing any part of it.

    c. **Take image and save it as a TIF.**

64. Look in your sample for a good example of "halos" around the cell(s).

    a. **Expose properly, take image, and save it as a TIF.**

65. Remove the aluminum-foil strip from the aperture stop, so that you have brightfield contrast.

66. Without changing anything else:

    a. **Properly adjust your exposure, take an image, and save it as a TIF.**

67. Adjust your focus slightly, and notice the following:

    a. As you move the focus in either direction, contrast should actually ***improve*** a little.

        i. This is important: for clear samples, you can usually see them better when you are slightly out of focus (being out of focus introduces some phase changes in the light at the image plane, which generates phase contrast like effects). As a result, if you can see a clear sample and brightfield, you are probably slightly away from the plane of best focus. [This is discussed nicely by Zernike; see Course Notes Appendix B for the reference to his article.]

    b. If you watch the nuclei or other dense features of the cells, they will be dark on one side of the plane of best focus, and light on the other side. This is a general characteristic of objects on either side of the plane of best focus – you can see the same thing looking at the image of sub-resolution beads. Sometimes one can use this transition to help find the plane of best focus: it is right in the middle of the transition.

68. Close down the aperture stop as small as it will go (~1 mm).

69. Using a balldriver (the zero-order mask will not work well with this objective), block the illumination in the back of the objective so you get a dark field image of the same cell.

    a. You will need a larger driver than before, since the focal length of the objective (and hence the magnification from the AS to the BFP) is a bit different. (For the same reason, the zero-order mask may be the wrong size for this objective.)

    b. You may need to use high Gain (under the Image tab on the Camera Settings menu) as well as a long exposure in order to be able to see things properly.

    c. **Expose properly, take image, save it as a TIF.**

        i. **How does the dark field image look compared to the phase image?**

DO NOT poke inside the objective with the driver! Just block the illumination.

70. Remove your cheek epithelial cell sample.

71. Turn the Gain back down, if you used it.

72. Set the AS back to the correct size for phase contrast.

73. Put your diatoms sample back in, and focus on it.

    a. Examine some diatoms in brightfield.

    b. Replace the aluminum foil strip properly in the AS, so you are imaging the diatoms in phase.

    c. **Do the diatoms look better in brightfield or in phase?**

    d. **Take images of the *same* diatoms in phase and brightfield, and save them as TIFs.**

    e. You can toggle between (roughly) brightfield and phase by lifting or moving the aluminum foil strip in and out of the light path.

V. Clean Up

74. Turn off your halogen light so it has time to cool before you put everything away.

75. **Carefully put away your resolution target in its special box.**

76. Put away any samples; **cheek epithelial cell samples go in the appropriate "glass disposal" box provided by your instructor.**

77. **Carefully disconnect the USB cable from your camera(s) by gently pulling the connector straight out.**

78. Screw a lens cap on over your camera tube lens and condenser.

79. Close the irises on the back of the objective and condenser (not super tight – just close it to keep dust out.)

80. Put your optical breadboard system away.

81. Copy your files off the computer onto your USB drive or cloud storage (remember – it is your responsibility to keep copies of your files!).

82. **<u>Shut down</u>** (not just "sleep") the computer.

Lab 8 Write-Up:

Basic Details:

o Turn in your write-up as a Word document, due date, etc. as specified by your instructor.

o **Number your pages "page X of Y" and have your name in the footer so it is clear if a page is missing if it gets printed out.**

In Microsoft Word (or whatever program you use) be sure to compress all your images before turning in the file; otherwise the files will be too large.

o After appropriately cropping and sizing images – not covering the whole page, not so small one cannot see the features discussed – reduce the image size so the document is not huge. In MS Office as of this writing, you do this by clicking on a picture, choosing the "Format" tab in the top menu bar, and then choosing "compress pictures" on the format menu toolbar. For submitting your assignment, click save and save-as-PDF. Be sure to check that the compression and the PDF conversion have not spoiled some feature in an image you were trying to point out in your lab write-up – this should not be an issue if you cropped and sized your images appropriately. If there is a problem, send the larger document so the image information is preserved.

Write-Up:

Darkfield (Part I)

1. Include images of the 0.2 μm or 0.5 μm beads you took in both brightfield and darkfield.

   a. The images should be cropped so only a single bead is visible – we do not need (or want) the entire image.

   b. This is why it was important to write down the pixel location of the bead that was in best focus; now you can still find it in ImageJ and crop appropriately.

2. For both beads, list the contrast ratio and the signal to background ratio in brightfield and darkfield, along with the pixel values you used to calculate them.

3. Explain why it was especially important to have the room lights off to get the darkfield data. What impact would having the room lights on have on the ratios you calculated?

4. How much better is the signal to background ratio for your darkfield image than for your brightfield image?

Aperture Stop and Resolution (Part II)

5. Paste in your two images of the diatoms taken with the aperture stop open and closed down.

6. Discuss the difference between images, and explain it in terms of the resolution formulae that you know from the "Equations to Memorize" sheet.

Aperture Stop and MTF (Part II)

7. Paste in your images demonstrating better contrast with the aperture stop slightly closed, and when it helped to have the aperture stop all the way open.

8. In a short paragraph, explain (using the concept of the MTF, including curves) why it is that closing down the aperture stop can sometimes be useful. On what sort of samples would it be helpful? On what sort of samples would it be problematic?

Absorbing Sample (Part III)

9. Paste in your two images of the tissue sample taken in brightfield and darkfield.

10. Discuss (briefly) the differences in the contrast between these two images; is one clearly better than the other? In this particular case, is brightfield useful for looking at the sample?

Phase Contrast (Part IV)

11. Paste in your three images showing cheek epithelial cells with:

    a. In-focus phase contrast

    b. In-focus brightfield (i.e., focal plane unchanged from that for phase)

    c. Slightly out-of-focus brightfield

        ➜   Make it easy to compare all three images here. Show the same cell, each cropped out of the larger image, arranged next to one another, with a caption.

    d. **Comment on the differences between images.**

12. Make a figure showing your phase contrast and darkfield images of the same cell (again, cropped out of the larger images).

    a. Comment on the differences between these images.

13. Include a (cropped out) image showing the "halos" you found.

14. Make a figure showing your phase contrast and brightfield images of the diatoms (crop out a single representative diatom from the larger images).

    a. Comment on the differences between these images; is phase contrast particularly useful for diatom samples?

    b. Regarding phase contrast:

        i. Is the phase shift of light going through a diatom small compared to the wavelength?

        ii. Diatoms reflect a lot of light; are they solely phase objects?

Lab 9 Notes: Fluorescence Microscopy

Optical Microscopy Course

Overview

In this lab, you will explore Fluorescence imaging, the last remaining contrast mechanism we will cover in this class, and a tool of immense important to modern biology (recognized in part by the awards of the 2008 and 2014 Nobel Prizes in Chemistry).

Contrast in fluorescence is generated by clever manipulation of light, but not as before where we altered what light passed through the objective BFP. Rather, now we will manipulate which colors of light pass through different parts of the optical system.

Lab 9 Reading & Preparation:

1. Lab 9 Lab Notes (this document).

2. Lab 9 Course Notes.

3. Watch the (short, and quite helpful) video on using the spectrometer software, "OSA Software Quickstart Guide" on the *Videos* tab at www.thorlabs.com/OMC.

4. Optional: Chroma Corp. *Handbook of Optical Filters for Fluorescence Microscopy*, pp. 2 - 9. See the *Reference Links* tab at www.thorlabs.com/OMC for the link.

Lab 9 Tasks

1. Remove the beamsplitter used to direct light to the BFP camera.

2. Set up Köhler illumination, with your aperture stop all the way open and the homebuilt objective with the BFP iris diameter set at 7.5 mm.

3. Set your camera frame rate to 4 fps; this may require reducing the "pixel clock" on the Settings menu.

    a. While you are at it, note that the gain (Settings / Image tab) has a checkbox underneath it for "Gain Boost." This just gives a tad extra gain beyond what you can get with the slider, and you may need all the gain you can get later on, so make a mental note of this checkbox.

4. Reduce the ND filters to 0 (no filter), and flip the green glass filter out of the light path. Remove the sample; in its place, hold a piece of white paper.

    a. **What color is the illumination on the paper? What wavelength is this, approximately?**

5. **Using your spectrometer (Thorlabs Item # CCS200), take a spectrum of the light from the lamp and save it.**

    a. In the interest of time, you can just paste a screenshot of the spectrum into MS Word instead of saving the raw data and then having to plot it separately. This is unusual: for almost all other purposes, it is a good idea to *always* save the raw data.

    b. Be sure the spectrum shows the 500 - 700 nm region well; you may want to rescale the X-axis before taking the screenshot.

    c. You can either grab a screenshot (Alt+PrtScn or Alt+Fn+PrtScn on most computers), or use the Thorlabs OSA / File tab / Save Image button.

6. **In the next step, DO NOT get your fingers on this filter, even with gloves on!**

7. Flip the excitation interference filter (Thorlabs item # FBH520-40) into the light path; this should be in the TRF90 flip mount.

    a. **What color is the illumination on the paper? What wavelength is this, approximately?**

8. **Using your spectrometer, take a spectrum of the light coming through the filter and save it as before.**

    a. Again, be sure the spectrum shows the 500 - 700 nm region well; you may want to rescale the X-axis before taking the screenshot.

9. Insert the pink plastic sample slide in the sample holder.

10. Hold some paper near the objective;

    a. **How much of the color of light (Step 7) that you are using to illuminate the sample is getting through the pink plastic slide?**

11. Remove the paper. Look carefully at the pink plastic slide right where the light hits it from the condenser.

    a. **What color (and thus wavelength) is the light you see, approximately?**

CAUTION! DO NOT get your fingers on this filter! It will ruin it and it costs over $160.

12. **Using your spectrometer, take a spectrum of the light from the slide, right where the illumination hits the slide, and save it as before.**

    a. You will need to get the end of the fiber right in next to the spot of light, aimed at an angle toward the lit spot on the slide so you do not just pick up a reflection from the condenser light (see Fig. 1, below). This requires a bit of messing around, and you may need to turn up the integration time on the spectrometer to get a nice spectrum. **Caution: Do NOT touch the fiber end face to anything or bend the fiber cable in a circle smaller than 24 mm (about 1") – these will damage it!**

    b. Again, be sure the spectrum shows the 500 - 700 nm region well; you may want to rescale the X-axis before taking the screenshot.
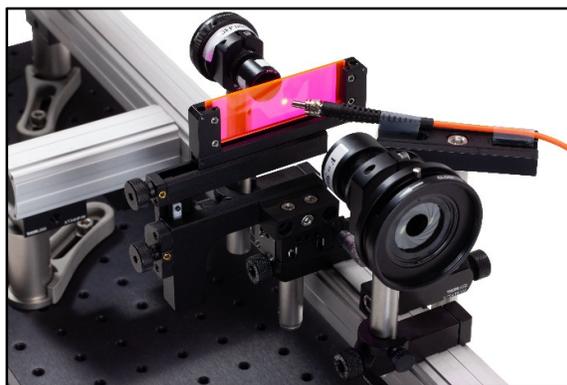
**Figure 1:** Fiber positioning to collect spectrum from illuminated area of pink slide.

13. **Stop now, and consider what you have seen and explain why, in each of steps 4 through 12, you see the color you do. Where does the wavelength in step 12 come from? Show the lab instructor your answers.**

    a. **Illumination light color without filters: why is the light this color? What wavelengths are present?**

    b. **Illumination light color *with* excitation filter: what wavelengths are present?**

    c. **What color illumination light is hitting the pink plastic slide?**

    d. **How much of that color illumination gets *through* the pink slide? Where is the light going?**

    e. **What color light do you see <u>on</u> the pink plastic slide, right where the illumination hits the slide? Explain what you see.**

14. Remove the pink plastic slide.

15. Color a bit of Kimwipe or Thorlabs lens paper (basically thin, fibrous paper) with yellow highlighter and a bit with pink highlighter, leaving a bit of it white as well.

    a. Wait for the Kimwipe to dry (this is important!), then tape it (by the edges) to a glass slide. Between the pieces of tape there should be an area of white (un-colored) Kimwipe, a bit of yellow Kimwipe, and a bit of pink Kimwipe:
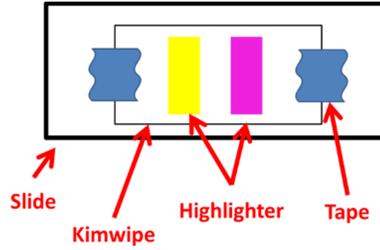
Figure 2: Kimwipe slide, showing highlighter color patches.

16. Insert this slide in the sample holder, and with the green filters flipped out, set up Köhler illumination while imaging the white area of the Kimwipe.

17. **In the next steps, DO NOT get your fingers on the filters, even with gloves on!**

18. Flip out the excitation filter if it is not already out.

19. Get an emission filter (Thorlabs item # FELH0550) from the lab instructor. **DO NOT TOUCH the face of the filter.**

20. **Using your spectrometer, take a spectrum of the lamp light coming through the emission filter.**

    a. Do not let the end of the fiber touch the filter – you do not need to have it close to the filter – hold it at least 1 inch away.

        i. **DO NOT touch the end of the fiber to anything – that messes up the (expensive) fiber face, and in this case will ruin the filter too.**

    b. Again, be sure the spectrum shows the 500 - 700 nm region well; you may want to rescale the X-axis before taking the screenshot.

    c. **Be careful to hold the fiber oriented along the optical axis, and the filter oriented so the light hits the filter face straight on (called "normal incidence").**

        i. **What happens to the spectrum you see if you angle the filter a bit with respect to the light?**

        ii. **Try looking through the filter at the lamp or some other light source; then angle it back and forth so the light hits the filter perpendicularly, and then at different angles.**

            1. **What happens to the colors you see? How does that match the spectral changes you saw in the earlier part of this question?**

                a. It should be obvious why it is important to get your filters mounted at the proper angle (usually perpendicular) to the incident light.
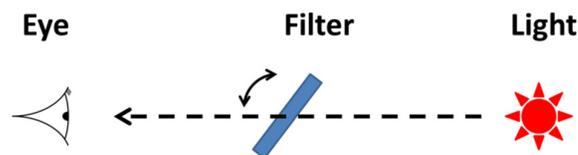
Figure 3: Filter orientation, illustrating how to tilt the filter.

21. Install the FELH0550 emission filter on your sample camera (screw the SM1 lens tube containing it onto the end of the camera lens tube).

22. Imaging the white area of the sample, optimize your camera settings, etc.

   a. **Take an image, and save it as a TIF.**

23. Flip the excitation interference filter into the light path. Optimize imaging, with the condenser aperture at its maximum diameter.

   a. **With the room lights *on*, optimize your camera settings, take an image, and save it as a TIF.**

   b. **With the room lights *off*, optimize your camera settings, take an image, and save it as a TIF.**

      i. **What is the difference between these images?**

      ii. **Explain why there is this difference.**

24. Flip the excitation interference filter out and flip the green VG-9 glass filter in.

   a. **With the room lights *on*, optimize your camera settings, take an image, and save it as a TIF.**

   b. **With the room lights *off*, optimize your camera settings, take an image, and save it as a TIF.**

      i. **What is the difference between these images?**

      ii. **Explain why there is this difference.**

25. **Using your spectrometer, take a spectrum of the light through the green filter and save it as before.**

   a. Again, be sure the spectrum shows the 500 - 700 nm region well; you may want to rescale the X-axis before taking the screenshot.

26. Switch back to the excitation (interference) filter.

27. Repeat Step 23.b while imaging the ***yellow*** area of the Kimwipe sample.

28. **Repeat Step 23.b while imaging the pink area of the Kimwipe sample.**

   a. **What is the difference between this image and the image you took in Step 22?**

   b. **What is the difference between this image and the image you took in Step 23.b?**

   c. **What is the difference between this image and the image you took in Step 27?**

29. Set the objective BFP aperture to 7.5 mm (it should be there already).

30. Change to a 4 μm bead sample (Position 1 or 6 of the ThermoFisher bead slide). Try to image these beads in both brightfield and fluorescence (they are very dim in fluorescence – it may be hard).

   a. It is best to image in brightfield, then shift to fluorescence, since it is easier to focus in brightfield, given the long exposures (and thus low frame rates) you may need to use in fluorescence.

   b. You will absolutely need to do this fluorescence imaging with the room lights off, and probably use very long exposure and high gain. **Be sure to be in focus on the beads in brightfield before you try shifting to fluorescence – if they are out of focus they will be too dim to see.**

   c. How bright are the 4 μm beads in fluorescence?

Step 23.b may be hard. Do the best you can.

d. **Save an image as a TIF; record your gain and exposure settings, as well as which filters you used and whether the room lights were on.**

31. Set the objective BFP aperture to 6.0 mm.

   a. **Adjust your camera settings and image the beads again. Save the image as a TIF.**

   b. Are the beads dimmer?

      i. **Why? Be quantitative.**

32. Change samples to a red-stained tissue slide (Carolina Bioscience Histology slides). The tissue is stained with so-called H&E stain, for <u>h</u>ematoxylin and <u>e</u>osin. Eosin is a reddish dye with a chemical structure similar to the rhodamine dye structure (shown in Fig. 5 of the Lab 9 Course Notes).

   a. Image this slide with the excitation filter flipped out. Get the sample into good focus.

   b. Flip the excitation filter in; readjust your imaging.

      i. You will need to have the room lights off in order to image well.

      ii. You may also need to use the maximum gain setting; do not forget about the extra "Gain Boost" toggle box on the Settings/Image menu.

      iii. It is very hard to focus once you go below 4 fps, so try to stay at 4 fps initially.

      iv. **Take an image and save it as a TIF.**

   c. Try different samples as you wish, and with different imaging settings.

      i. Can you notice a difference between a lower exposure with maximum gain, and a longer exposure with no (or little) gain?

   d. This stain is not actually a "fluorescent" stain. This fluorescence is inadvertent side effect of the red eosin dye. Many things (other than just dyes) fluoresce that you do not expect to; the amino acid Tryptophan is an example. In cells unexpected fluorescence can be a major source of background light, and is one reason you do not want to use excitation if you do not need to (for instance, never use excitation at wavelengths your dye does not excite at – all you will get is increased background).

Clean up:

33. Turn off your halogen light so it has time to cool before you put everything away.

34. **Carefully put away your resolution target in its special box.**

35. **Carefully put away your bead target in its special box.**

36. Carefully put away all other samples in their proper boxes.

37. **Carefully disconnect the USB cable from your camera(s) and spectrometer by gently pulling the connector straight out.**

38. Put away your spectrometer; **be sure to replace the little cap on the end of the fiber.**

39. Screw a lens cap on over your camera tube lens (and onto the color camera too).

40. Close the iris on the back of the objective (not super tight – just close it to keep dust out).

41. Put your optical breadboard system away.

42. Copy your files off the computer onto your USB drive or cloud storage (remember – it is your responsibility to keep copies of your files!).

43. **Shut down** (not just "sleep") the computer.

Lab 9 Appendix: Filter Naming Conventions

- Interference filters are often labeled by their center λ and the width of their transmission band, so e.g. a "600/60" filter would transmit between 570 nm and 630 nm – a 60 nm wide (i.e. ± 30 nm) window centered at 600 nm.

- Often such filters will have an "m" or an "x" attached to them – these stand for e**M**ission and e**X**citation. So a 600/60 m is designed as an emission filter. Be careful about dichroics – their naming conventions vary, and sometimes a "D" in the filter name means the filter is designed to work at 45° light incidence and sometimes it means the filter is designed for 0° ("normal" incidence)… and filters will not work properly at the wrong angle. Beyond this, every company uses different letters to signify different filter properties, so you usually have to look it up in the product's specifications.

  Note: Although it might seem not to matter whether filters are designed for excitation or emission (and, in theory, it should not), in practice it can. Excitation filters are often constructed slightly differently from emission filters. For excitation filters, it does not matter so much if the filter fluoresces a bit, or the filter is not flat and hence introduces aberrations – after all, this is just in the illumination light path, not the imaging path, and also any fluorescence will be emitted isotropically (in all directions) and so is not that likely to get all the way to the camera. For the emission filter, on the other hand, it matters a lot if the filter is flat, since you are imaging through it. Often the emission filter also is thicker than others, having UV or IR absorbing glass built in that results in even better blocking outside the filter transmission band. Dichroics in an epi-illumination configuration must have especially low "autofluorescence," since they are excited by the illumination and you are imaging through them, so that light would contribute badly to background in your image; in addition, they need to be thin to avoid shifting the image too far sideways (since they are at an angle to the light path, they shift the rays sideways a bit), and (like the emission filter) they must be very flat in order to avoid degrading the image.

- Usually there is a little arrow (→) on an edge of the filter, pointing toward one side. This matters a lot: it is important to have the interference coating facing toward the direction the light is coming from in order to get best performance from the filter – one wants to reflect the bulk of the light from the first surface to reduce the intensity of reflections between the front and back of the filter substrate, as well as any fluorescence generated in the filter substrate by the excitation light. This is similar to the reason it is preferable to have the metalized side of the ND filters facing the light. Filter labeling conventions vary; sometimes the arrow points toward the coated side (at which point you want that side facing the light source), but sometimes it faces away from the coating (so you want the arrow facing away from the source of light). Be careful, and check the specifications.

- For example, **for our filters (from Thorlabs), the arrows are supposed to point in the direction the light should be transmitted through the filter**. This generally means that the arrow faces away from the coating, since you usually want the interference coating to be the first thing the incident light hits.

Figure 4: Bandpass filter (we use this as an excitation filter). The light should go through the filter in the direction of the arrow (note arrow on right in the label, right after the "–40").

Lab 9 Write-Up:

Basic Details:

o Turn in your write-up as a Word document, due date, etc. as specified by your instructor.

o **Number your pages "page X of Y" and have your name in the footer so it is clear if a page is missing if it gets printed out.**

o **Paste in only the relevant portion of an image** – for instance, do not past in the entire image if you are only discussing a small part in the lower left corner; in that case crop the image down to just the area of interest to keep the total image smaller.

o After appropriately cropping and sizing images – not covering the whole page, not so small one cannot see the features discussed – reduce the image size so the document is not huge. In MS Office as of this writing, you do this by clicking on a picture, choosing the "Format" tab in the top menu bar, and then choosing "compress pictures" on the format menu toolbar. For submitting your assignment, click save and save-as-PDF. Be sure to check that the compression and the PDF conversion have not spoiled some feature in an image you were trying to point out in your lab write-up – this should not be an issue if you cropped and sized your images appropriately. If there is a problem, send the larger document so the image information is preserved.

> **NOTE:** This is a very short write-up on purpose, to give you time to work on the upcoming filter-selection problem set.
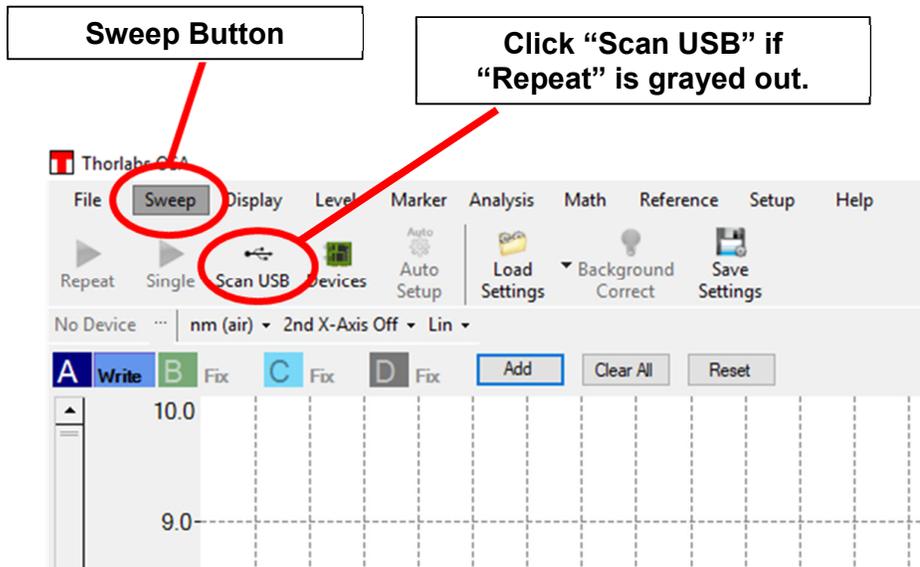>
> Be sure to work on the problem set early, so you have time to use office hours and lab time to ask questions.
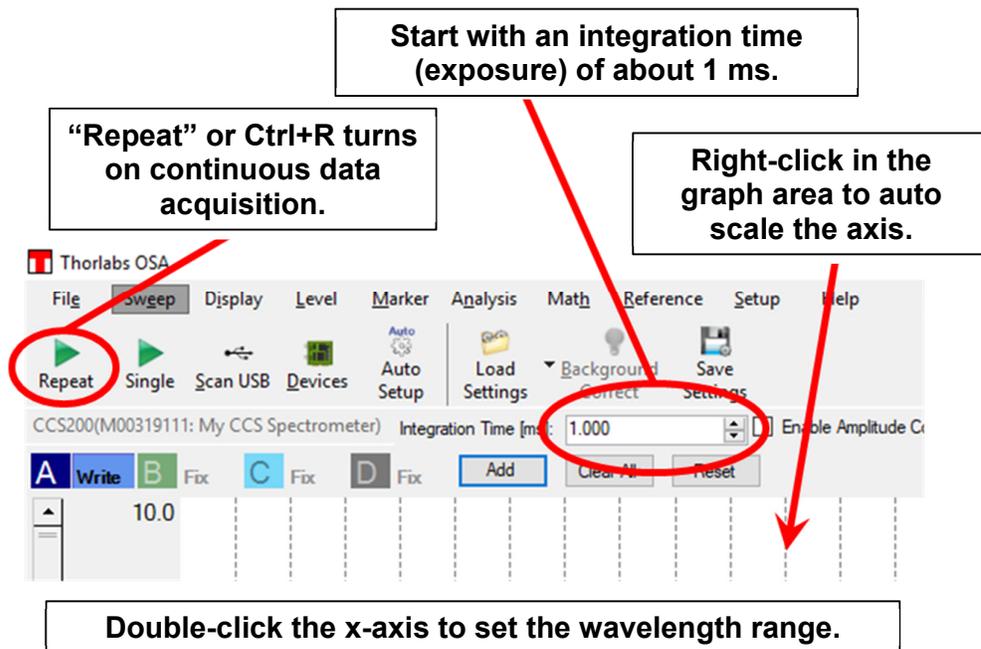
Write-Up:

1. Answer the questions from Step 13.

    a. Paste in your spectra to help with your explanations.

2. Answer the questions from Steps 23, 24, and 28 (you need not get into Step 27; that was for in-class investigation).

    a. Definitely use the spectra you took to help explain the differences in the images, including your spectrum from Step 20.

3. For Steps 30 and 31, explain

    a. Why you could see the beads.

        i. Include your image(s).

    b. Quantitatively, why they got dimmer.

4. For Step 32, include your image.

In Microsoft Word (or whatever program you use) be sure to compress all your images before turning in the file; otherwise the files will be too large.

Thorlabs CCS200 Spectrometer with Thorlabs OSA Software (The Basics)

Sweep Button

Click "Scan USB" if "Repeat" is grayed out.

You may then have to select your spectrometer from a list, depending on whether a different one is attached or has been used.

Start with an integration time (exposure) of about 1 ms.

"Repeat" or Ctrl+R turns on continuous data acquisition.

Right-click in the graph area to auto scale the axis.

**Double-click the x-axis to set the wavelength range.**

Thorlabs CCS200 Spectrometer with Thorlabs OSA Software (Additional Features)

To use the Auto Setup when taking data:
- **Stop acquisition ("Stop" or Ctrl+R).**
- **Click "Auto Setup".**
- **Start acquisition ("Repeat" or Ctrl+R).**

Note: If the light into the spectrometer is very dim, Auto Setup can take a very long time. Only use it if there is a fair amount of light.

Double-click the x-axis to set the wavelength display range.
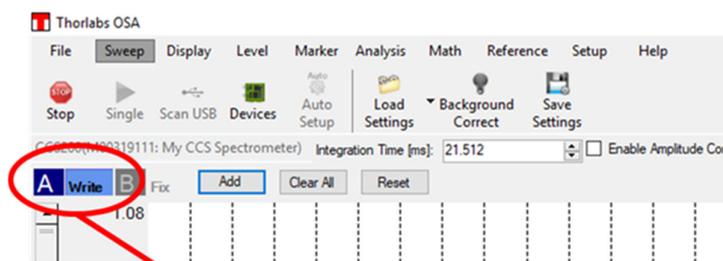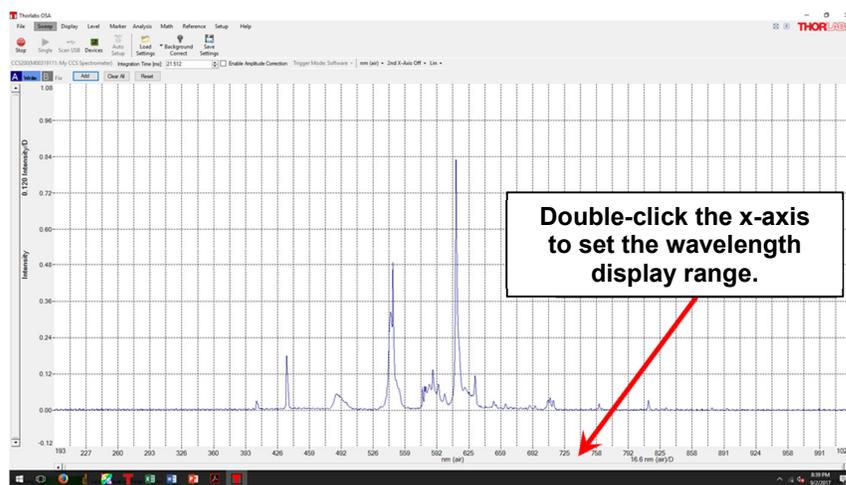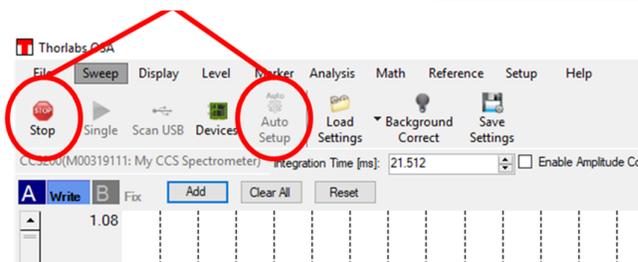
Averaging: Right-click "A" and choose averaging from the menu.

Lab 10 Notes:
Spectra and Filters

**Optical Microscopy
Course**

Overview

This lab involves a deeper exploration of spectra and the data your spectrometer can collect. There are several reasons for this; one is to help you gain more familiarity with spectrometers, one of the workhorse instruments in any optics lab. Another is to help underline the behavior of different filters and an important limitation of interference filters. Lastly (and optionally) the lab investigates the meaning of the Quantum Efficiency of your detectors (and cameras) and how spectral data is actually collected, so that you can collect it yourself when need be, or understand its origin when using it from other sources (e.g. company websites).

Because we want you to focus on the details of filter selection (the Lab 10 Problem Set), this lab will be more self-contained than usual: what you need to know will be outlined below. The connections to the reading in the Lab 10 Course Notes and the Filter Selection problem set should be fairly obvious.

Lab 10 Reading & Preparation:

1. Lab 10 Lab Notes (this document).

2. Lab 10 Course Notes.

3. Read the Chroma Corp. *Handbook of Optical Filters for Fluorescence Microscopy*, pp. 2 - 9. See the *Reference Links* tab at www.thorlabs.com/OMC for the link.

4. Review the screenshots and instructions for the CCS spectrometer (at the end of the Lab 9 Lab Notes).

5. Optional but highly recommended: Watch the (short, and very helpful) "OSA Software QuickStart Guide" video on using the spectrometer software, on the *Videos* tab at www.thorlabs.com/OMC.

Lab 10 Tasks

I. Wavelength Calibration

A common task when using a spectrometer is checking its wavelength calibration. One can buy "calibration lamps," which are gas discharge lamps much like the fluorescent ceiling lights, but with special mixes of gas that provide highly specific, i.e. sharp spectral lines. One takes a spectrum of one of these, and then compares the wavelengths measured with the known wavelengths put out by the lamp, and adjusts the spectrometer (usually in software) so that the wavelengths are reported correctly.

For instance, fluorescent lights are almost always mercury (Hg) based, and the most common ones have fluorescent materials (the white stuff on the bulb, often Terbium and Europium in Yttrium oxide, Y_2O_3) that absorb some of the light emitted by the Hg and re-emit it at other wavelengths to get "whiter" looking light.

The main lines of Hg are given in Table 1; you can use these to check your spectrometer wavelength calibrations:

1. Plug in your spectrometer.

2. Open the Thorlabs OSA spectrometer software.

3. Carefully remove the black protective cap covering the fiber face.

    a. Do this by pulling the edge with your fingers/fingernails; squeezing causes it to stick.

    b. Put the cap in the spectrometer box so it does not get lost.

4. Point the fiber at a fluorescent light and capture a spectrum.

    a. You may need to adjust the integration time (100 ms is plenty; less may be fine – you can adjust the angle of the fiber to change the amount of light getting in.)

    b. Take a spectrum of the fluorescent light.

5. Stop data acquisition (using Ctrl+R, the repeat button, or by right-clicking on the Trace letter and choosing "Fix").

    a. **Using the OSA software, find the wavelengths of the main peaks in your spectrum (the mouse scroll-wheel zooms in on traces).** For each major intensity peak in the data, find the wavelength of the peak and enter it into Table 1:

Line	True λ, nm	Measured λ
Hg I (neutral Hg)	404.7	
Hg I (neutral Hg)	435.8	
Terbium (Tb^{3+})	~ 543-544	
Hg I (neutral Hg)	546.1	
Europium (in Eu^{+3}:Y$_2$O$_3$)	~ 611	

Table 1: Measured Spectral Peaks

See www.thorlabs.com/OMC for the *Spectrum of Mercury* link. Notes: The Eu and Tb lines are only listed so you know what they are; use the Hg I and II lines for "calibration."

!

DO NOT touch the fiber face to anything!

6. **How good is your spectrometer wavelength calibration[7]?**

   a. **Is it off by a constant factor? If so, what is it, roughly?**

   b. **Is it off by a factor that is proportional to wavelength?**

   c. **Look up the absorption spectrum of GFP (Green Fluorescent Protein). A common fluorescent microscope light source is an Hg arc lamp, which has a spectrum similar to the fluorescent lights. Would it work well for GFP?**

   d. **Explain your answers to your lab instructor, and have them check off your table.**

7. **Save a screenshot of the fluorescent light spectrum for your lab report.**

II. Transmission Spectra

Often we may not care what the exact intensity spectrum of some illumination looks like; rather, all we may want to know is how much of the light at each wavelength gets through an object (a filter, for instance). In that case, we can take a spectrum without the filter in place, and then another spectrum with the filter in place and divide to get the relative transmission:

Equation 1:   Transmission T = Filter Raw(λ) / Illumination Raw(λ)

where "Filter Raw(λ)" is the raw spectrometer data taken with the filter in place, etc.

Question 1: What is the transmission, T, if you take a "filter" spectrum with *no* filter in place?

Separately, one often wants to know the absorption of a material (e.g., a fluorescent dye) and one can get that in a similar way, by taking the same spectra (i.e., with and without the material in the light path) and applying the appropriate manipulation:

Question 2: What is the formula to use to calculate absorption? Figure it out and fill it in:

Equation 2:   Absorption A(λ) =

Note: All detectors register some signal (called the "dark signal") even when there is not light on them. Furthermore, there can be background light from room lights, etc., that are not from the sample, and show up in a constant way on the detector and are often lumped in with the dark signal. It is often important to subtract these signals off, so that one only sees the true data. Doing this, Eq. 1 becomes:

Equation 3:   Transmission T = [Filter Raw(λ) – Dark(λ)] / [Illumination Raw(λ) – Dark(λ)]

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[7] Note: Obviously this is an exercise; a fluorescent light is not an ideal calibration source – one would typically use a multi-line low-pressure gas calibration lamp instead.

Question 3: If the dark signal shows up on both the top and bottom of the equation, why cannot it just be ignored? What transmission would you get using Eq. 1 if there was actually no (zero) light transmitted, but there was a significant dark (or background) signal?

> Note: It is important **not to change the optical setup** (except for inserting or removing the object to be measured) between taking each spectrum; otherwise the two measurements may differ for reasons other than the transmission of the object, and the calculated transmission may be inaccurate.

While one can calculate the transmission, absorption, etc., using a spreadsheet, it is easier to set it up on the spectrometer so you can see the results live.

8.   Set up your optical rig on the desk.

9.   Make sure there is sufficient room between your ND filters and condenser; if there is not, move the green filters and the ND filter wheel closer to the field stop. You do not want to bend the fiber abruptly to make it fit – it can break or get damaged.

10. Insert a rail carrier with the fiber-mount on it and the spectrometer fiber attached, as shown in Fig. 1, below:

DO NOT touch the end of the fiber! If you scratch it, it will be ruined.
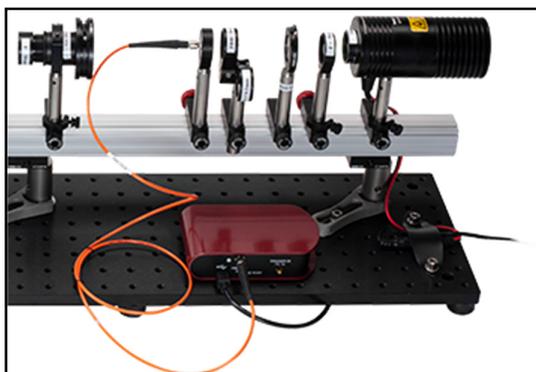
Figure 1: Spectrometer fiber mounting and positioning

11. Flip all filters (green, ND, etc.) out of the optical path.

  a.   Also remove your IR filter from the lamp. **Please**:

    i.   Unscrew the IR filter from the collector lens mount / its current location.

    ii. Screw it onto the end of your color camera lens tube (just as a place to keep it safe).

Reference (Illumination) Spectrum

12. Turn on your lamp.

13. Acquire data with the spectrometer.

  a.   Start data acquisition (e.g. using Ctrl+R).

b. Set integration time properly to maximize signal without saturating.

   i. Integration time should be roughly 0.1 - 1 ms; however, it is extremely sensitive to your fiber angle and position with respect to the light path. Try rotating the fiber mount back and forth, and raising and lowering it while watching the trace – you should be able to maximize the height of your trace fairly easily this way.

   ii. Note: It is important to then lock down the fiber mount securely; otherwise it will move around and your intensity will change during your measurements, messing up all your results.

   iii. Right-click on the trace to be sure autoscale is showing your entire trace.

   iv. You may need to adjust the height and angle of the fiber plate to maximize the amount of light getting in to the fiber.

   v. Double-check that your signal is not saturating; if it is, adjust your integration time appropriately.

c. Right-click Trace A and select averaging; choose 100+ averages.

   i. Even more averages (even 1000) is actually good here, even if it is slow – we will only use this curve for reference, so it will not always need to update: long averaging will not bother us later, but will make the curves we derive from this one less noisy.

14. Cover the fiber end with your hand again **(without touching the fiber!),** so the spectrum is dark.

a. Wait until the averaging time is over – this could take a while if you set the averaging high.

b. From the Sweep menu, click "Background correct"

   i. Now the background is being subtracted from the trace when it is being displayed.

**Background
Correct Icon**

c. When you remove your hand to allow light onto the fiber again, the trace should now rise from zero. If it does not, repeat the steps, since your background is wrong or the fiber has moved.

15. Wait for your averaging time to be over, then right-click Trace A and choose "Fix," so values stay constant for your reference spectrum.

16. Right-click the left vertical axis, and set the axis to go from 0 to something close to the maximum of the curve, and check the "Lock Axis" box so it will not keep autoscaling.

Question 4: Do you see much IR power in the spectrum, compared to what you would expect for this temperature lamp (2800 K)? Compare to Figure 2 on page 10-14; the hot filament is surely putting out a huge amount of IR light. How do you account for what you see in the spectrum?

Measurement Spectrum

17. Right click Trace B, and choose:

    a. Averaging at 50 (much lower than the Reference – you can change it later if you want).

        i. Defaults: If you do not change Background Correct, it will stay on, which is what we want; similarly, if you do not change the exposure it will stay the same as Trace A was, which is also what we want.

    b. You should see Trace B superimposed on Trace A on your display. If you do not see this then go back and repeat the steps above.

Transmission Spectrum

18. Right-click Trace C (or "Add" if you need to create another trace to get Trace C).

    a. Move to Secondary Axis.

19. The transmission is just the data spectrum B divided by the reference spectrum A. To set that up:

    a. Right-click Trace C, then choose Calculate - Transmission - B/A [%].

        i. Using Calculate - Quotient - B/A also works.

    b. Close the box.

20. Right-click the right vertical axis, and set the axis to go from 0 to 110 (to leave room for noise taking you over 100% "transmission"), and check the "Lock Axis" box so it will not keep autoscaling.

21. You should see a transmission curve at 100% on the same plot as your data and reference; if you do not, revisit the steps above.

Question 5: Why is there so much noise in the Transmission spectrum at short wavelengths (≤400 nm)? Hint: look at that region in your reference spectrum (Trace A).

Practicalities: Copying Spectra to Excel (or Other Spreadsheet)

The spectral measurements provide intensity values at each wavelength, so a natural way to work with them is in a spreadsheet like Excel. This is fairly straightforward: copy the spectral data so you can paste it into a spreadsheet. If you want to do this,

22. Acquire the spectrum you want.

23. Right-click the Trace letter for the trace you want, and choose "Copy to Clipboard;" choose "Tab separated."

    a. See screenshot on the last page of these notes.

24. Click on your spreadsheet and use Ctrl+V to paste the data in.

25. If you already have wavelength data in a column, delete the repeated / new wavelength column.

III. Filter Transmission Spectra

26. Flip in your excitation filter (FBH520-40) and examine the transmission spectrum.

    Does this look as you would expect? At what wavelength regions is the Transmission curve noisy? <u>Why</u> is it noisy there?

    a. Right-click your transmission Trace (probably Trace C) and copy your data (choosing "Tab separated").

        i. **Paste it in to your spreadsheet, labeling it "Ex filter transmission"**

27. Switch out your excitation filter, flip in your VG9 green glass filter, and get a spectrum.

    a. Right-click your transmission Trace (probably Trace C) and copy your data (choosing "Tab separated").

        i. **Paste it in to your spreadsheet, labeling it "VG9 filter transmission."**

28. Flip both filters out.

29. **CAREFULLY, without touching the filter with your fingers:**

    a. Unscrew the emission filter from your monochrome (sample) camera.

    b. Hold it carefully in front of your fiber so that the surface is perpendicular to the light path.

        i. You could also screw the filter onto the fiber mount.

    c. Get a spectrum and pause acquisition (Ctrl+R) so the data does not change.

    d. **Remove the emission filter from the light path and screw it back onto the monochrome camera.**

30. Right-click your transmission Trace (probably Trace C) and copy your data (choosing "Tab separated").

    a. **Paste it in to your spreadsheet, labeling it "Em filter transmission".**

31. Make a plot in Excel (or other spreadsheet) with all three (Emission, Excitation, and VG9) filter transmissions displayed.

    a. **Note**: If your trace has some noise (e.g. at short wavelengths, in the UV), then Excel can autoscale the plot to the (sometimes very large) noise values, leaving the main trace looking practically flat. Be sure to set the vertical scale of the plot so that you can see the main data – the axis should not go far below 0, and if you do not see what you expect, try adjusting the maximum as well. What is a reasonable value for your highest transmission?

    b. **Save this plot for your lab report.**

        i. The plot should have a title, and have both axes properly labeled. The horizontal axis should be "Wavelength, nm," and the vertical axis should be "Transmission."

32. Make new spreadsheet columns where you calculate what the transmission would be through:

    a. Both the Excitation and Emission interference filters in series.

        i. **Make a plot of this and save for your lab report.**

    b. Both the VG9 and Emission filters in series.

        i. **Make a plot of this and save for your lab report.**

    c. **Is there a problem using the VG9 filter and Em filter for doing fluorescence, instead of the Ex and Em filters?**

        i. **What is the problem?**

        ii. **What contrast mode would you have if you used this pair?**

IV. Filter Behavior – Angular Dependence

33. Open a word-processor (e.g. MS Word) document.

    a. Save the file with a descriptive name.

34. Restart spectrometer data acquisition (Ctrl+R) if you need to.

    a. If you have not already, <u>**carefully**</u> **remove the emission filter from the light path and screw it onto the monochrome camera.**

35. Flip in your excitation filter, and get a transmission spectrum as before. Make sure the filter is normal (perpendicular) to the optical path.

36. Take a screenshot (Alt+PrtScn or Alt+Fn+PrtScn) of the spectrum window, or alternatively use the Thorlabs OSA software File / Save Image / Copy to Clipboard.

    a. You can use the Ctrl+R first, to freeze the spectrum.

    b. Paste it into your document.

    c. Title it "Ex Filter, 0 deg'.

37. The FWHM (Full Width at Half Maximum) is the distance between the points on the curve where transmission hits 50% of maximum. When entering your data in Table 2, below, the Center λ is just halfway between the Red and Blue edges of the transmission curve, and the FWHM is the distance between those edges.

Ex. Filter Angle	Blue λ Edge, nm	Red λ Edge, nm	Center λ, nm	FWHM, nm
0°				
15°				
30°				
45°				
Nominal filter transmission band (from filter label):				

Table 2: Filter transmission as a function of incident angle.

In Part IV, SAVE OFTEN using Ctrl+S so you do not lose your data if the program crashes.

Note: The "blue edge" may not actually be at a wavelength that is blue in color; "blue edge" simply means "the shorter wavelength edge of a curve." Similarly, "red edge" means "the longer wavelength edge of a curve" and not necessarily a wavelength that is red in color. "Red edge" and "blue edge" are sort of like saying "right" and "left" – they are relative statements about direction along the spectral (horizontal) axis. This phrasing is standard in spectroscopy.

38. Repeat this (save a screenshot, fill out the table) with the filter rotated so that the light hits it at roughly the following angles (does not need to be exact):

    a. 15°

    b. 30°

    c. 45°

39. Write down the nominal filter transmission band (listed on label on the filter mount).

40. Take the filter out of the rail carrier and look through it at a white object (e.g. a piece of paper on the desk, or the ceiling lights, or whatever).

    a. Rotate the filter so you are looking through it at an angle (say, 15°, 30°, or 45°…); does what you see match the spectra you got, above?

41. **Would it be worse if your <u>excitation</u> filter or <u>emission</u> filter were rotated 15°?**

    a. **Why?**

42. **How does what you see when rotating the filter correspond to the 'Center λ' data in Table 3?**

    a. **Which direction (in wavelength) does the filter transmission band shift when you rotate it away from normal (perpendicular) to the light path?**

43. Replace the filters in their rail carrier (if you have not already). Then flip out the excitation filter, flip in the green VG9 filter, and take a transmission spectrum.

    a. Rotate the filter while watching the transmission spectrum; does the transmission spectrum change location?

44. Flip the VG9 filter out and the excitation filter back in, and get a transmission spectrum.

    a. Rotate the filter while watching the transmission spectrum; does the transmission spectrum change location?

        i. **This should help make it clear what the change in center wavelength means.**

V. Filter Transmissions Revisited

45. Restore your excitation filter to 0° incidence (perpendicular to the optic axis).

46. Get a transmission spectrum of the filter; do not pause acquisition.

47. Unscrew (again, carefully!) the emission filter from the camera, and hold it between the fiber and the excitation filter (or screw it onto the excitation filter mount).

    a. What does the transmission spectrum (through both filters) look like?

    b. Are the spikes in the UV real?

    c. How much light is getting through?

    d. Does this look like your plot from Part III?

    e. **Get a lab instructor and show them your results.**

48. Flip out the excitation filter; remove the emission filter temporarily from the light path. **(Do not put it down; hold it carefully!)**

49. Flip in the VG9 green filter and get a transmission spectrum; do not pause acquisition.

50. Hold the Emission filter between the fiber and the VG9 filter.

    a. What does the Transmission spectrum look like now?

    b. How much light is getting through?

    c. Does this look like your plot from Part III?

    d. **Get a lab instructor and show them these results as well, and explain why this matters for imaging.**

51. **Carefully screw the Emission filter back onto the monochrome camera.**

VI. Interference Effects

52. Take a stack of 2-3 fresh glass slides from the box.

53. Flip out your interference filter, and take a new reference spectrum. Also retake (and set) the "Background Correct" spectrum as before

54. Hold the slide stack near the fiber face, in the light path between the fiber and the lamp.

    a. Try to hold the slides with their faces perpendicular to the optical axis.

        i. This can be easiest if you hold the slides flat up against the side of the ND filter wheel, over the hole the light goes through. You can then slide the slides up and down, and back and forth (while always perpendicular), looking for the best modulation in your transmission curve.

    b. You should see some oscillations / modulation. If you do not, look at them (by eye) in the reflected room lights to see if you see colored fringes. If you do not, or they are inconsistent, grab a different pair of stuck-together slides (slides from newer boxes of slides tend to work better).

55. **Take a spectrum, and pause acquisition (e.g. using Ctrl+R).**

    a. **Save a screenshot of this spectrum for your lab report.**

    b. Restart acquisition (Ctrl+R).

56. **Do you notice the bumps in the transmission curve?**

    a. **Try squeezing the slides together a bit – do the bumps in the transmission curve change spacing?**

DO NOT pull the slides apart! You want them all stuck together. (Ideally, you want just two slides, stuck together – the slides tend to stick like this because they are polished very flat.

If all else fails, you can do this step with the plastic colored filter sheet in the Roscolux filter book, though the fringe spacing is quite different.

57. Hold the slides up toward the fluorescent room lights (or any light source) and look through them a bit off to the side of the lights.

    a. Do you see colored fringes?

    b. Try squeezing the slides together a bit – does anything change?

    c. What you have created is a "Fabry-Pérot interferometer," sometimes called a "Fabry-Pérot Etalon." An etalon is just two parallel mirrors or pieces of glass, and for wavelengths for which the round-trip distance for a reflection between two plates is m * λ, with m = integer, there is constructive interference and the transmission goes up. For wavelengths where the round-trip spacing is (m + ½) λ, you get destructive interference and the transmission goes down. Try squeezing the slides to change the spacing and make the fringes move.

        i. It is easy to get *accidental* etalons, e.g. between the flat side of a PCX lens and a flat sample, or due to the window in front of a camera or detector. This mainly matters when using highly collimated / coherent light (e.g. lasers), but it is something to be aware of since it can cause a lot of confusion and trouble.

            1. The trick for avoiding this is to either use antireflection coatings (with reduced reflections and thus reduced etalon effects) or (cheaper and easier) to slightly tilt the plane of the optical surface – say a PCX lens – such that one surface is not parallel to the other. Some so-called "optical flats" have a few degrees of wedge built into one side for exactly this reason.

        ii. The etalon effect lies behind antireflection coatings on lenses and the interference filters (which are just fancy (anti-)reflection coatings) we use for fluorescence imaging.

        iii. In the (optional) following section, the bumps in the QE curve for your detector are due to interference effects between layers of the silicon detector (surface electrodes and silicon, etc.) For front-side-illuminated detectors, where the light goes through a lot of layers, the QE is often bumpy like this.

58. **Advanced/Optional (I):** Try holding the slides close to the lamp instead of close to the fiber. Do you still see the bumps? Can you explain why the effect is different? Hint: look at the slides by eye and see if the fringes are the same everywhere – is the spacing of the slides constant? What *area* of the slide does the light that gets into fiber go through if the slide is near the fiber? Near the lamp?

59. **Advanced/Optional (II):** Measuring thickness using etalon fringes:

    a. Hold one of the plastic Roscolux filters (from the book of plastic filter sheets) in the light path. Choose a red filter, since the transmission spectrum is less noisy at longer wavelengths.

    b. While watching the transmission spectrum, rotate the filter from normal to the light path to different angles. Do you see fringes? Do they disappear? If so, when do they disappear?

    c. Hold the filter against the ND filter wheel hole (just as a way to keep the plastic sheet normal to the optical path / optical axis).

        i. The fringes should be reasonably large / visible; if they are not, check the angle of your ND filter wheel.

60. Right-click your transmission Trace (probably Trace C) and copy your data (choosing "Tab separated").

    a. **Paste it in to your spreadsheet, labeling it "Etalon fringe spacing for plastic filter."**

Etalon Fringe Spacing

Transmission will be a maximum when the round-trip path in the filter is an integer number of wavelengths, resulting in constructive interference. The wavelength in the filter plastic is

      Equation 4: $\lambda_{plastic} = \lambda_{vacuum} / n$

where n = index of refraction (n ~ 1.47 for cellophane), and λ_{vacuum} is the wavelength you read out with your spectrometer. With this, the relation for the peaks of transmission becomes

      Equation 5: $2d = m * \lambda_{plastic} = m * \lambda_{vacuum} / n$

where d is the thickness of the plastic sheet and m = integer ≥ 0 is the number of wavelengths in a round-trip.

61. In your spreadsheet, make a list of the peak wavelengths for a sequence of adjacent fringes.

62. In the column next to this list, number the peak wavelengths starting at 0 for the longest wavelength and going up as the wavelengths get shorter (since more shorter wavelengths will fit into the same distance 2d). Title this column, "Fringe number."

63. Make a spreadsheet cell for m_0, the base number of wavelengths.

64. In a column next to your peak wavelengths, calculate the plastic thickness d for each wavelength assuming some value for m_0. This formula should use the fringe number as well as m_0.

    a. Tip: in your formula, you will want the m_0 value to be the same for all formulae when you copy your formula down. You can achieve this by adding "$" signs to the cell address: cell reference A4 will change when you copy down, but A4 will stay constant. Function key F4 automatically adds the $'s to a cell, or you can do it by hand.

65. Adjust your value of m_0 until all the calculated d values are as close to the same as you can get them. When that is true, m_0 will be the base number of round-trip wavelengths in your plastic sheet (i.e., in thickness 2d).

How thick is the plastic? Typically plastic film is a few thousandths of an inch (10's of μm) thick.

Optional: Relative Intensity Measurement and Quantum Efficiency

The following section is **optional**; only do these tasks if your instructor has required it, or you have extra time. The following pages cover the theory of relative intensity and quantum efficiency measurement, followed by two related sets of lab exercises.

Background

So far you have taken spectra using your spectrometers simply by pointing the fiber at a light source and taking the raw spectrum data using a screenshot or pasting into a spreadsheet.

While this is fine for quick work, you may have already noticed a problem with the data: when you point the fiber at the lamp and take a spectrum, you do not see much IR light even with the IR filter removed (try it!). Yet, you may recall from the early labs that we can block *all* visible light from your cameras and yet still image well; clearly the lamp is putting out a lot of IR light (which should make sense – after all, radiated heat is simply long-wavelength infrared, and the lamp is hot).

How to fix the discrepancy between our spectrum and what we know to be ample IR light? There are two ways: either pay a lot to get a "calibration spectrum" for the spectrometer, or you do it yourself. It is better, but a lot harder, to do it yourself (and critical if you are doing serious measurements); we will cheat a bit to simplify things.

Intensity

Usually spectrometers are not too far off in wavelength calibration, and for many optical microscopy purposes we do not care that much whether we are plus or minus a couple of nanometers in wavelength as long as it is consistent every time we measure. We usually care much more *how much* light we have at different wavelengths. This also needs to be calibrated, and usually spectrometers are *very* far off on this, since their cameras do not detect all wavelengths equally.

Detectors in general are not equally sensitive to all wavelengths; in the case of silicon, which makes up the CMOS and CCD detectors used in all non-specialized cameras, the Si is increasingly transparent as λ approaches 1.1 μm (corresponding to a photon with the same energy as the semiconductor band-gap of Si), so of course these detectors do not detect light past ~ 1100 nm at all; it just goes straight on through. Conversely, they absorb UV light so well that the photoelectrons are created on the surface of the pixel structures, away from the inside where they can be efficiently collected, and so the electrons usually recombine with positive charges before being collected for readout. So Si detectors are poor detectors in the UV as well as the IR.

How do we account for this in our measurements? We need another calibration source to use to check our camera (or the Silicon CCD used in the spectrometer). Luckily, quantum mechanics tells us (in one of the most profound accomplishments of science) exactly what the intensity of emitted light from a hot body is at various wavelengths. This is known as the Planck blackbody distribution, where the power radiated by a hot object, in kW per m² of surface area per nm of wavelength, is:

Equation 6: $$P(\lambda, T) = \frac{8\pi h c^2}{\lambda^5} \frac{1}{e^{\left(\frac{hc}{\lambda k_B T}\right)} - 1} * 10^{-12} \text{ (to get units into kW/(m² nm))}$$

Of course, the QTH10 incandescent tungsten/halogen lamps contain a hot tungsten filament. Very hot, in fact: 2800 K, or ~2500 ºC, per the manual. And this is the genesis of the term "color temperature" – the color is produced by the range of light intensities at different wavelengths produced by a hot body of a given temperature. The noonday sun has a color temperature of ~ 5600 K (though it does not fit the theoretical curve very well, due to atmospheric absorption and other issues).

The Planck distribution P(λ, T) is the intensity (power per unit area) emitted by a hot object *per unit wavelength*. So the actual intensity you measure is I = P(λ, T) dλ, which should make sense: as you let more wavelengths (Δλ, or "dλ") in, you expect more total power. In the equation above, h is Planck's constant, 6.626e-34 J·s, c is the speed of light, 3e8 m/s, k_B is Boltzmann's constant, 1.38e-23 J/K, and T is the temperature in Kelvin. It is worth noting that since the energy of a photon is

Equation 7: Energy of a photon $= E_{phot} = \dfrac{hc}{\lambda}$

and the average thermal energy is given by

Equation 8: Average thermal energy $= E_{Therm} \sim k_B\,T$

The Planck distribution (in power per square meter per unit wavelength) can be rewritten as

Equation 9: $P(\lambda, T) = \dfrac{8\pi hc^2}{\lambda^5}\left[\dfrac{1}{e^{\left(\frac{E_{Phot}}{E_{Therm}}\right)}-1}\right]$

Note that at short enough wavelengths, when the photon energy is >> the average thermal energy (i.e., E_{phot} >> E_{therm}), then the right-hand factor becomes very small because the denominator is a huge exponential. Similarly, at very long (large) wavelengths the left-hand factor becomes very small, and as the photon energy becomes << thermal energy, the left-hand term becomes close to E_{therm}[8]. In between these limits there must be a hump, and there is; see Figure 2.

---

[8] The Taylor series is given by $e^x = 1 + \dfrac{x}{1!} + \dfrac{x^2}{2!} + \dfrac{x^3}{3!} + \cdots$

so for x << 1, $e^x \approx 1 + x$ and $e^x - 1 \approx 1 + x - 1 = x$

Thus the term $\dfrac{1}{e^{\left(\frac{E_{Phot}}{E_{Therm}}\right)}-1} \approx \dfrac{E_{Therm}}{E_{Phot}}$. Plugging this into Eqn. 9 gives

P(λ, T) $\approx \dfrac{8\pi c}{\lambda^4}\,E_{Therm}$. As related in Stone's beautiful book *Einstein and the Quantum*, the proportionality of this exponential factor to E_{Therm} is almost certainly what led Planck to try using the factor in the first place, thus leading to quantum mechanics.

Blackbody Curves
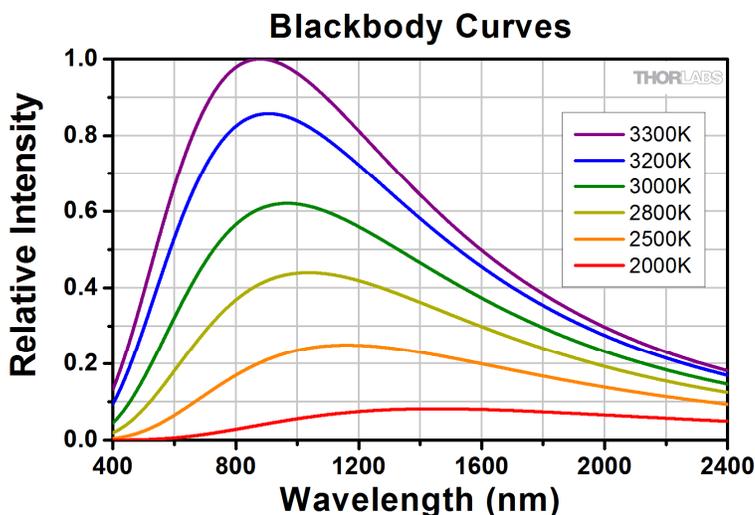
Figure 2: Planck distributions for various temperatures. Note how the peaks move toward the blue (shorter wavelengths) and overall intensity rises at every wavelength as the temperature increases. The peak intensity is at $\lambda_{max} = 0.2\,[\,hc\,/\,k_BT\,]$, known as Wien's Law.

It is worth looking at those curves a bit. It should start to make sense why the coals in the center of a hot wood fire look white (so-called "white hot") – more short wavelength light is being emitted where the coals are hottest, and the curve covers blue, green, and red… a combination of all visible wavelengths, which is what white light is. Similarly, when you turn down the burner on an electric stove, it goes from orange-ish (800 °C / ~500 K) to deep red (600 °C / ~400 K) to black (really, infrared that your eye is not sensitive to <500 °C / ~300 K) as it cools and the curve peak shifts further into the IR. And if you want to have a lot of UV light, to excite certain fluorophores, you could just turn up the lamp temperature to 6,000 or 7,000 K… except that tungsten filaments melt at 3,695 K… which is why one uses arc-lamps for those applications, though those are usually far from perfect blackbodies.

As you may have noticed: did your fluorescent lamp spectrum (which is similar to a Hg arc lamp) look like the Planck blackbody spectra plotted above?

Given that we know exactly what intensities are emitted by a hot material[9] at some given temperature, we can calibrate our spectrometers simply by taking a spectrum of our lamp (assuming we know the filament temperature), and then adjusting things so the output of the spectrometer is what it *should be* at each wavelength.

How do we adjust it? We want:

Equation 10:
Calibrated Spectrometer output = Calibration(λ) * Raw(λ) = P(λ, T) = Planck spectrum

---

[9] Someone is sure to point out that the Planck distribution only holds for "blackbodies"… and our lamp filaments are not perfect blackbodies. This is quite correct – they actually emit an intensity $I = \epsilon(\lambda) * P(\lambda, T)$, where $\epsilon(\lambda)$ is the emissivity of the substance at each wavelength. One can deal with this by buying a more perfect source, or (in the case of this lab, and not too bad a guess if you don't have a fancy blackbody source) assuming ϵ = constant in the visible. The case of ϵ = constant < 1 is known as a "graybody" (ϵ = 1 is a blackbody, by definition).

From this you should be able to figure out what the calibration function – i.e., Calibration(λ) – should be, based on a measurement of the lamp spectrum and a known lamp temperature:

Equation 11: Calibration(λ) =

Since we know the power from a blackbody, P(λ, T), from theory, if we illuminate with a blackbody at a known temperature then we can pick the calibration such that it converts the measured values into the ones we know must be correct. Then our output should look like a Planck blackbody distribution for the given temperature, T, known as the "color temperature."

Optional Lab Tasks

Relative Intensity

A. Reference and Data Curves

1. Set up just as you did in Part II, above, for the transmission spectrum. In particular, set up so that you have:

    a. The fiber facing the lamp with no filters in place; optimize fiber height and angle to get maximum light into the fiber.

    b. Trace A: Reference spectrum – a properly-exposed (~ 1-5 ms), highly-averaged (100+), background-subtracted trace.

        i. Once you get Trace A set up, right-click Trace A and choose "Fix".

        ii. Remember, the Traces should rise starting from zero. Otherwise repeat the steps, since your background is wrong or the fiber moved.

    c. Trace B: Data spectrum – a less-averaged (10-50), background corrected trace.

2. Right-click the left vertical axis, and set the axis to go from 0 to something close to the maximum of the curve, and check the "Lock Axis" box so it won't keep autoscaling.

B. Planck Spectrum

3. Click on the Math tab, then click the "Synthetic Spectrum" icon and choose "Black Body Emission."

    a. Set the temperature to 2800 K.

    b. Save to Trace C.

    c. Scale to Trace A (for simplicity).

    d. Click OK.

4. Right-click Trace C and choose "Move to Secondary Axis" (so scale is on the right-hand axis).

5. Right-click the right vertical axis, set the axis to go from 0 to something close to the maximum of the curve, and check the "Lock Axis" box so it will not keep autoscaling.

    a. Note: the power units here are only relative, not truly absolute!

6. If you cannot see your reference (Trace A), data (Trace B), and Planck (Trace C) spectra at the same time, right-click on any Trace and choose "Show All Traces."

7. You should see Trace B superimposed on Trace A on your display, along with the Planck blackbody spectrum for a lamp with the same temperature as yours. If you do not see this then go back and repeat the steps above.

Question 7: The Planck curve (and our tests imaging with infrared light in the first few labs) indicate that there should be a lot of IR light at longer wavelengths, above 850 nm. Why does the trace from the spectrometer show the light intensity falling at those wavelengths?

We would like to fix this artificially low IR spectrum; we *know* there is light there, but it is not showing up in our measurement. We can do this by:

    a. Taking a reference spectrum with very low noise (hence all the averaging above), then dividing all our subsequent spectrums by that. This is a lot like a transmission or absorption measurement: the result should be a value

---

DO NOT touch the fiber face when blocking the light to the fiber!

of 1 at all wavelengths, assuming we do not put a filter in the way and change one of the spectra. This will compensate for the low output of the sensor at wavelengths where its quantum efficiency is low, i.e. we are "normalizing" the output by a known reference.

b. Multiplying that "1" by a Planck distribution – which is what we *know* the lamp *should* be putting out – to get the actual *relative* power from the spectrometer at all wavelengths. This is called a "relative intensity measurement." It is not an absolute measurement because we do not know the exact powers – that would require painstaking calibration of the exact sensor quantum efficiency (QE), the reflectance of all surfaces, the precise temperature of the lamp filament, the collection efficiency of all the optics, etc. But we do know the *relative* powers now, since we have compensated for the varying QE of the spectrometer sensor. For a rough correction, this is all one really needs.

c. Now, if we look at a blackbody we should see the blackbody curve we expect! Better than that, if we look at anything else, we should see the <u>correct</u> relative powers – so if we dimmed the lamp, the curve would change correctly, and all spectra would be properly compensated for the varying QE of the spectrometer sensor.

C. Relative Intensity Measurement

8. Right-click Trace D (or "Add" if you need to create another trace to get Trace D) and choose:

   a. Move to Secondary Axis (so it displays on the same scale as the Planck distribution)

9. To set up the relative intensity measurement, we need to do the calculation just outlined:

   a. Right-click Trace D, then choose Calculate / User Defined

   b. Enter B * C / A, and click Calculate

      i. This makes Trace D = Planck(λ) * Raw(λ) / Reference(λ)

   c. Close the box

You should now see the Reference (A) and data (C) traces superimposed on your display, along with the Planck (B) and Relative Intensity (D) as well. Your RI (Relative Intensity) should be fairly low-noise and overlie the Planck spectrum quite closely. Otherwise you need to go back and repeat some of the steps above, since some of your spectra must be off (probably the reference Trace A, if you have to guess – sometimes the fiber moves while you are working).

10. **Does the plot look like the Planck distribution shown in Fig. 2, above?**

11. **Copy the spectrum graph and paste it into your Word document for your lab report.**

The software has subtracted the dark spectrum, calculated the Planck spectrum, and done the calibration calculations for you. Nice!

HINT: For Question 8, look at (display them if you are not already) spectrometer Traces A and B. What are the values of the dark-subtracted signal at short and long wavelengths?

12. Explore how this affects spectra; try looking at something that has spectral information both near the middle of the sensor QE (middle of the Trace A peak) and near either the long- or short-wavelength ends where the QE is low.

    a. A cellphone flashlight/camera flash LED works well for this, as will an LED flashlight.

13. You should be able to see both the raw data (Trace B, which will have peaks at different places) while your RI measurement (Trace D), which will have peaks in roughly the same places[10], but with different heights. Look for sources that make this distinction clear to you.

    a. **Take a screenshot of these combined spectra for your lab report.**

Quantum Efficiency

It is often desirable to know the quantum efficiency (QE) of a detector, i.e. the average amount of signal (often in photoelectrons) generated by an incident photon. Measuring the absolute Quantum Efficiency is a bit complicated[11]. However, the *Relative* QE – i.e., the QE within some overall constant multiple – is not so hard, and is very useful – after all, you can choose a fluorescent dye that emits where your camera is most sensitive, even if you do not know the exact numerical sensitivity. For our purposes, the QE is given by:

Equation 12:   Quantum Efficiency QE = (# photoelectrons) / (# incident photons)

We are already halfway there, as the value we read out for each pixel on a silicon detector is proportional to the number of photoelectrons. Then all we need is the number of photons… and we almost have that: Eq. 6 gives us the *intensity*, which is the power / unit area; we just need to convert that to photons. Again, it is convenient that we are only interested in a *relative* answer, since we do not need the actual number of photons collected. Rather, we just need something proportional to the number of photons. Each photon has an energy E_{phot}, so if we divide total energy received by the individual photon energy ($E_{phot} = hc/\lambda$ from Eq. 7) we will get the number of photons it takes to get the total energy. And the power is just energy/time, so all we have to do is divide the power (or intensity) by E_{phot} and we are set. We know the power, $P(\lambda, T)$, from Eq. 6 or from Eq. 9, so our photon count becomes:

Equation 13: $\text{\# photons} \propto \dfrac{Power}{E_{phot}} = \dfrac{\lambda}{hc} * P(\lambda, T) \propto \lambda * P(\lambda, T)$

We can also use the fact that

Equation 14: $\text{\# photoelectrons} \propto Raw(\lambda)$

i.e., the raw signal is proportional to the collected photoelectrons. Using Equations 10, 13, and 14, Equation 12 then becomes:

---

[10] Shifts in peak location will be small, since the Planck spectrum is not strongly peaked in the visible given the 2800K filament temperature.

[11] Take a look in the book *Photon Transfer*, by Janesick, for an excellent introduction.

Equation 15: Relative QE $\propto \dfrac{Raw(\lambda)}{\lambda*P(\lambda,T)} = \dfrac{Raw(\lambda)}{\lambda*Calibration(\lambda)*Raw(\lambda)} = \dfrac{1}{\lambda*Calibration(\lambda)}$

> This is the important result of all the discussion above. It is usually nice to scale this so it has a maximum = 1, even though the units are arbitrary. Often the maximum absolute QE is listed in a datasheet; if we have scaled to max = 1, it is then easy to multiply in your head.

In some spectrometer systems the values for the calibration are not available – they are only used internally to provide the relative intensity measurement. In our case, we cannot multiply by λ in software, so we must do it ourselves in Excel, as we will in the following section.

Relative Detector QE

Note: in the following, you want to set your spreadsheet up similar to Fig. 3, below.

	A	B	C	D	E	F	G	H	I	J
7	Wavelength, nm	Raw	Dark	Raw DS	Normalized Raw DS	Planck	Normalized Planck	Calibration	RQE	Normalized RQE
1	Week 10 Lab, Optional RQE calculations									
2				6.63E-34	h, Js					
3				3.00E+08	c, m/s		Split-View Divider			
4				1.38E-23	k_B, J/K					
5				2800	T, K					
6										
7	Wavelength, nm	Raw	Dark	Raw DS	Normalized Raw DS	Planck	Normalized Planck	Calibration	RQE	Normalized RQE
8	350	200.85	192.24	8.61	2.21E-03	1.18E-01	1.34E-02	6.06E+00	1.65E-01	5.55E-02
9	351	201.2	191.89	9.31	2.39E-03	1.21E-01	1.38E-02	5.76E+00	1.74E-01	5.84E-02
10	352	201.69	191.59	10.1	2.60E-03	1.25E-01	1.42E-02	5.46E+00	1.83E-01	6.16E-02

Figure 3: Example spreadsheet layout. Note use of 'split view' divider so column headings are always visible.

1. Move all filters out of the light path.

2. As in Part II for Transmission, get a good spectrum, properly exposed (with peak value large but not saturating). Do **not** use Background Correction, though.

   a. Set the averaging so the trace is not too noisy; more is better.

3. Copy and paste the spectrum into your Excel sheet.

4. Block the light and take another spectrum; this is your Dark spectrum.

5. Copy and paste the spectrum into your Excel sheet.

   a. Delete the extra wavelength column you get, and title the intensity "Dark."

6. Make a new column in which you calculate the dark subtracted raw signal (*Raw DS* as shown in Figure 3).

   > a. **NOTE**: Always be sure to subtract the 'Dark' data off your spectra before using them in any calculations. With the Thorlabs CCS200 you could do this automatically, but here it is better to see all the steps in your spreadsheet.

7. Title a new column titled "Planck," and enter the formula from Equation 6, and copy the formula down the column.

  a. Use a temperature of 2800 K.

  b. For reference, h = 6.626e-34 J·s; c = 3e8 m/s; k_B = 1.38e-23 J/K.

    i. It can be handy to use the function key F4 to select absolute cell references for h, c, k, and T (i.e. to add "$"'s to the cell reference so that cell reference does not change when you copy the formula down the column.)

  c. **To make things nice, make a new 'Normalized Planck' column which is the Planck data divided by the maximum of the Planck column, so the maximum = 1.**

    i. It can be handy to use the "max" function in Excel to find the maximum, along with F4 to make the cell selections absolute.

8. Title a column "Calibration" and use the formula for the Calibration(λ) you figured out in Eq. 11.

  a. **Be sure to subtract the dark data from the Lamp spectrum before dividing.**

  b. **Use the 'Normalized Planck' data instead of the actual Planck calculation.**

  c. Optional: Plot your Calibration(λ) vs. λ, and consider why the curve looks as it does (compare it to the Raw(λ) spectrum and the Planck curve).

9. Title a column "RQE" and use Eq. 15 to calculate the Relative QE (RQE) for the detector, using the Calibration(λ) data you have from previous step.

  a. Make a new column and normalize your RQE to max = 1.

10. Add a new column with the normalized, dark-subtracted Raw signal.

11. **Plot the normalized RQE on the same plot as the normalized, dark-subtracted raw lamp data and the normalized Planck distribution.**

  a. **Is it clear why the Raw data from the lamp looks as it does?**

    i. Note that bumps in the RQE may well be due to interference effects between layers / features on the sensor.

12. Find the QE (or Sensor Sensitivity curve) for the Thorlabs monochrome camera we use (Item # DCC1545M). Get the manual online from the Thorlabs website, and look under the Specifications section[12]. How does this compare with the sensor in the CCS200 spectrometer?

13. **Show your plot to a lab instructor.**

  a. **Save the file and plot for your lab report.**

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[12] Page 446 of the DCC1545M manual.

Clean up:

1. Turn off your halogen light so it has time to cool before you put everything away.

2. **Carefully disconnect the USB cable from your spectrometer by gently pulling the connector straight out.**

3. Put away your spectrometer; **be sure to replace the dust cover on the end of the fiber.**

4. Carefully put away your any samples or targets **in their special box(es).**

5. Put away any samples; **cheek epithelial cell samples go in the special "glass disposal" box.**

6. **Carefully disconnect the USB cable from your camera(s) by gently pulling the connector straight out.**

7. Screw a lens cap on your camera tube lens and condenser.

8. Close the irises on the back of the objective and condenser (not super tight – just close it to keep dust out).

9. Put your optical breadboard system away.

10. Copy your files off the computer onto your USB drive or cloud storage (remember – it is your responsibility to keep copies of your files!).

11. <u>**Shut down**</u> (not just "sleep") the computer.

Lab 10 Write-Up:

Basic Details:

o Turn in your write-up as a Word document, due date, etc. as specified by your instructor.

o **Number your pages "page X of Y" and have your name in the footer so it's clear if a page is missing if it gets printed out.**

In Microsoft Word (or whatever program you use) be sure to compress all your images before turning in the file; otherwise the files will be too large.

▶

o After appropriately cropping and sizing images – not covering the whole page, not so small one cannot see the features discussed – reduce the image size so the document is not huge. In MS Office as of this writing, you do this by clicking on a picture, choosing the "Format" tab in the top menu bar, and then choosing "compress pictures" on the format menu toolbar. For submitting your assignment, click save and save-as-PDF. Be sure to check that the compression and the PDF conversion have not spoiled some feature in an image you were trying to point out in your lab write-up – this should not be an issue if you cropped and sized your images appropriately. If there is a problem, send the larger document so the image information is preserved.

Write-Up:

The main part of this lab is the spreadsheet-based problem set; see below for details. The Lab 10 write-up is intended to be short to give you time to work on the problem set. For the write-up itself:

1. Part I: Copy and paste in your data from Table 1.
    a. Answer the questions:
        i. How accurate is your spectrometer wavelength calibration?
        ii. Is it off by a constant factor? If so, what is it, roughly?
        iii. Is it off by some factor that is proportional to wavelength?
2. Part II: Answer Question 4.
3. Part III:
    a. Paste in your plot of the different filter transmission spectra.
    b. Paste in your plots of:
        i. Your calculated combined Ex filter and Em filter transmission.
        ii. Your calculated combined VG9 filter and Em filter transmission.
    c. Answer the questions:
        i. Is there a problem using the VG9 filter and Em filter for doing fluorescence?
        ii. What is it?
        iii. What contrast mode would you have if you used the VG9 and Em filters to image?
4. Part IV:
    a. What happens to the filter FWHM as the incident light angle increases?
    b. What happens to the Center wavelength?
        i. Which direction does it shift?

c. **IMPORTANT: Would it be a good idea to put the Excitation filter between the condenser and the objective? Why or why not? Explain in detail.**

d. Would it be worse if your <u>excitation</u> filter or <u>emission</u> filter were rotated 15° from perpendicular?

   i. What would happen to the total transmission if only the Ex filter were rotated 15°?

   ii. What would happen to the total transmission if only the Em filter were rotated 15°?

5. Part VI: Paste in the plot of your Transmission spectrum from the stacked slides showing the interference fringes.

6. Optional Lab Tasks:

   a. Paste in your plot of the Relative Intensity lamp spectrum from Step 11 (and 13 if you did that).

   b. Paste in your plot of the normalized RQE, dark-subtracted Raw lamp, and normalized Planck spectra.

Lab 10 Filter Selection Problem Set

As discussed in the Course Notes, choosing better filters is the least expensive and easiest way to get dramatic improvement in your fluorescence imaging – often it is possible to get a factor of 2 - 3 X longer (before photobleaching) or brighter (for the same exposure time) imaging for < $500.

This is the only problem set we have for this course; the point is to walk you through how to do these calculations, so in the future you can choose your own filters if you want to.

The specific problem we pose for you comes from a real-world design issue: fluorescence microscopy for diagnosis of tuberculosis. As a brief background, tuberculosis (TB) is a vicious disease that, until 1900, was responsible for close to one out of every three deaths in Europe. Even two generations ago in the United States, most families had one or more family members with the disease; that only ended with the era of modern antibiotics in about 1960. Today, about 1.5 million people die every year due to TB, and some 10 million people have active TB. The antibiotics used to treat TB are expensive, must be taken for 6 - 9 months, and are not good for you if you do not need them; so it is important to be able to diagnose people to see if they have TB and thus need antibiotic treatment and monitoring.

A standard test is fluorescence microscopy, examining stained sputum smears to look for TB bacilli stained with the fluorescent dye Auramine O. Since most people with TB are poor, the diagnostic test must be inexpensive: you are designing a low-cost LED-based fluorescence microscope to help diagnose TB in low resource areas. Making this work will require optimizing every part of the system. Here you will focus on the filters.

For the problem set, you will examine filters suggested by Chroma, an optical filter company, and also become familiar with their (very handy) spectra-plotting web utility. You will t**hen choose filters of your own and** see if you can do better than the set suggested.

To do this, you will need to:

1. Get the "Lab 10 Filter Selection Problem Set" Excel spreadsheet from the Thorlabs website; see www.thorlabs.com/OMC for the link.

    a. This spreadsheet has two tabs:

        i. An example of filter selection (for eGFP), including plots, that you can examine.

        ii. A spreadsheet with instructions and the start of your data columns.

            1. The data for the Thorlabs DCC1545m camera QE is included. Assume you will use that camera.

2. Find the emission spectrum of the appropriate high-power LED. Until recently, the 455 nm LEDs were the best available for this work; however, recently 430 nm LEDs have become available, and may eventually be better for this application. Do your calculations for a 430 nm LED, the Thorlabs M430L4.

    a. Find the M430L4 430 nm high-power LED at the Thorlabs website; see *Mounted LEDs* under the *Reference Links* tab at www.thorlabs.com/OMC for the link.

    b. Display the spectrum, and then download it.

    c. Find the spreadsheet tab that has the spectral data in 1 nm steps (which will match other data you can find), and paste it into your "Lab 10 Filter Selection Problem Set" sheet.

3. Find the emission and absorption spectra for the fluorescent dye Auramine O. This sort of data is easiest to find at the University of Arizona Spectra Database; see www.thorlabs.com/OMC for the link.

    a. Go to this site, and find the Auramine O spectral data. Display this data.

    b. Figure out how to download the spectrum:

        i. It is easiest to "Save Spectra" and then open it in a spreadsheet like Microsoft Excel.

    c. Open the spectrum.

    d. Cut and paste the data into your "Lab 10 Filter Selection Problem Set" sheet.

        i. Note: If the data is not already in 1 nm steps (this occasionally changes for online sources), then for simplicity in this lab we may include it in the Problem Set Excel spreadsheet for you – check for the data in the Problem Set sheet if the wavelength step size is an issue (you may have to scroll down in the sheet to see where the data starts).

4. Plot the effective excitation and emission (fill in any required steps; we have included only the camera QE in the Excel sheet)

    a. **Note: The camera QE column <u>does</u> have data in it; the first few rows are blank because the data is not reported at those wavelengths. If you scroll down, you will see the data starting at 390 nm.**

5. Go to the Chroma Corp. website (Chroma and Omega are both large (and excellent) manufacturers of interference filters for specialty use; Chroma happens to have an Auramine set, so we chose them). Search for "Auramine", and look at the suggested filter set (19008 - AT). See www.thorlabs.com/OMC for the link.

    a. Download the data for the excitation and emission filter, and put it in your spreadsheet; this will be the set you use as "standard". (For simplicity, we will assume a transmission geometry, and thus ignore the dichroic filter).

6. Set up your spreadsheet to calculate:

    a. The brightness for this filter set, given the camera, LED, and dye.

    b. The fraction of total emitted fluorescence that you collect using this filter set.

    c. The total detected leakage allowed by this filter set.

7. **Choose another pair of filters. Calculate the relative brightness, fraction of total emission collected, and leakage for the filters you chose, and compare them to the standard set.**

    a. You may use filters from any company; Omega, Chroma, Semrock, and Thorlabs are excellent places to look.

        i. Since you have already been to the Chroma website, consider looking for more filters there: Chroma / Products will give you a choice to look for bandpass, longpass, or shortpass filters, and

the subsequent pages give ways to select for different wavelengths. See what you can find that suits your needs.
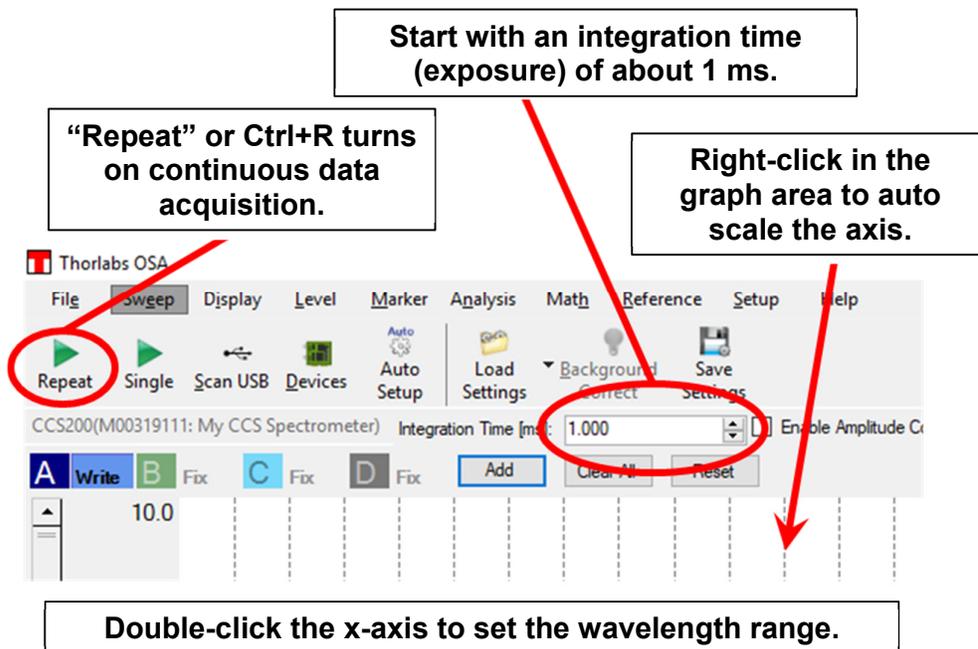
    b.   Download the filter transmission data for your choices and enter it in your spreadsheet.

    c.   Calculate the brightness, fraction of total fluorescence emission collected, and leakage for the stock filters and for *your* filter choice. Compare these: how much better did you do for each?

        i.   **Explain (briefly, as we do in the red text in the spreadsheet example) WHY your results differed as they did – why were your filters better (or worse) in each category?**

       ii.   Note: the fact that you can get better performance *does not* indicate a problem with Chroma's stock filter set; it is a fine set, but was clearly designed assuming a different light source than we are using. Which is the entire point of this exercise: sometimes you want to do something that is not standard!

8.   **NOTE: If you are using this exercise as a starting point for your own application, you may well need to include a dichroic filter as part of epi-illumination. If so, be sure to include the dichroic filter in all your calculations! That will require adding columns to the spreadsheet and adjusting some of the formulae.**

Thorlabs CCS200 Spectrometer with Thorlabs OSA Software
(The Basics)
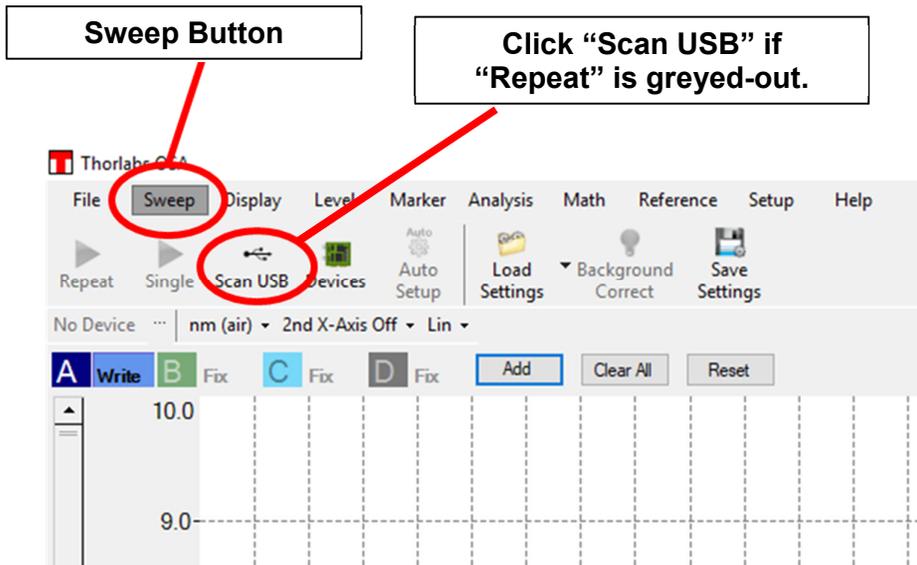
Sweep Button

Click "Scan USB" if "Repeat" is greyed-out.

You may then have to select your spectrometer from a list, depending on whether a different one is attached or has been used)

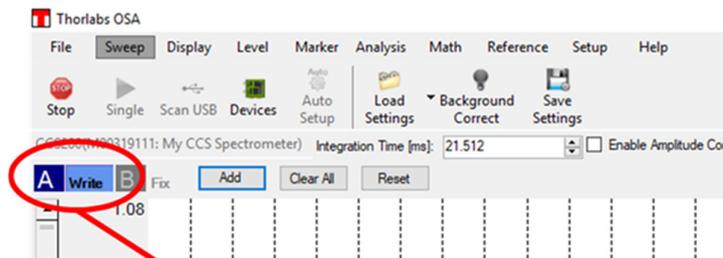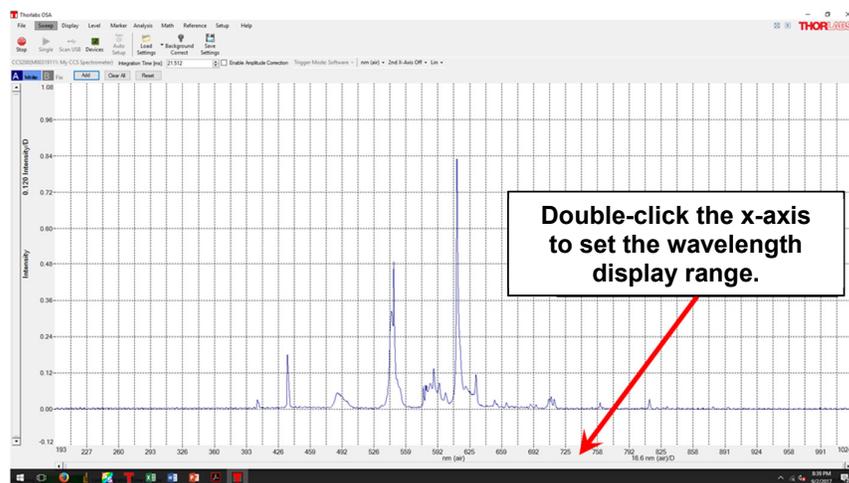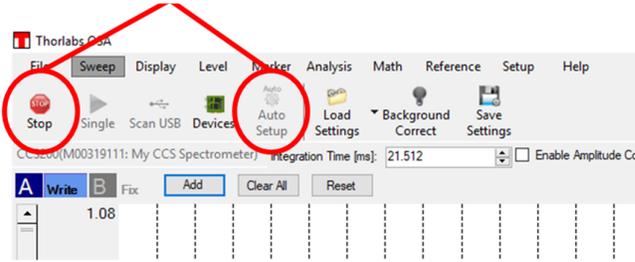**Start with an integration time (exposure) of about 1 ms.**

"Repeat" or Ctrl+R turns on continuous data acquisition.

Right-click in the graph area to auto scale the axis.

Double-click the x-axis to set the wavelength range.

Thorlabs CCS200 Spectrometer with Thorlabs OSA Software (Additional Features)

Note: If the light into the spectrometer is very dim, auto setup can take a very long time. Only use it if there is a fair amount of light.

To use the Auto Setup when taking data:
- **Stop acquisition ("Stop" or Ctrl+R)**
- **Click "Auto Setup"**
- **Start acquisition ("Repeat" or Ctrl+R)**

Double-click the x-axis to set the wavelength display range.

Averaging: Right-click "A" and choose averaging from the menu.

Right-click "A" and choose averaging from the menu.

Important Options:
- **Set as Active**
- **Move to Secondary (Right) Axis**
- **Average**
- **Fix (Stop Acquiring)**
- **Calculate (Do Math)**
- **Copy to Clipboard (Choose Tab Separated)**

Appendix A:
Lab Write-Up Template

**Optical Microscopy
Course**

Appendix A Lab Notes:
Lab Write-Up Template

Lab Write-Up Instructions:

This is an example of a lab write-up; the idea is to show you what we expect for your write-ups in this Optical Microscopy Course. Do not worry if you do not understand the technical contents of this document; we made up some lab questions just to illustrate a write-up without giving away questions from actual labs.

It is useful for you to know how to present your results; in industry (and in academia) you will frequently be called upon to put together some document – often called a "White Paper" – on some work you have done or topic your team needs to understand the basics of. Your lab reports need to reflect an ability to do that, and are, frankly, practice for it. We will not require you to write introductions/conclusions/hypotheses/etc., but we do expect that your answers – sentences, charts, images – will look appropriate for such a document and contain the information such a document should have.

Some of the main items to pay attention to:

Most Important:

- Be sure to **actually answer the whole question**. If we ask you to compare two numbers and comment on the relationship between them, then do not just list the numbers; comment meaningfully (with an explanation, and/or a quantitative comparison with some formula – not just some statement that "X seems bigger than Y. How odd.")

General:

- Submit as a PDF

- Compress images before generating a PDF; check that final document image quality is sufficient.

- Title the document with the 'Week X' of the lab.

- Include a footer on **every** page with 'Page X of Y', the date, and your name.

- Questions: Make it clear what question you are answering by using the same numbering as in the Writeup instructions for the week. You do not have to get into great detail like "A.3.a.i.i," but all the answers for question A.3 should be under A.3, or at least under 'A'.

Formatting:

- **Equations**:
    - Any equation of substantial size (e.g. the lens equation) should be numbered and on a separate line, with text big enough to see if you use subscripts. E.g.:
        - Equation 5: $1 / f = 1 / S_o + 1 / S_i$
    - Small equations can be put in-line with your text; e.g.: "The resolution we obtained did not match what we expected from the Rayleigh criterion given by $\delta = 0.61 \lambda / NA$; this may be due to fingerprints on the lens."

- **Graphs**:
  - o Always label both axes, with units (e.g.: 'Resolution, μm'). Numbers and labels should be easy to read with sufficiently large fonts, etc.
  - o Always number figures, plots, and images, with a title and caption. E.g.: "Figure 3: Resolution vs. Illumination Wavelength."
    - ▪ It is better if your caption has some additional information, e.g.:

      Figure 3: Resolution vs. Illumination Wavelength
      Note that resolution improves linearly with decreasing wavelength in the visible; data point in the near IR does not fall along the theoretically predicted line, likely due to chromatic aberration.

- **Figures, Including Images**:
  - o Figures and images should not be larger than necessary; crop out areas irrelevant to what you are trying to explain or show. For instance, if using a resolution target image, show the area with the small resolution bars, blown up so they can be seen as well as possible, rather than the whole target with the small bars as a tiny fraction of the image area.
  - o Always number figures, with a title and caption, as with the graphs.
  - o After appropriately cropping and sizing images – not covering the whole page, not so small one cannot see the features discussed – reduce the image size so the document is not huge. In MS Office as of this writing, you do this by clicking on a picture, choosing the "Format" tab in the top menu bar, and then choosing "compress pictures" on the format menu toolbar. For submitting your assignment, click save and save-as-PDF. Be sure to check that the compression and the PDF conversion have not spoiled some feature in an image you were trying to point out in your lab write-up – this should not be an issue if you cropped and sized your images appropriately. If there is a problem, send the larger document so the image information is preserved.

Quantitative Issues:

- **Numbers and Units:**
  - o Significant digits matter. In this class, we will do almost nothing further than 2 - 3 significant digits, so "resolution was calculated to be 8.35475485 μm" is not appropriate. For our purposes it does not matter if you use 3 instead of 2 digits, but using way too many is extremely poor form.
  - o Use easy units: 532 nm is much better than 0.000532 mm. Be consistent, though – if most of your wavelengths are in nm, do not then mention the last one in microns.
  - o Be sure to list all variables you use – for instance, if you say "we calculated the Rayleigh resolution $\delta = 0.61\ \lambda\ /\ NA = 5.6$ μm," you should also list what λ and what NA you were using. If there is any doubt about the NA (e.g., if it was not printed on the barrel of the objective), you should explain that too – for instance, "NA = 0.25 was obtained using NA = r / f, where r = 12.5 mm and f = 50 mm for our lens."
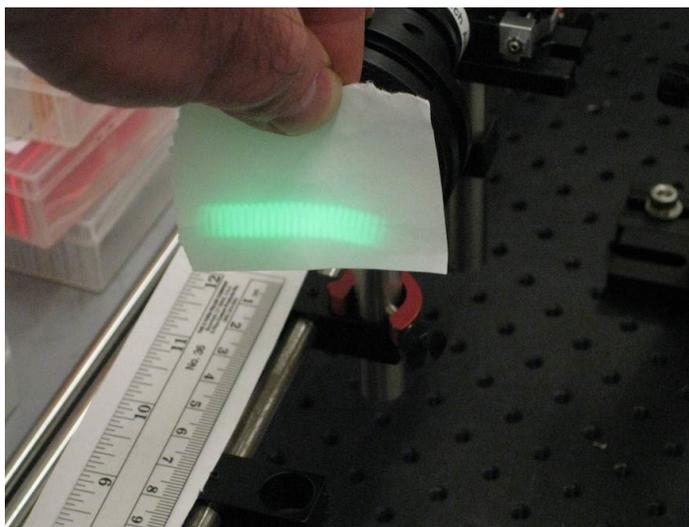
The template follows on the next page.

Question 1: *Show your image of the lamp filament, and explain what results you got for the distance over which the lamp filament was in focus near the aperture stop, and discuss whether the depth of field explains your results.*

Figure 1: Our lamp filament as seen near the aperture stop.

Our collector lens has f = 50 mm, and we measured it to be approximately ~ 57 mm from the lamp filament; this results in an image at the aperture stop ~ 40cm away. Using the thin lens formula,

Equation 1. $1 / f = 1 / S_i + 1 / S_o$

gives an image distance S_i = 40.7 cm for that object distance S_o, which matches fairly well given the difficulty of estimating S_o due to the filament being inaccessible in the lamp housing.

We measured the distance over which the image of the filament seemed to be in focus to be S_i ~ 36 – 44 cm, or almost 8 cm of travel near the aperture stop (AS).
This distance is much larger than the expected depth of field given by

Equation 2. $DoF \sim n \lambda / NA^2$

where I am using the low-NA formula since the NA on the image side of this lens is quite low: the lens is 25 mm dia., while the image distance is ~ 40 cm, giving an NA ~ r / f ~ 0.03. We are imaging in air, so the index n = 1. We used green light at λ ~ 550 nm, so the expected depth of field from Equation 2 is ~ 0.5 mm, much smaller than the 8cm we measured. As a result, it is unlikely that the depth of field is the reason the filament image is clear over such a large distance near the aperture stop.

It is possible that the issue is more related to the finite diameter of the lamp filament coil; looking into the bulb, it appears that the filament coil is ~ 1 mm dia.

Using Equation 1 and plugging in S_o = 56.5 and S_o = 57.5 mm (for the front and back of the filament) results in expected image distances of ~ 435 and 383 mm respectively, or a range of

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5.1 cm. This is much closer to the 8 cm measurement we obtained; as a result, I believe the finite diameter of the lamp filament coil is the most likely reason the filament appears in focus over such a distance near the aperture stop – essentially, we are not noticing the blurriness much until both sides are out of focus, which is an extended distance since the sides of the filament image to different locations S_i along the optic axis.

Since the filament is such a large object that it is hard to tell exactly when the edges become blurry; this makes it seem likely that the actual distance over which the filament is technically in best focus is somewhat smaller than what we measured, placing it closer to my 5.1 mm estimate.

Question 2.
-   *Plot your spectra for the high-powered blue LED.*
-   *A friend asks if they need to use an excitation filter with their LED excitation source when doing fluorescence imaging. Based on your plot, what do you suggest, and why?*
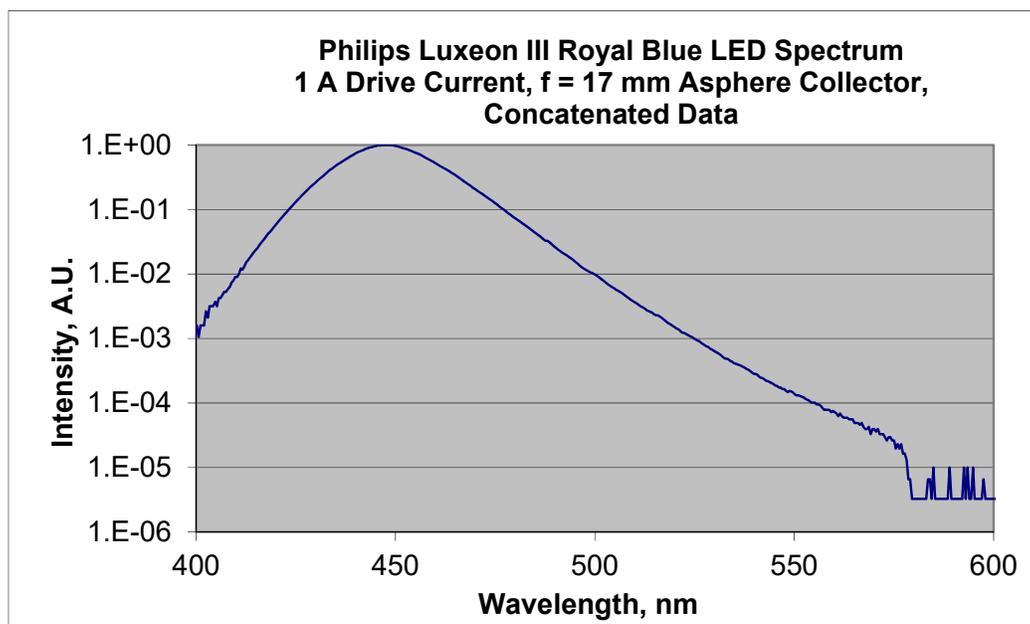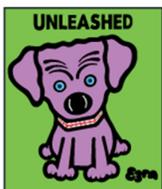

Figure 2. Spectrum of our blue LED; only relative intensity was measured, hence the units on the Y-axis are arbitrary (Arbitrary Units).

Although the LED intensity is falling, it is still significant at a long distance from the peak wavelength. For example, in our system, where the excitation filter is a 520/40 (i.e., transmits from 500 - 540 nm), our emission filter begins to transmit at ~ 550 nm (it is a 550 long pass). We see some background even with our inexpensive cameras, and the filters we use block at OD6 outside their transmission band… if we used the LED above without an excitation filter, the excitation intensity from this blue LED would be down to only the equivalent of OD4 (10^{-4}) at 550 nm, despite the fact that its peak is at 450 nm, not 550 nm. As a result, the background would be 100X worse than what we already have. If we assume the fluorophore used with this excitation would have an emission peak closer to the excitation, say at ~ 500 nm, then the situation is even worse – equivalent to only an OD2 reduction in excitation intensity.

As a result, I would tell my friend that an excitation filter would almost certainly be necessary.