MODULE 4: MICROSCOPY AND IMAGE RESOLUTION

This lab module retraces the famous experiments of Ernst Abbe, which showed how diffraction of light by a specimen (and interference with the illuminating light) gives rise to an image and how collection of diffracted light defines the resolution of the microscope. These concepts are demonstrated by using a diffraction grating as a specimen and visualizing and comparing the diffraction pattern in the back focal plane as well as the image in the image plane.

I. Why Build Microscopes?

To observe the microscopic world, it is often taken for granted that a compound microscope is necessary. Let us challenge this idea with a thought experiment- what is the highest magnification possible using a magnifying glass and your eye? Further, let us use the simplest approximations, geometrical optics and the thin lens approximation. In geometrical optics, light is treated as rays propagating in straight lines, which may be subject to refraction or reflection at an interface. This model is valid inasmuch as the wavelength is much shorter than any optics encountered and phase can be ignored (therefore no interference or diffraction).

A. Brief Review of Lenses[†]

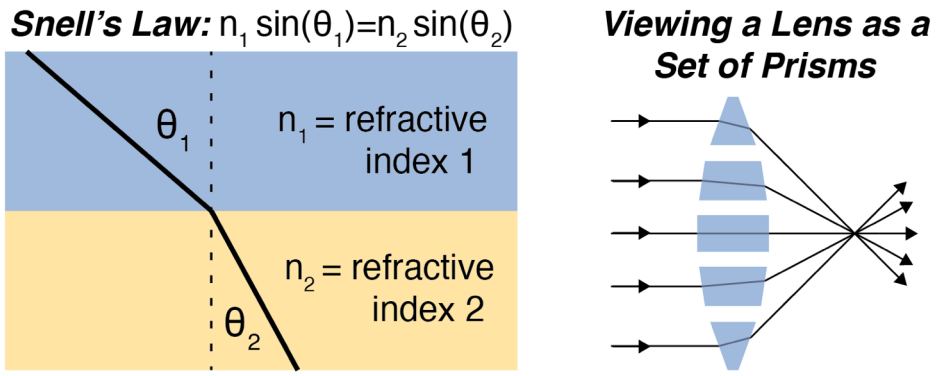


Figure 1. (left) Diagram showing Snell's Law of Refraction. (right) A refractive lens can be understood as a discrete stack of prisms.

Figure 1 shows how the defining feature of a lens – rays of light incident parallel to the optical axis pass through the focus - can be understood using Snell's Law of Refraction. Light incident on the lens is refracted at the air/glass interface, propagated through the glass, and refracts again at a

[†] For a more descriptive review, see the e-book *Handbook of optics Vol.1*, Chapter 1: General Principles of Geometrical Optics and Chapter 17: Lenses. Free access is available to the Yale community.

second glass/air interface. The thickness clearly matters; imagine stretching the lens in Figure 1 horizontally without changing the curvature at the ends. Depending on the wavelength and focal length, the aberrations resulting from the thickness of the lens may be significant, but we shall ignore them for the time being and use the thin-lens approximation.

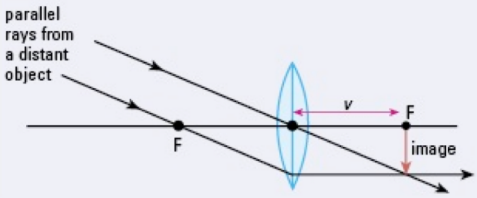
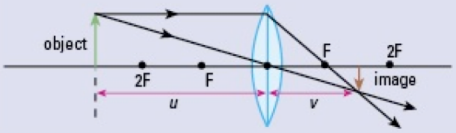
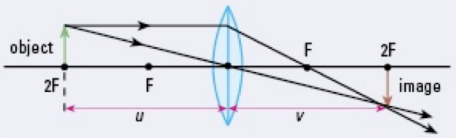
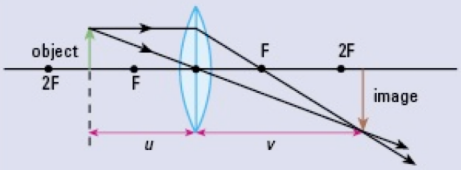
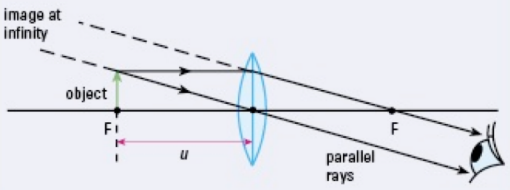
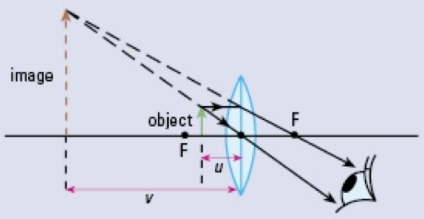
Object distance (u)	Ray diagram	Type of image	Image distance (v)	Uses
$u = \infty$		- inverted - real - diminished	$v = f$ - opposite side of the lens	- object lens of a telescope
$u > 2f$		- inverted - real - diminished	$f < v < 2f$ - opposite side of the lens	- camera - eye
$u = 2f$		- inverted - real - same size	$v = 2f$ - opposite side of the lens	- photocopier making same-sized copy
$f < u < 2f$		- inverted - real - magnified	$v > 2f$ - opposite side of the lens	- projector - photograph enlarger
$u = f$		- upright - virtual - magnified	- image at infinity - same side of the lens	- to produce a parallel beam of light, e.g. a spotlight
$u < f$		- upright - virtual - magnified	- image is behind the object - same side of the lens	- magnifying glass

Figure 2. Examples of thin lens image formation. *"Positive thin lenses," is licensed under CC BY-SA 3.0*

Various examples of the imaging qualities of a lens are shown in Figure 2, and all can be summarized in the thin lens equation,

$$\frac{1}{u}+\frac{1}{f}=\frac{1}{v}, \tag{1}$$

where u is the object distance, f is the focal length, and v is the image distance. Note that with this sign convention, u is negative in Figure 2. These examples show that magnification occurs (namely there is an image that is larger than the object) when the object distance is less than $2f$ away from the lens.

B. The Eye as a Detector

To continue with our thought experiment, we need to consider the imaging properties of the human eye. Your eye has two modes of operation – relaxed and tense. In relaxed mode, the imaging scheme resembles $u = \infty$ in Figure 2 with $f \approx 20$ mm, about the distance between your lens and retina. When you focus on closer objects, your ciliary muscles contract to make the lens wider in the center and give it a shorter focal length. This scheme resembles $u > 2f$ in Figure 2. The closest object that most healthy eyes can focus on is 25 cm away (the near point). We can use the thin lens equation to determine the focal length in this scheme and find $f \approx 18$ mm. In optimal use of a magnifying glass, the object is f away from the lens and the image is 25 cm away, as shown the $u < f$ case in Figure 2. This gives a combined magnification of

$$M = \frac{25\ cm}{f}, \tag{2}$$

To replace a microscope with a magnifying glass, apparently we need lenses with as small of an f as possible.

C. Ball Lenses

As one wishes to increase the refraction of a lens to shorten the focal length, the limit is reached where the lens is nearly a sphere. In the limit that the lens is a sphere, the focal length, f, is determined by the radius, r, and index of refraction, n, by the ball lens equation,

$$f = \frac{nr}{2(n-1)}. \tag{3}$$

If we were to use a ball lens like a magnifying glass, we would combine *(2)* and *(3)* to find that

$$M = \frac{50cm\ (n-1)}{nr}. \tag{4}$$

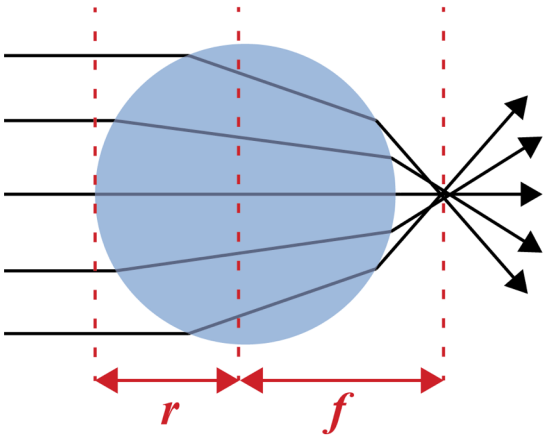


Figure 3. Ball lens (note spherical aberration)

Equation 4 is maximized when n is large and r is small. The index of refraction for optically transparent solids ranges from 2.4 (diamond), 2.15 (cubic zirconia), 1.45 (fused silica) to 1.31 (ice). If we take r to be 1mm for a perfect diamond sphere (f = 0.86 mm), this yields a magnification of 292. Manufacturing considerations aside, this would magnify a 700 nm to 0.2 mm- barely a speck but technically recognizable!

The above train of logic mirrors some of the history in the development of microscopes. Antonie van Leeuwenhoek (1632-1723) pushed the craft of lensmaking close to the limit we derived above, and in doing so opened up the new science of observing microscopic organisms and features in human physiology. The next major advance came with the refinement of compound lens microscopes, which in addition to correcting many of the aberrations inherent to the Leeuwenhoek approach made microscopy tremendously more practical (imagine performing your research by peering through a 1mm diamond sphere and recording what you see).

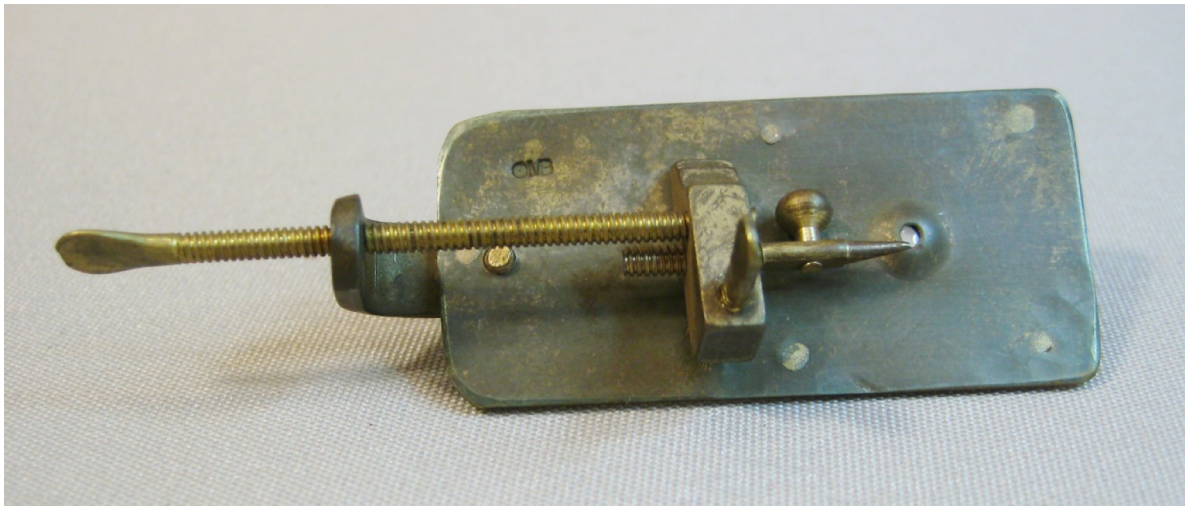


Figure 4. van Leeuwenhoek Microscope Replica, The University of Queensland, Physics Museum

II. Köhler illumination

One issue that was apparent to early microscopists is that magnification inherently makes an image dimmer. Collecting the light from a small region and magnifying it does not also increase the amount of light. As better lenses were used for magnification, lenses also came to be used for illumination. The simplest idea is to use a lens to collect and focus a large, bright light source on the region of interest. The main problem with this approach is that focusing results in image formation. You will see in this lab, as early microscopists did, that this illumination scheme results in uneven illumination- a focused spot of light is brighter at the center than the edges. In the worst case, this can result in an image of the light source superimposed on the microstructure of interest.

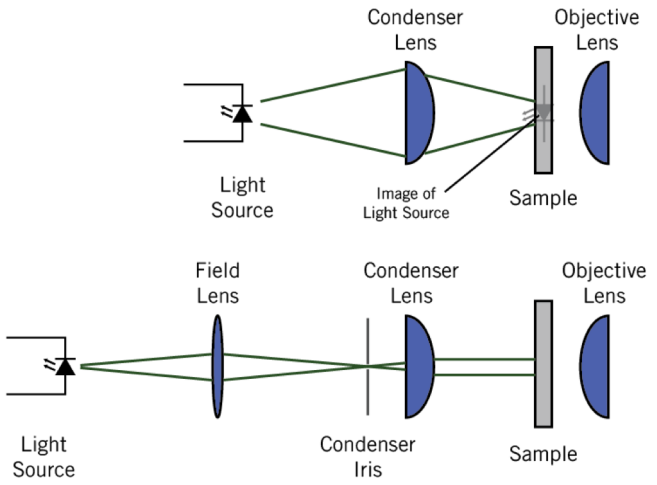


Figure 5. Köhler Illumination. Rather than focusing the illumination onto the sample (above), the illumination is focused on an iris by a field lens, which provides a point source that is collimated by the condenser (below).

An elegant solution to this issue was introduced in 1893 by August Köhler of the Carl Zeiss corporation. In this scheme, collimated light – rays of light that are neither converging or diverging – are incident upon the sample. This scheme provides even illumination. The image of the light source would be at the position of the condenser iris, which is f away from the condenser lens. This position is important enough to deserve its own name – the *back focal plane*.

If you are familiar with the math of Fourier transformations, you can recognize that the lens is performing a momentum-position Fourier transform; each ray of light incident on the lens at a different angle (momentum) emerges with a distinct position. The Fourier transform of an iris pinhole is a flat plane of light – a collimated beam.

III. Compound Lens Systems

A fundamental limitation of single lens imaging systems is chromatic aberration; due to the physical origin of the refractive index, it is wavelength dependent.‡ Thus, it would be impossible to record a

‡ For those interested, see for example The Feynman Lectures on Physics, Vol I., Ch. 31

color image in perfect focus. While this was the early motivation, microscope objectives are typically constructed with several lenses utilizing glasses with different refractive indices to correct chromatic aberration, spherical aberration, and flatness of the image (Figure 6).[§] In this lab you will characterize these aberrations in your microscope, and in the next modules, we will use a commercial objective for optimal image collection.

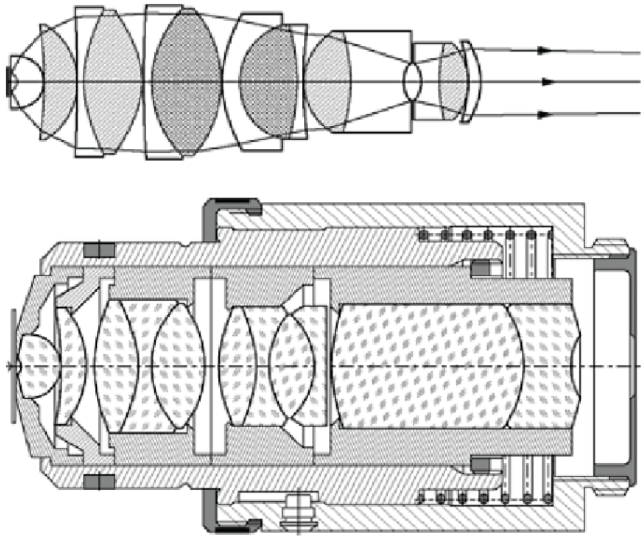


Figure 6. (top left) Design of Nikon Plan Apochromat oil-immersion objective with 60× magnification and 1.40 NA. (bottom left) Carl Zeiss Infinity Color-Corrected 63/1.4 Plan Apo objective. *From* *Handbook of optics Vol.1*, Chapter 28: Microscopes (right) Illustration of image distortions

In addition to allowing for the correction of aberrations, a compound lens system allows for increased magnification (Figure 7). Wherein the ball lens example, we found that the limit could be pushed to get a magnification of ~300x, this can be accomplished more simply by combining a 30x and 10x lens pair. As commercial objectives with 100x magnification as readily available, pairing with a 16x ocular lens can yield 1600x nominal magnification. Why is it not possible to image atomic nuclei structure just by combining the magnification power of a series of lenses?

§ *Handbook of optics Vol.1*, Chapter 28: Microscopes and Handbook of Biological Confocal Microscopy, Chapter: Objective Lenses for Confocal Microscopy

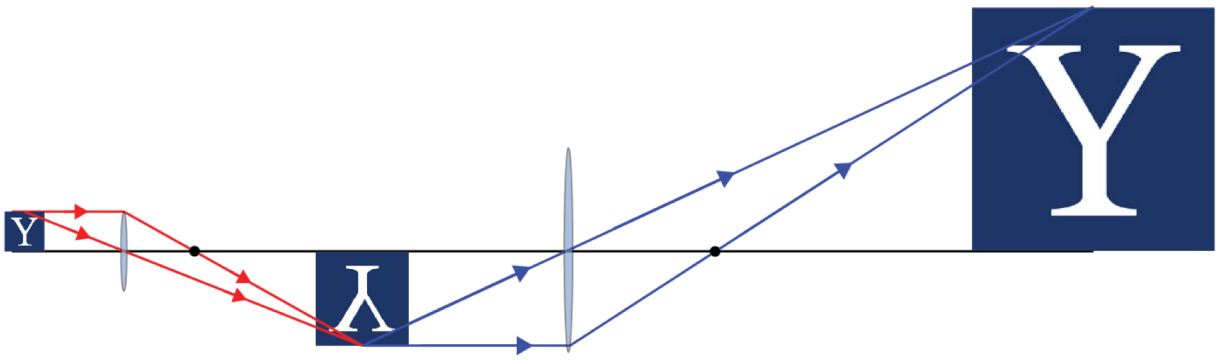


Figure 7. Magnification by a pair of lenses can be treated with the same ray tracing as shown in Figure 2 by using the image from the first lens as the object for the second lens.

IV. The Diffraction Limit

As lensmakers realized that technology was opening up a new world for investigation, they worked to improve magnification while reducing aberrations. However, it was not understood whether the barrier to perfect imaging was due to practical issues (and therefore worthy of devoting resources to solve) or something fundamental.** Ernst Abbe developed a criterion for imaging based on systematic microscopy of diffraction grating samples. In 1873, he wrote "No microscope permits components (or the features of an existing structure) to be seen separately if these are so close to each other that even the first light bundle created by diffraction can no longer enter the objective simultaneously with the non-diffracted light cone."†† What does this statement mean?

In addition to use diffraction grating samples, Abbe correlated image quality with an observation of the back focal plane. The idea is illustrated in Figure 8. By imaging the sample plane, a view of the grating is obtained – as expected, a series of dark lines on a glass slide. Abbe noticed that finer gratings diffracted light to larger angles and gave wider images in the *back focal plane of the objective*. If this intensity at the edges was not captured by the objective lens, the grating was blurry or unresolvable. From this, he determined a resolution limit (d), as a function of the wavelength of light (λ), the refractive index of the imaging medium (n), and the half angle over which the objective can gather light from the specimen (θ). The combination $n \sin(\theta)$ is known as the numerical aperture (NA),

**See also Handbook of Biological Confocal Microscopy, Chapter: Fundamental Limits in Confocal Microscopy

†† Innovation: The Magazine from Carl Zeiss. In Memory of Ernst Abbe.

$$d = \frac{\lambda}{2n\sin(\theta)} = \frac{\lambda}{2\,NA}. \quad (5)$$

This relation began as an empirical observation and microscopists accepted it as a fundamental limit before it was theoretically derived from a Fourier transform theory of optics. In modern language and Figure 8, what Abbe called the "first light bundle created by diffraction" is the first diffraction order and the "non-diffracted light cone" is the zeroth order.

What do diffraction gratings have to do with real samples? It was recognized that fine features, whether they come from the periodic edges of a diffraction grating or from sharp edges in the sample plane, appear in the edges of the back focal plane. Diffraction gratings in the sample plane act as a model system to systematically study how features of different sizes are imaged in microscopes, and real systems can be thought of as linear combinations of diffraction grating patterns.

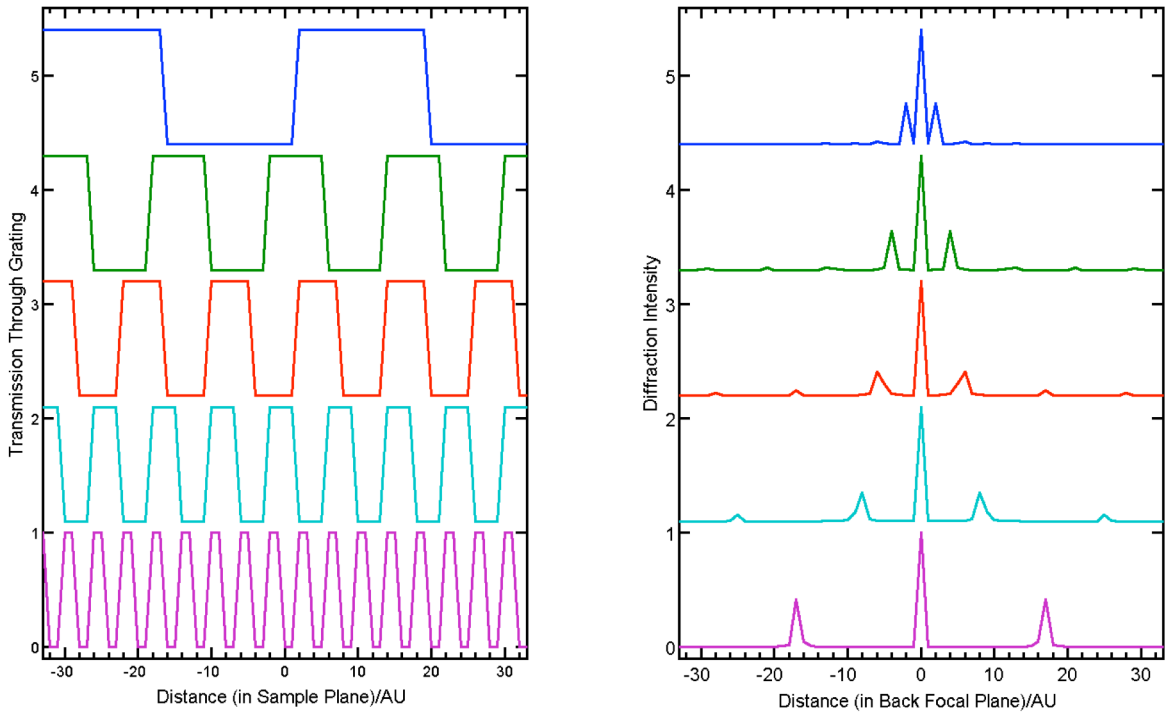


Figure 8. (left) Transmission through five diffraction gratings with varying line spacings. All depicted gratings transmit either 0% or 100% of the light depending on where in the sample the light is incident, and the gratings are vertically offset for clarity. (right) Intensity of the corresponding diffraction grating samples in the back focal plane of the objective. Note that a zeroth order peak is always present in the center, while the flanking first-order diffraction peaks approach the center as the grating becomes wider.

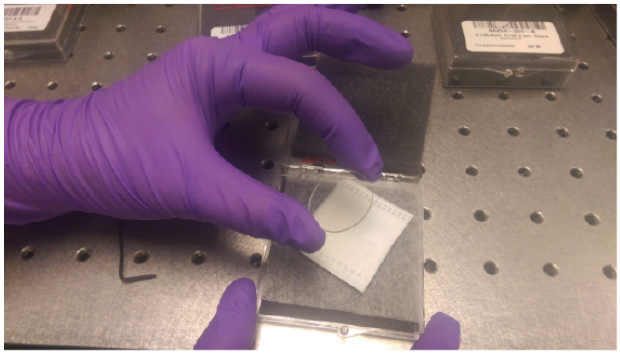
V. Safety and Proper Optics Usage

The goal of this laboratory module is to introduce you to the general working principles of microscopy, but along the way we hope to familiarize you with good optical practice to enable you to understand and build your own optical devices in research.

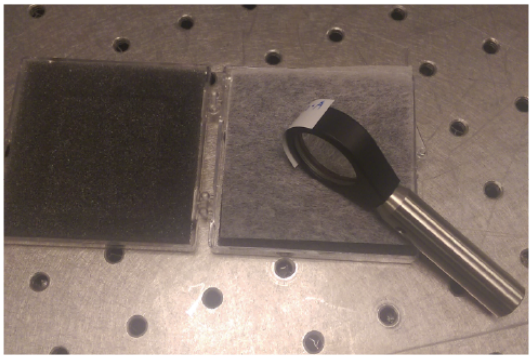
As perhaps the first time practicing these new skills, appreciate that there is a learning period required. How tight should I make this screw such as to keep the optic in place but not damage anything? My image is slightly blurry, how much does a slight adjustment of the lens *feel like* in my hands? When I let go of this unrestrained mount, is it balanced or will it fall over? Am I about to bump something with my second hand? Needing to keep a mind towards safety (for you, your lab partner and TA, as well as the equipment) while practicing new optical alignment skills and trying to complete the requirements of the lab module is certainly not easy, and we ask for your focus and patience. To facilitate this process, this lab has been designed such that *nothing needs to be rushed*. Feel free to work as slowly as you need to remain comfortable with understanding at each step. We believe that as you gain experience in the first hour or two, you will find this unfamiliar world soon becoming more intuitive and have plenty of time over the two lab periods to complete all the requirements. We hope you find the experience as enjoyable and as inspirational as the instructors did when they were in your place.

- Don't touch optical surfaces with bare hands or with gloves
    - Handle optics by the side with gloves
- Always wear gloves while handling
    - To keep optics safe from oils on your skin
    - Some optical materials are hazardous
- Always hold optics a few inches over the breadboard
    - (Imagine they could be dropped at anytime)
- Always block the light source before inserting an optic into the beam path
    - Similarly, do not wear shiny jewelry such as watches and rings while aligning optics
- Always align at lowest light powers then turn up for data acquisition
- Never clean optics (leave it to instructors, some coatings are sensitive)
- Proper eyewear when lasers on (none in this lab)
- Do not have your eyes at beam height with or without goggles
- Store optics sideways on lens paper (never flat, which collects dust)

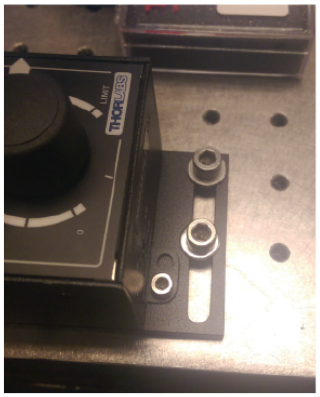
GOOD IDEA



If necessary handle optics directly above the table by the side while wearing gloves

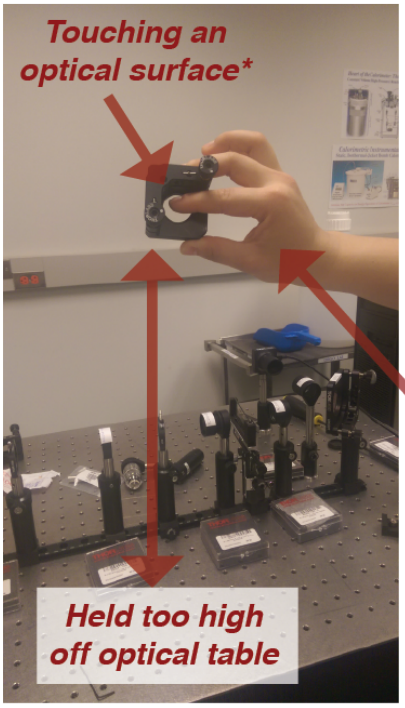


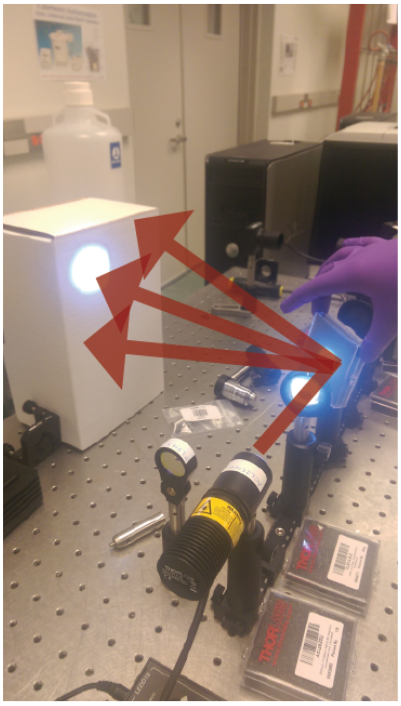
Store optics sideways on lens paper when not in use (to avoid dust collection)



Extend the life of our optomechanics by using washers with screws where possible

BAD IDEA





Do not insert optics into an open beam; uncontrolled reflections are dangerous

***No optics or retinas were harmed in creating this warning.**

VI. Day 1 - Microscope Construction

Your goal for today is to build a microscope. Figure 9 diagrams the alignment procedure. Try to get the sharpest, clearest image that you can. If you have plenty of time left over, you may think about beginning to characterize your microscope, but that is largely the task for Day 2.

Step 1. Align Tube Lens and Camera

a) Make sure camera and tube lens are at same height
   - A good beam height is ≳130 mm; some optics cannot be adjusted any lower
   - Make your life easy and set all optics to this height

b) Position tube lens to focus an image from infinity to the image plane camera

c) Start ThorCam and choose C1284R13CC
   - ..CC is the color camera, ..MM is monochrome you will use later

d) Under the camera settings (gear icon) set the exposure time, gain, and possibly RGB balance to get an image to your standards

e) Record this image of an object "at infinity"

f) Cover camera with a lens cap

Step 2. Collimate LED

a) Place LED and collimating lens on optical rail

b) Move lens to produce a minimally diverging beam
   - Proceed by moving the lens until you see an image of the LED grid pattern, then systematically move this image towards "infinity away"
   - Good results achieved when image of LED (focused) in front of camera (roughly 2' away)
   - See Figure 3 for examples of uncollimated and well-collimated beams
   - At this point, your setup should resemble Figure 9A
   - You may use a set of two irises to check your collimation (see Figure 9B)

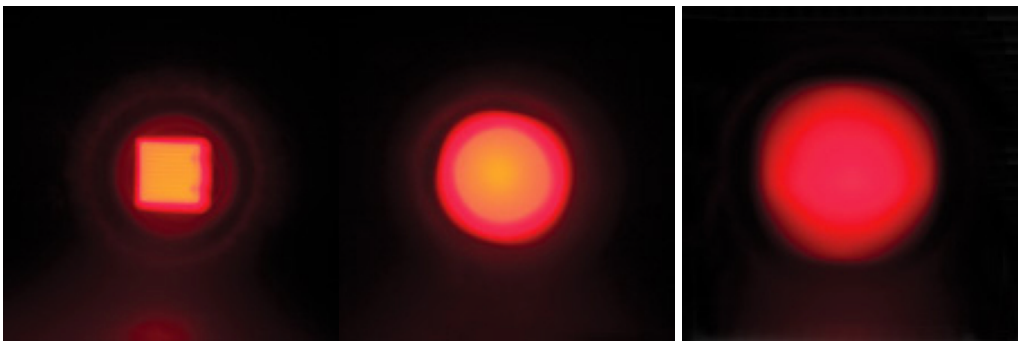


Figure 3. (left) Image of the LED. (middle) Uncollimated beam. (right) Collimated beam. "Collimating the LED," Copyright 199-2015 Thorlabs, Inc.

Step 3. Image LED to camera

a) Adjust LED assembly to same height as camera and mount to rail

b) Inset beamsplitter
c) Acquire an image of the LED with the image plane camera
d) Turn down LED until both the image at infinity and the LED are superimposed
   - At this point, your setup should resemble Figure 9C

Step 4. Align objective to sample

a) Insert objective and sample holder as shown in Figure 9D
   - Distance between the objective/sample and LED and tube lens is not critical
   - Objective should sit very close to end of rail to facilitate Step 7a
b) Insert a grating sample in the sample holder
c) Adjust the objective until a focused image is obtained.
   - Integration time may need to be reset.
   - Setup should resemble Figure 9B
d) Record image showing an overlay of the sample and "infinity" object
   - Note that this illumination scheme (no condenser) wastes quite a bit of light
   - This image demonstrates that the tube lens-camera distance is correct, the beamsplitter is correctly aligned, and the objective is focused on the sample

Step 5. Align condenser

a) Insert condenser as shown in Figure 9E
b) Place the field iris in front of the LED (exact position not critical at this point)
c) Close down the field iris such that you can see the focused LED from the condenser on the sample
d) Image plane camera should show a bright spot
e) Record an image demonstrating uneven illumination with focused light

Step 6. Köhler Illumination

a) Insert condenser iris as shown in Figure 9F, exactly f away from condenser
b) Insert field lens such that image of LED focused on condenser iris
c) Insert field iris and adjust edges of the field iris are sharp
   - Line profile overlay in ThorCam is very useful here
   - May need to adjust condenser position
f) Record final result image showing edges of field iris

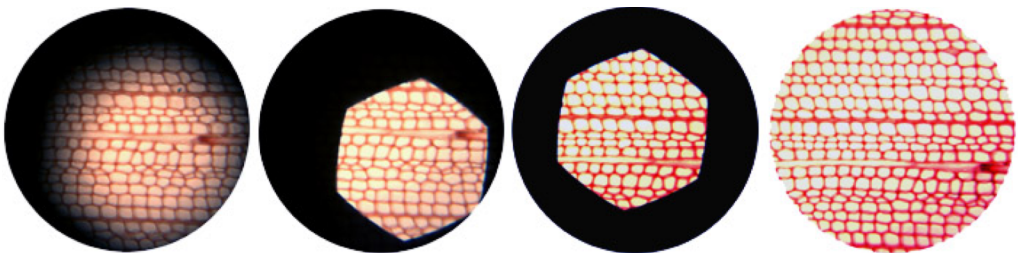


Figure 5 Köhler Illumination Steps. Adjustments are made until the edges of the field iris are sharp and centered. This results in optimal illumination. Copyright © 2005 University of Victoria.

Step 7. Operate the Image Plane and Back Focal Plane Cameras Simultaneously

a) Insert projection lens and the back focal plane camera as shown in Figure 9G
b) Make sure both cameras are plugged in
c) Start Micromanager Software
   - Micromanager should load the monochrome camera automatically

Step 8. Acquire image of back focal plane

a) Narrow both irises down all the way, reduce power to minimum visible
b) Insert the bandpass filter in the beam path (its position is not critical) to make monochromatic, green light
c) Make sure that the grating is clearly visible on the image camera
d) Look for an image that resembles three horizontally displaced white dots on the back focal plane camera and adjust it to focus
   - Record two images showing the image plane and back focal plane for the same sample

Optics Parts List

Part	Description	Part Number*
Tube lens	f = 150 mm, Ø25mm, Achromat	AC254-150-A
Objective lens	f = 30 mm, Ø25mm, Achromat	AC254-030-A
Condenser lens	f = 50 mm, Ø25mm, Achromat	AC254-050-A
Field lens	f = 150 mm, Ø25mm, Achromat	AC254-150-A
Collimating lens	f = 20 mm, Ø25mm Aspheric	ACL2520
White light LED	6500K color temperature, 800 mW	MCWHL5
Beamsplitter	Ø2” 50/50 @λ=450-650 nm	EBS2
Projection lens	f = 50 mm, Ø25mm, Achromat	AC254-050-A
Image plane camera	USB CMOS, 1280 x 1024, Color	DCC1645C
Back focal plane camera	USB CMOS, 1280 x 1024, Monochrome	DCC1545M
Vertical grating sample	Variable Line Grating Test Target, 3" x 1"	R1L3S6P
Bandpass filter	Transmits light only with λ=514.5 ± 3 nm	FL514.5-3
Radial grating sample	Sector Star Test Target, 1"sq, 72 Bars	R1L1S3P
Horizontal/vertical resolution sample	NBS 1952 Resolution Target, 3" x 1", 2.4 to 80 lp/mm	R1L3S10P
Point grid sample	Grid Distortion Target, 1.5" sq, 1000 µm Grid Spacing	R2L2S3P4
f =focal length, Ø=diameter, λ=wavelength *ThorLabs part numbers given		

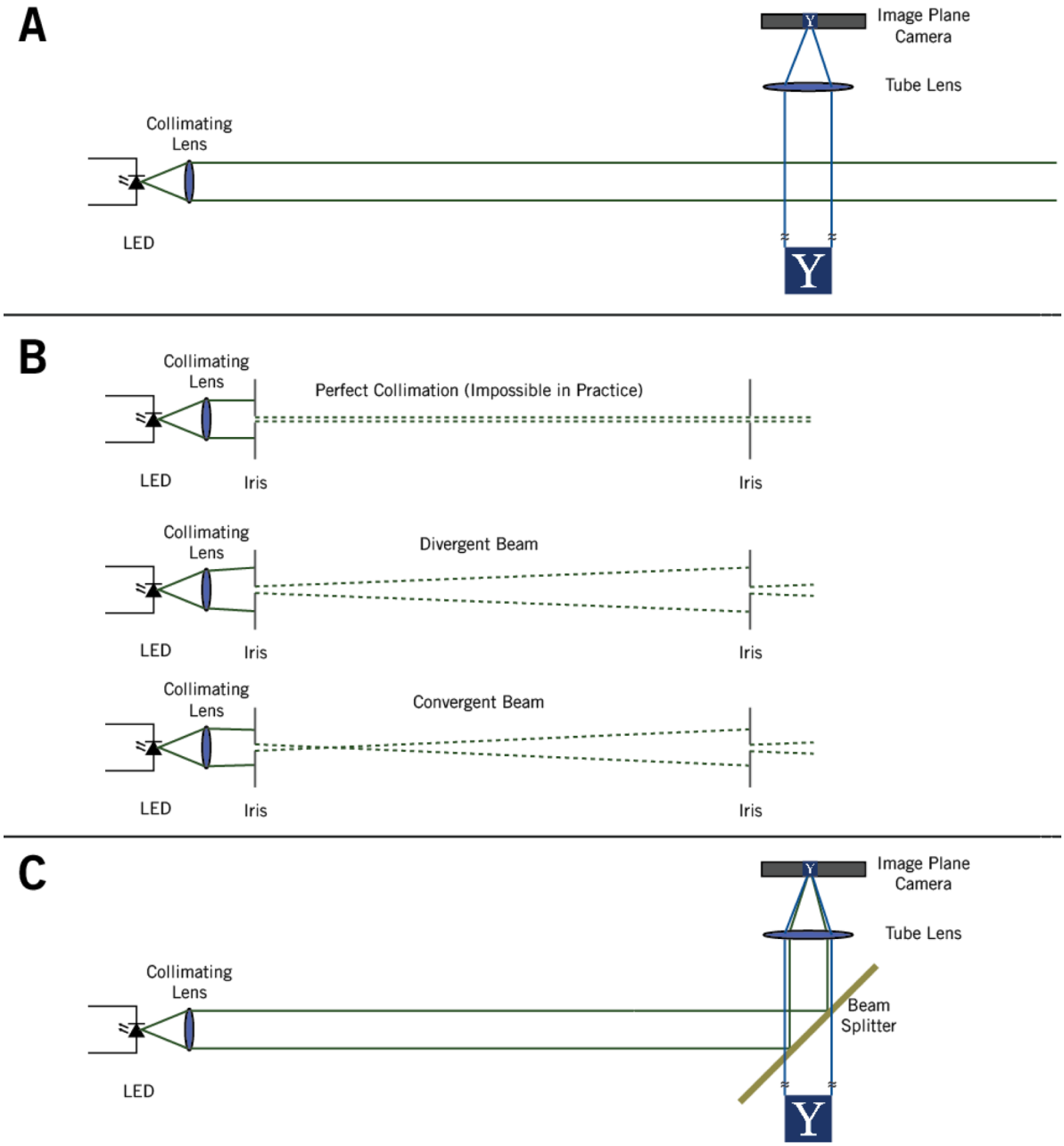


Figure 9. Steps in Microscope Construction and Alignment.

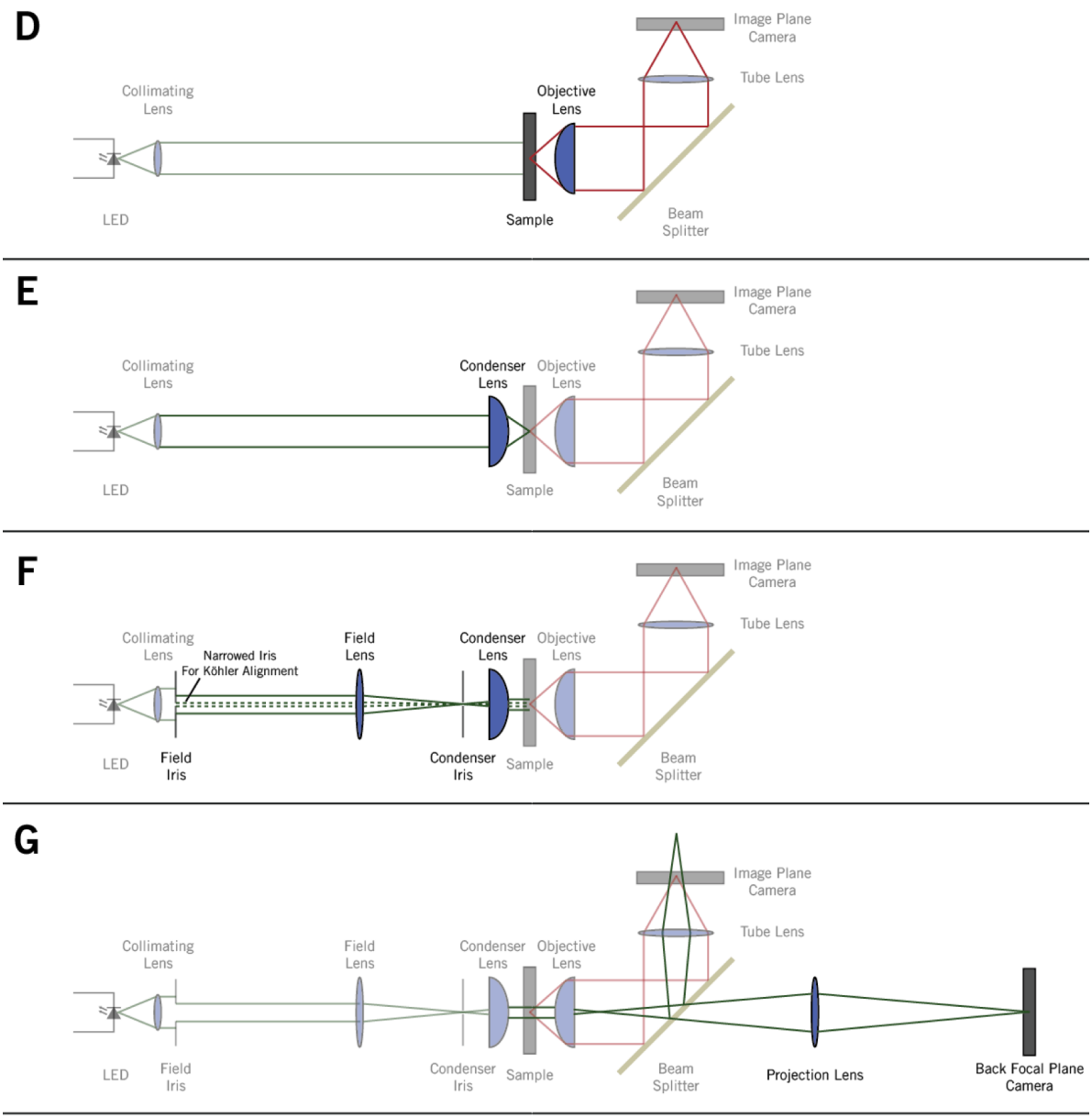


Figure 9. (continued). Steps in Microscope Construction and Alignment.

VII. Day 2 - Microscope Characterization

Characterize resolution *(can be done on Day 1 or Day 2)*

a) Using the calibrated ruled grating sample (Figure 10), take an image of the grating and use it to determine a pixel to mm conversion
b) Based on observing fine structures in the test samples, estimate the best resolution of your microscope
c) With this objective and tube lens combination, what is the magnification? What resolution would you expect?

Characterize gratings in the sample and image plane

a) Using the variable line grating (Figure 10), obtain a series of images in the object plane and in the back focal plane of different grating spacings
b) (Optional) Use the ImageJ software to Fourier transform the object plane and back focal plane images, which will give you four images to compare for each grating spacing
c) (***Awesome Optional Experiment*** devised by A.S. and A.E. in S17 Class) Place the *color camera* in the back focal plane and acquire an image with and without the bandpass filter.

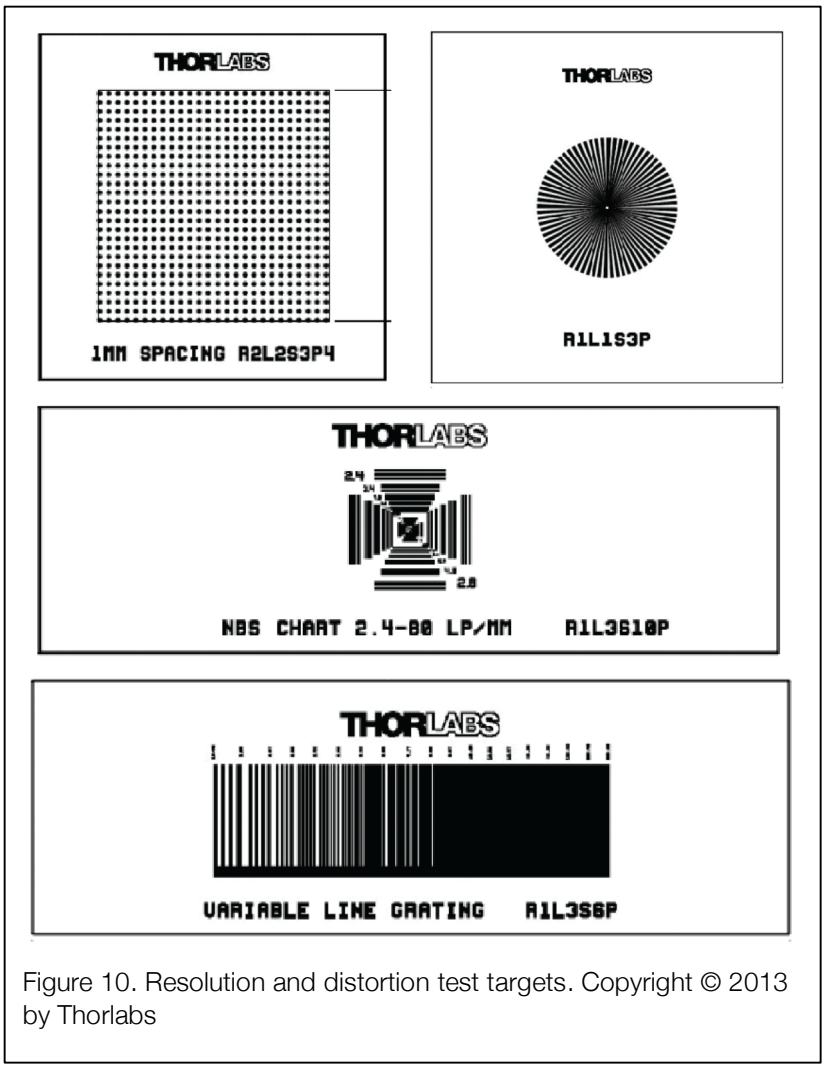


Figure 10. Resolution and distortion test targets. Copyright © 2013 by Thorlabs

Characterize Aberrations

a) Collect a series of images demonstrating the extent to which the following aberrations are present in your microscope using the available test targets

- Spherical aberration
- Chromatic aberration
- Non-flat image plane
- Asigmatism

VIII. Lab Write-Up Questions

A. Please include all images specified in the Day 1 and Day 2 instructions
B. Discuss any correlations in the sample plane and back focal plane images with diffraction grating spacing
C. Calculate your image resolution and compare this to diffraction limit
D. You went through several alignment steps (Figure 9D-G) with different illuminations. Please discuss any observations.
E. Why is the bandpass filter necessary? Describe what the back focal plane image would look like with white light (i.e., a broad spectrum spanning 400-800 nm)
F. Use your images to point out any imaging aberrations. How would you quantify these? (You do not need to do so.)
G. (Optional) Discuss whether you could recover the sample image via Fourier transform of the back focal plane image (and vice versa)
H. (Optional) Calculate your field of view. You may need the following detector specifications:
    i. Monochrome detector size is 6.656mm x 5.325mm, with 0.0052mm square pixels
    ii. Color detector size is 4.608mm x 3.686mm, with 0.0036mm square pixels

IX. Acknowledgements

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