

Hands-on Curriculum in Optics of Microscopy

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ABSTRACT Optics is an important subfield of physics required for instrument design and used in a variety of other disciplines, especially subjects that intersect the life sciences. Students from a variety of disciplines and backgrounds should be educated in the basics of optics to train the next generation of interdisciplinary researchers and instrumentalists who will push the boundaries of discovery or create inexpensive optical-based diagnostics. We present an experimental curriculum developed to teach students the basics of geometric optics, including ray and wave optics. The students learn these concepts in an active, hands-on manner through designing, building, and testing a homebuilt light microscope made from component parts. We describe the experimental equipment and basic measurements students can perform to learn these basic optical principles focusing on good optical design techniques, testing, troubleshooting, and iterative design. Students are also exposed to fundamental concepts of measurement uncertainty inherent in all experimental systems. The project students build is open and versatile to allow advanced projects, such as epifluorescence. We also describe how the equipment and curriculum can be flexibly used for an undergraduate level optics course, an advanced laboratory course, and graduate-level training modules or short courses.

KEY WORDS imaging; resolution; image analysis; active learning; project-based learning; CURE course

I. INTRODUCTION

Optics is the study of how light interacts with matter. For centuries, people have been able to manipulate materials to bend and reflect light. For the past 800 years, humans have shaped lenses from high-index materials to enable magnified imaging of objects. The combination of lenses into instruments resembling microscopes dates back 500 years. Despite the long history of microscopic measurement, advances in microscopy techniques are still being made today. For instance, the 2014 Nobel Prize in Chemistry was awarded for groundbreaking super-resolution techniques, and the 2018 Nobel Prize in Physics was awarded for the optical tweezer and its application to biologic systems (1, 2). Inexpensive, massively fabricated microscopes, such as the Foldscope, enable anyone to make microscopes from paper to examine the microscopic world or check their water supplies for parasites (3).

For the boundaries of instrumentation to be pushed in all directions, students need to be trained in basic optics concepts. Yet optics is often only taught in physics courses and is often dropped from the curriculum due to time and personnel constraints. Not only

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should optics be taught, but it also can easily and quickly be taught with modern methods of active learning, resulting in better learning outcomes in a short time. Luckily, students are motivated to learn optics to better understand their optical equipment and because optics allows for hands-on discovery and tinkering. Here, we describe an optics curriculum composed of mostly active learning: students work through guided activities to provide basic skills for designing and building a microscope. This curriculum is versatile, and we have offered it in a variety of educational venues, including a semester-long optics course, a senior laboratory course, and graduate-level short courses presented at summer schools and conferences.

II. SCIENTIFIC AND PEDAGOGIC BACKGROUND

A. Approach

The basis of the curriculum we describe is active learning, with a goal of replicating more realistic research experiences in a laboratory course. The course is not a full course-based undergraduate research experience (CURE), characterized by having a high level of each of these 5 elements: scientific relevance, collaboration, iterative approach, practicing science, and discovery (Fig 1A; 4). The curriculum we describe here has several elements that are CURE-like, in particular, collaborative nature, iterative approach, and practicing skills needed in real scientific research. There are also some elements of scientific discovery in the course. The researchlike parts of the curriculum are highlighted throughout the text.

For collaborative research, students work in small groups to build a light microscope from component parts. Prior to starting the project, students work through initial guided experiments to give them the foundations to build a microscope. The guided hands-on work and project all have rubrics for establishing expectations, learning goals, and for easy grading. Rubrics focus on student mastery of optical concepts and skills. For the project of building a microscope, students must test the specifica-

tions of their systems, leading to trial-and-error iterative learning that reinforces optical concepts and a deeper understanding of fundamental science concepts, such as measurement accuracy, uncertainty, and reproducibility. The project can, likewise, be extended to add on advanced projects for students to create designs, build and rebuild the systems, and evaluate the effectiveness of the systems. The higher level learning goals of analyzing, evaluating, and creating have been shown to lead to better learning outcomes and longer retention of concepts (5).

B. Assessment

One struggle when having students work in groups is how to properly assess individual learning gains. For a graduate-level short course, in which there are no grades, this does not matter so much, and students often self-regulate based on interest. In a semester-long majors' course, we have developed several mechanisms to individually assess students in the course.

Although we focus on the laboratory curriculum to build students' experimental skills, there is also a more traditional, content-driven curriculum that accompanies this work. That content-driven curriculum can be taught in a variety of ways, such as reading and doing homework, lecture, in-class group work, and exams. We use self-assessment, competency-based exams, in which each student must pass competency on 10 different areas of optic concepts. These exams are completed individually and give students the opportunity to demonstrate individual knowledge, despite working in groups.

For the lab curriculum, some of the grades are group grades, and some are individual. Group grades are mostly described for hands-on work in lab modules and the project, with each having a rubric. The entire group gets the same grade for these components of the lab. Individual grades are given for how well students work in their group and in a hands-on practical assessment used as the final exam. To grade the students' ability to work in a team, students assess themselves and the group

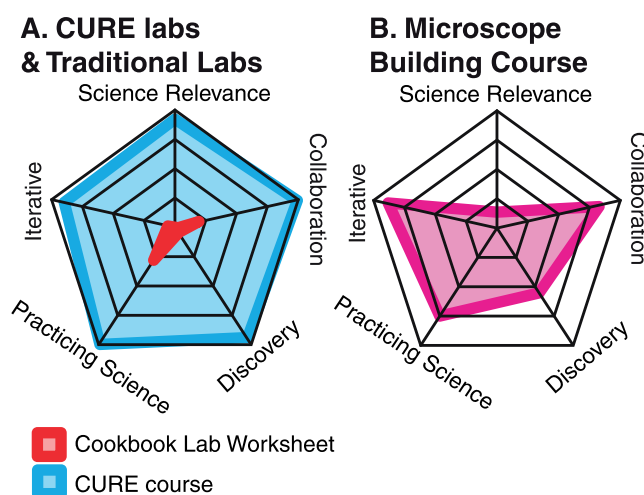


Fig 1. (A) Radar plot of the 5 parts of a course-based undergraduate research experience (CURE): science relevance, collaboration, practicing science, iterative approach, and discovery. CURE seeks to create research experiences for students that encompass all these elements (blue). Traditional, “cookbook” labs do not encompass these elements (red). (B) The course presented here encompasses some of the elements of CURE but not all (pink). Images modified from (1).

members and assign a grade. The group work grade is worth 10% of the total grade for the course, providing positive incentive to work together well. When the students are first assigned groups, they spend time talking about group expectations for participation, communication, and peer support. Based on the guided discussions, each group creates a rubric to grade the performance at the end of the semester. At the end of the semester, we return the rubrics to each group. Each group is given the opportunity to update the rubric, in case they did not consider an important aspect. They must agree to any updates as a group. Based on the agreed-upon rubric, each student scores himself or herself and the team members. The student-assigned scores are aggregated to assign a grade. In general, this process works well to ensure that expectations within each group are articulated early and judged fairly. Students have responded positively to this approach in end-of-term evaluations.

The second individual assessment for the laboratory skills is the final exam, which is given as a hands-on practical assessment. In this assessment, students are given an hour to design and build an optical configuration based

on a set of articulated needs. The question is articulated as if they are an optical engineer for a company, and the instructor is a customer who needs an optical system built. For instance, the customer might have a laser with a diameter of 2 mm, but they need it to have a 10-mm diameter. Students are graded on the ability to address the question by designing and building an optical system that solves the customer’s problem. There is a rubric given to students beforehand to make sure they are demonstrating good design skills, such as ray tracing and building skills (properly mounting optics, and testing and trouble-shooting skills), to ensure the design works as expected. Student evaluations have shown that students enjoyed the practical assessment and felt that they were able to demonstrate individual laboratory skills. Several students have commented that the practical assessment was “the most realistic exam” they had in college.

C. Resources

Hands-on courses often take a commitment of time and human resources. For this curriculum, the amount of time and human resources depended heavily on the venue in which it was offered. For the undergraduate course for interdisciplinary majors, the hands-on building components took about 50% of the course time (total of about 26 h in-class) to allow for more tinkering time. The human resources were one instructor and one teaching assistant for about 12 students (4 groups of 3). A smaller version, for example 6 students, could be taught without a teaching assistant. For the graduate-level short courses, we devoted only a 1 to 1.5 d (8 to 12 h) to the curriculum, but it was mostly hands-on, with only 20% of the time used for lectures. In these courses, we often had 8 groups of 4 students, and each group had an individual instructor to offer guidance. These instructors were faculty of the short course, including academic and industrial scientists from microscope companies, who presented other aspects of the course.

Overall, the supervision is mostly needed for guidance and feedback to student-led tinkering. For courses that need to move faster, more

instructors are recommended so that student misconceptions and missteps are corrected faster. For courses that have the luxury of time, fewer instructors allow students to take misconceptions and missteps further down the rabbit hole, often allowing them to correct themselves. The student-to-faculty ratio is flexible and can be adjusted to fit your needs.

III. MATERIALS AND METHODS

A. Experimental equipment

For students to design microscopes for their project, they need access to a variety of optical components. We have created a portable kit of optical equipment for students to use. Table 1 outlines the equipment needed; images and part numbers are from ThorLabs (Newton, NJ; used by permission and not meant to endorse a particular vendor) and possible substitutions. Because the parts are durable and basic, reused, recycled, or even 3-dimensional (3D)-printed parts could be used (6–8). Indeed, we have done just this for a recent 1-d short course presented on the Sunday before the American Physical Society March Meeting (9). We encourage instructors to use creativity to find inexpensive substitutions for the parts described. For instance, breadboards could be made in machine shops, or smaller models can be used to reduce cost.

The optical breadboard with 20 tapped holes (0.25 in) in a 1-in² grid was used as a platform to build the microscope if an optical table was unavailable. Using small breadboards saved space and allowed for portability. One dimension needed to be at least 2 ft long so that the optical train was long enough to achieve the correct magnification, but the other dimension could be as thin as 6 in for a standard transmitted light microscope. Rubber feet were used under the breadboard to elevate and stabilize the system against vibration. Using breadboards allowed the systems to be packed away during alternate semesters or transported for other labs to use.

To create the optical train, we chose to use dovetail rails that were 0.5 in wide. Rails are common in other prefabricated optical labora-

tory systems for student labs, such as those that can be purchased from PASCO, because they allow easy positioning of the optical components along the same optical axis for students who are new to aligning optics. Unlike large, bulky systems from PASCO, the 0.5-in rails are inexpensive and are authentically similar to what one might find in a research laboratory. Further, other rail systems, including inexpensive homebuilt systems (6) or those printed with new 3D printers, would also work (7).

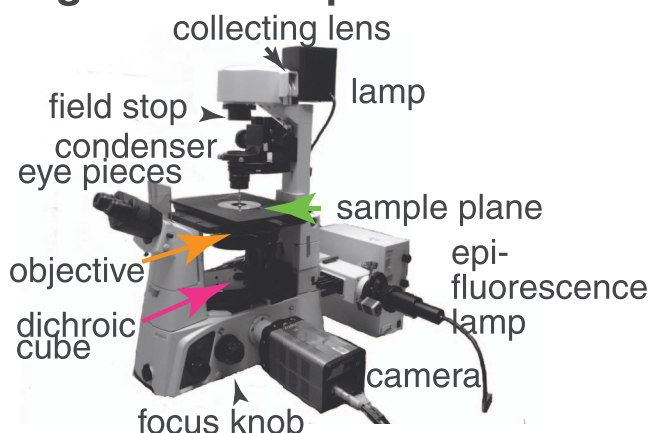
We chose rails over cages for confining the optical train because cages are more difficult to add and remove new components. Because the students experience substantial trial and error, the cage system becomes hindering to ad hoc additions or subtractions of components. We also chose rails over affixing the components to the optical board with kinematic mounts because new students often have difficulty aligning freely mobile optical components. The rail is more rigid and easier to align for novice users. Thus, although there are a large number of options for optical systems, the rail system is preferred. Optical components were mounted to the rail on 0.5-in posts with postholders attached to dovetail rail cars. Short posts, 2 in long, are preferred for stability, but we have used 3- and 4-in posts without detrimental effects on the stability of the system. The ability to use what is available without negative effects makes this laboratory easily duplicable, adaptable, and able to reuse surplus parts.

The light source was a white light-emitting diode (LED) with a variable power supply. The variable power supply is a simple potentiometer that allows the intensity to be changed. The LED does not come with a collecting lens because we want the students to use it as a point source without a lens. A collecting lens is a crucial component of the condenser they need to design and build, so purchasing one with the lens attached would defeat the purpose of part of the exercise. The LED serves as a source for lab modules on image formation, as well as the lamp for the transmitted light path on the microscope. Although we do not use other filters, such as

Table 1. Optical components for laboratory curriculum.

Item description	Image	Part number	Use	Alternatives
LED lamp and controller		MWWHL4	Illumination of samples	Incandescent lamps, various
LED power supply		KPS101	Power LED	Available online at other retailers
CMOS camera, cable, and software		DCC1545M	Record image data via USB	Available at other retailers
Optical Breadboard and feet		MB2436, AV4	Stable building surface, minimizes vibrations, portable	Homebuilt
12" rails (x2), 6" rail		RLA1200, RLA0600	Mounting optics using rail carriers. Flexible positioning	3D printed
Rail carriers (x8)		RC1	Mount post holders and slide along optical axis	
Post holders (x8)		PH2	Hold posts with adjustable heights	3D printed
Posts (x8)		TR2	Mount lens holders and other optical components	3D printed
Slip ring		SM1RC	Holds LED	3D printed
Lens holders (x10)		LMR1	Mount lenses	
Lenses: 25.4 mm, 30 mm, 50 mm, 75 mm, 100 mm, 150 mm, 200 mm		LA1255-A	Building optical systems	Surplus versions
Tube retaining rings		SM1RR	Holds lenses in lens mounts	
Sample Holder (stage)		XYF1	Holds sample with adjustable positioning in x-y	Pressure mounts can be used
Objectives, 4x, 10x, 20x		RMS4X	Objectives for microscope project	160 mm focal length, infinity-corrected
Translation stage		DT12XY	Fine focus control	
Objective adapter ring		SM1A3	Mounts objective to lens holder, RMS	
Irises		SM1D12	Acts as field stops and aperture stops	
Quarter-20 screws		SH25S075	Most common screw needed for mounting	Found at local hardware store
Ball driver set (yellow wrenches)		TC2	Needed to tighten optical components	Hex wrench sets can be purchased from local hardware store
Spanner wrench (red wrench)		SPW602	Needed to place and tighten retaining rings to hold lenses	

A. Modern, Inverted Light Microscope



B. Microscope Light Path

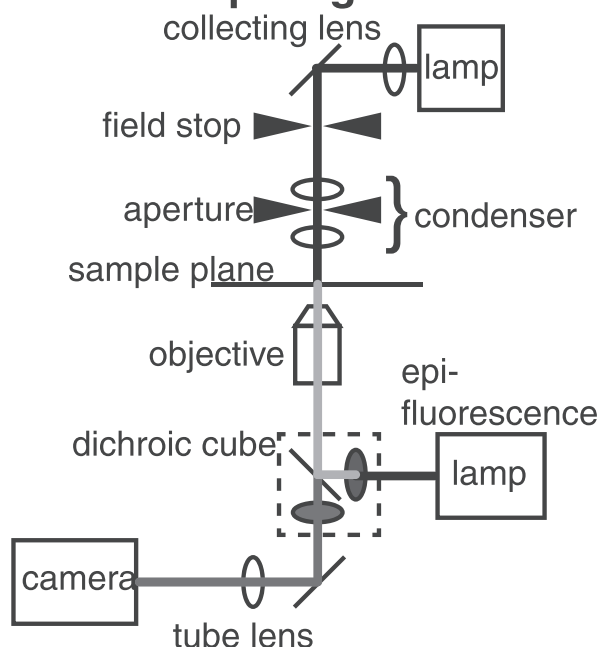


Fig 2. (A) Image of modern, inverted light microscope with optical components noted. Components are inside the black box, interrupting the ability of students to understand how the microscope works. (B) Light path for the same modern light microscope with optical components shown.

neutral density filters, a monochromatic interference filter, or a diffuser, those components are often used in modern condensers and can be added to alter or add to the iterative approach to the design and construction of the condenser.

Planoconvex and achromatic lenses of various focal lengths were purchased from Thor-

Labs or Edmund Optics to allow students to tinker with different lenses. Extra lenses with focal lengths preferred by the students, such as 30, 50, and 100 mm, were also purchased to ensure there were enough for playing and trial and error. All lenses were 1 in in diameter and held in 1-in lens holders mounted to 0.5-in-diameter posts.

Objectives of various magnifications were donated from prior laboratory courses on microscopy for use in this course. Both infinity-corrected and 160-mm-focal-length objectives were used to give students exposure to both types. A special adapter was purchased to mount the objective with Royal Microscopical Society threads to the lens holders. The sample holder can be adjustable (Table 1) or a simple pressure clip. A small translation stage with 0.5- to 1-in range can be used for fine focus motion. The translation stages can be placed under the objective or the sample holder on the rail.

Modern light microscopes use charge-coupled device or complementary metal oxide semiconductor (CMOS) cameras to capture images (Fig 2). We chose inexpensive but small and powerful CMOS cameras to capture images with a universal serial bus link to a student's laptop. The cameras are simple bare arrays of photodetectors (pixels). We do not recommend that the students use other cameras, such as the camera from their cellular phone, because those have lenses built in, to allow imaging of objects at a distance.

Many modern microscopes still include eyepieces, as scientists have come to expect them. Thus, eyepieces are not necessary for most modern microscopy applications. The microscope designs we describe here do not have eyepieces and do not allow the students to directly observe the image with their eyes. Instead, images can be observed by projecting onto cards or imaging onto the camera detector array. This is mainly because we are building the system horizontally on the bench, and leaning over to put a face in the beam path is unsafe. These safety issues should be stressed and repeated during the work. Here, we do not present any systems with lasers, but the system we develop can easily be modified to an optical

tweezer, confocal imaging, or total internal reflection fluorescence microscope when lasers are used. When lasers are implemented, laser safety guidelines must be followed, including keeping your eyes out of the beam path, which should stay horizontal to the breadboard.

An instructor could have students use their camera phones to capture the images, although we have not. Note that it should be stressed to the students that such cameras already have a lens in front of the detector. This unknown lens before the camera detector takes light that is collimated and focuses it onto the detector. For educational purposes, the phone's camera system can be likened to the eye that also has a built-in lens (cornea) in front of the detector (retina). To use a phone camera or the eye as a detector, the students must add optics to collimate the image light into the camera. This is similar to adding an eyepiece to the microscope. The eyepiece that is added can alter the magnification of the system and should be included in the discussions with students about calibration, magnification, and resolution (see the following).

The cameras we use need to be run by computers to display and record the images. They come with a software provided by the vendor that runs on personal computers (PCs). A student often provides his or her own computer, but computer workstations or laptops can also be provided. Instead of using the provided software, it is also possible to use Micro-Manager, an open-source microscopy software that can be used on Mac or PC (10).

Analysis software to make quantitative measurements from the images is also needed. We recommend FIJI–ImageJ for data viewing, manipulation, and quantification. ImageJ is a free Java-based software that works on both PC and Mac platforms. FIJI is a package for ImageJ with many useful additional plug-ins that we highly recommend. The students can open images, regardless of the format of the images, adjust the brightness and contrast, zoom in or zoom out on the image, crop the image, and save in a variety of file formats with FIJI–ImageJ. We recommend using FIJI–ImageJ because it is free, open to both Mac and PC

platforms, has a number of powerful measurement tools and plug-ins to allow sophisticated analysis of images included, and has more tools and help that can be found in online forums, making it incredibly accessible and powerful. Quantitative data can be exported for manipulation in other programs, such as MATLAB, Python, or Origin.

B. Methods

1. Guided exploration: laboratory modules

For students to productively and efficiently work on a longer self-directed project, they need some basics of optical skills and empiric understanding of how lenses, apertures, and detectors work (Table 2). To that end, we have designed 3 guided exploration exercises on these topics. The activities are hands-on with scaffolding worksheets to encourage students to investigate how optics work and to develop good practices for handling and placing optics. Each module can take anywhere from 1 to 4 h, depending on how much guidance is given in the scaffolding worksheet and the instructor support in the room. For instance, for graduate-level short courses, these can be performed in about 1 h with one instructor assigned per group. The worksheets can serve as both the guidance and the report or product for the activity. The worksheets have a short initial reminder of the learning objectives for the activity, followed by open-ended questions with room for students to sketch diagrams, draw pictures, and write equations to answer the questions. These questions encourage exploration and tinkering to help students develop an intuition for how the optical components function. Students have a rubric, which assigns points based on the open-ended output, not if they have the “right” answer.

2. Project: build a microscope

Using the skills and concepts gained during the guided lab modules, students plan and build a basic working microscope. Students are asked to test the magnification and resolution of their system to enhance iteration and learn about measurement concepts. The project has a rubric and scaffolding worksheet to help them to understand how to test their complet-

Table 2. Timing of laboratory curriculum.

Order	Curricular element	Learning goals or broad objectives
1	Laboratory module 1: lenses, focal lengths, and creating images	(a) How to find the focal point of an unknown lens; (b) how to create an image with a lens; (c) how to collimate light from a point source with a lens; (d) how to use a series of lenses to expand or magnify an image; and (e) increase your comfort level with lenses and how they work to make images such as those used in a microscope.
2	Laboratory module 2: aperture stops, field stops, focal planes, and numerical aperture	(a) How to manipulate light using irises; (b) acquaint students with field stops and aperture stops to give some physical intuition about the wave nature and Fourier optics used in optics and microscope design; (c) physically demonstrate the differences between field stops and aperture stops; (d) build and tinker with a multilens system with a field stop and aperture stop and discuss different focal planes within that system; and (e) discuss the connection between the aperture size and the numerical aperture of a lens system.
3	Project: building the condenser	Build a microscope condenser.
4	Laboratory module 3: detectors (cameras)	(a) Help students understand what cameras detect; (b) show students how to make an image on a camera; (c) have students become acquainted with the camera software and use FIJI–ImageJ to quantify images; (d) give students training and intuition on the meaning of the gray scale of the camera and how the gray scale changes with exposure time, averaging, binning, gain and offset, and other settings; (e) discuss measurement and quantification of light using a detector with sources of noise, measuring dark counts, discussing saturation, and quantifying the linear range of the detector.
5	Project: using an objective to make an image on the camera	Complete the microscope with camera.
6	Project: testing the magnification and resolution	Test the microscope.
7	Discovery: open-ended advanced project	Student-driven, open-ended project is presented.

ed system. In conversations with instructors, they are encouraged to try, test, assess, and correct repetitively, giving the work trial-and-error iteration. The ability to successfully try, fail, and correct helps students to create longer term learning gains. Each section of the project can take 1 to 6 h, depending on how much time is given for students to struggle.

3. Discovery: open-ended advanced project

Depending on the venue, the microscope project can include an option to build an additional optical system onto the basic light microscope. The point of this additional project is to give students the opportunity to research a scientific question, propose a possible experimental approach to the problem involving microscopy, research equipment parts and prices to create a budget, and iteratively design and build the new approach to address the scientific question. As with other parts of the course, the assessment is directed by a rubric that guides the student, because there is very

little scaffolding or worksheets that can be given a priori. The advanced project can take anywhere from 4 to 10 h of class time, depending on how ambitious the projects are, and the level of design, construction, troubleshooting, and testing needed.

IV. RESULTS AND DISCUSSION

A. Laboratory modules

To teach some basic optics prior to the students jumping into the project of building a microscope, we take students through several short, scaffolding modules to learn basic concepts. The worksheets provide both the scaffolding for the work, as well as the report and rubric for earning points (all scaffolding worksheets are available upon request). The structure of each worksheet is as follows:

- (a) Background section that briefly describes the fundamental concepts and principles needed for the hands-on activity. This can

be as long or short as each instructor wants. It can also be used to guide relevant content that the instructor may choose to deliver before the module. Alternatively, the instructor may want to use the module as an introduction to the content. The flexibility of this laboratory curriculum allows for any option.

- (b) A specific set of learning objectives for the lab module. This helps students orient their learning toward the hands-on design and building aspects. The modules also use concepts, and those can be included in the learning objectives, depending on how the instructor designs their course.
- (c) A set of miniexperiments for the students to work through in the groups. These are detailed in the sections that follow, with additional pointers for instructors about typical student misconceptions and how to enable student learning through tinkering.

1. Laboratory module 1: lenses, focal lengths, and creating images

In the first hands-on module, students are introduced to lenses and the idea that we can shape materials that can bend light to converge (focus) or diverge light rays, which can ultimately be used to create images. This laboratory module should be done before the project is started by students. The background section includes some reminders of the basic principles and concepts, including Snell law and the thin lens equation. This section can include the equations, required reading from optics textbooks (11, 12), or even short conceptual questions to get students warmed up.

After the background section, the worksheet lists the learning goals for the module: (a) how to find the focal point of an unknown lens; (b) how to create an image with a lens; (c) how to collimate light from a point source with a lens; (d) how to use a series of lenses to expand or magnify an image; and (e) how to increase your comfort level with lenses and how they work to make images, such as those used in a microscope.

The hands-on activity about lenses and images has 4 parts: (a) finding the focal length for an unknown converging lens, (b) collimating light with a lens, (c) creating real and virtual images with lenses, and (d) making images with a 2-lens system and testing the magnification of the multilens system.

(a) Finding the focal length of an unknown lens. As described previously, the kit of optics provides several lenses and mounts. Depending on the amount of time available for this work, students can either practice mounting 4 different converging lenses with unknown focal lengths (long format course) or start with lenses already mounted (short course). The lenses are labeled A through D, instead of with focal lengths so that the students can practice finding the focal length of an unknown lens. This is a skill that is often needed in real research labs in which, after a few years, optics do tend to become disorganized and misplaced. As with all optical techniques, there is more than one way to make this measurement. Students can bring collimated light in to see where it focuses or try to collimate a point source. The easiest method is for students to make an image of a bright, very distant object, such as the scene outside the laboratory window or down the hall.

After each group has come up with the focal lengths that is best, each group needs to know the actual focal lengths of the lenses for later use. If all groups have the same 4 lenses, they can compare with each other or report the empirically measured focal lengths to the group. Often, the focal lengths will be measured slightly differently from the actual focal lengths and each other. This is a good opportunity to stop and discuss measurement error, such as systematic or statistical errors in length measurement. Many of the short focal-length lenses are very thick, so it can be difficult to determine where to start the measurement of the focal length. Adding these discussions will help students understand measurement uncertainty and allow them to understand that the measurements were not “wrong,” but simply different, and this needs to be accounted for in scientific discussions.

(b) Collimating light. Next, students use a 50-mm focal length to collimate the light from an LED light source. This is a difficult yet important skill that offers an opportunity for trial-and-error iteration. After students think they have it collimated, the worksheet asks how far away from the lens it remains collimated or if the light converges or diverges. Because the LED is not actually a point source, but does have finite size, the light will often begin to form an image ~ 200 mm away from the lens and is significantly larger than the actual LED. A discussion can be had here with students to question if this result is reasonable or not.

As part of the discussion, instructors can remind students that the rays from a source “at infinity” (or very far away) could converge at the focal point to make an image. Collimating the light is just running the system in reverse, so the lens should make an image of the source at the focal length, and the image location should be at infinity. The result gives an opportunity to discuss the question, “What is ‘infinity’ for an optical system?” For most lenses, infinity is really just about 5 times the focal length of the lens. This is not about the lenses being imperfect or the student not being able to collimate properly—it is actually a true property of the wave nature of light. The rays that are collimated convolve back into the image at infinity or just ~ 5 focal lengths away from the lens.

(c) Creating real and virtual images. Now that students have created collimated light, they can use that to illuminate a sample that can be imaged. In this case, a good sample is a clear plastic ruler. Using a second 50-mm lens, students are guided through placing the lens at different locations with respect to the ruler to observe the image of the ruler and determining if the image is either real or virtual. This can be confusing because there is also an LED, and the second lens will also make an image of the LED at a different location from the location of the image of the ruler. If this is becoming too confusing, we often encourage students to use the phone as the source for the sample. When turned up to full intensity, modern phone screens produce enough light

that images can be made of them with lenses, especially in a darkened room. Most importantly, the sample needs spatial structure that can be measured on the object and the image of the object.

When the lens is closer to the sample than 1 focal length, a real image cannot be formed onto a screen. Instead, the students can look into the lens and see a magnified virtual image of the samples. When the lens is farther than 1 focal length, a real image of the sample can be formed on a screen. Students can use a second ruler to measure the distances between the source and image to compare the results to the thin lens equations:

$$\frac{1}{s} + \frac{1}{i} = \frac{1}{f} \text{ and } M = -\frac{i}{s} = \frac{h_i}{h_s}, \quad (1)$$

where s is the source distance, i is the image distance, and f is the focal length. The magnification, M , can be determined from the lateral size of the image, h_i , and source, h_s , or from the image and source distance.

(d) Creating a 2-lens magnifier system. The last task of this activity is to use 2 lenses to create a magnified image of a sample (source) and measure the magnification. They will be creating a 2-lens magnifier, such as an objective. Students are instructed to place the 50-mm lens 50 mm from the source and a second 200-mm lens 100 mm from the 50-mm lens (Fig 3).

With the 2-lens system, an image should be created that is 200 mm from the second lens and magnified by $4\times$. Of course, students are encouraged to play with this system, changing distances and lenses to see how that changes the image location and magnification of the final image. One specific opportunity is to discuss “infinity spaces” with students. These are locations in which the light is collimated within an optical system, and the light acts the same way within that space. In this 2-lens system, the space between the 2 lenses acts as an infinity space for the rays from the sample (Fig 3B).

Working with students on the board to sketch the ray diagram will demonstrate the infinity space and that sometimes the same ray

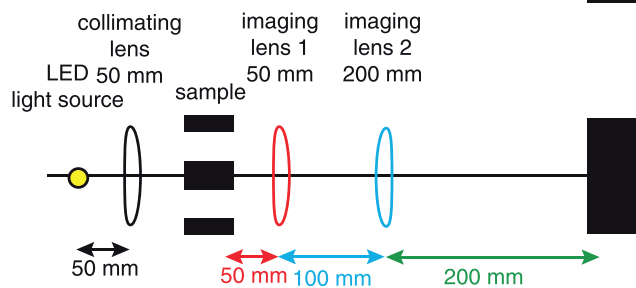
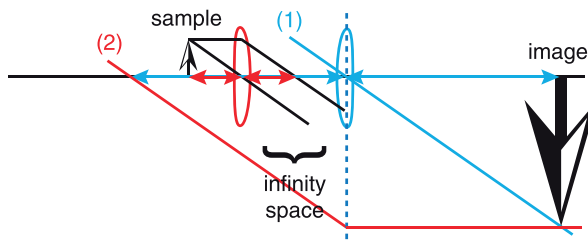
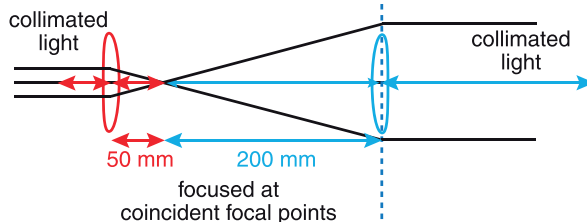
A. Two-lens optical system: 4x magnifier**B. Ray tracing: 4x magnifier****C. Ray tracing: 4x beam expander**

Fig 3. Two-lens system. (A) Schematic of the 2-lens system to create a 4 $\times$ magnifier (telescope and beam expander). If the sample is placed 1 focal distance from the first lens (50 mm), the image will be at the focal distance from the second lens (200 mm), and the magnification will be the ratio of the focal lengths (200/50 mm = 4 $\times$). (B) Ray tracing through the 2-lens system as set up shows a space between the 2 lenses where the rays are collimated (parallel) and angled. The ray that runs through the middle of the second lens (1, blue) and the ray with the same angle that emanates from the focal distance of lens 2 (2, red) can be used to find the height of the image. (C) Same 2-lens system altered to create a 4 $\times$ beam expander or 4 $\times$ telescope. The main difference is that the distance between the 2 lenses must equal the sum of the focal lengths ($f_1 + f_2 = 50 + 200 \text{ mm} = 250 \text{ mm}$). In the ray tracing, the collimated light focuses at the coincident focal points and is recollimated by the second lens with a 4 $\times$ larger width.

cannot be propagated through the entire system to determine the image. For instance, to find the image location, 2 new rays must be used for the second lens, namely, the ray that

passes through the center of the second lens (Fig 3B; 1) and the ray that would emanate from the focal point of the second lens (Fig 3B; 2). These rays all have the same angle in the infinity space, but the ray that appears to come from the focal point bends at the location of the lens. Additionally, the lens axis is extended to help find where the lens should be for ray tracing (Fig 3B; dashed). This example is useful for reiterating to students that the rays of ray tracing are not what the light is truly doing but rather a helpful tool to find the image of a given source.

For the 4 $\times$ magnifier described, the sample is placed at the focal plane of the first lens, but a similar system can be a beam expander or a telescope, with minor adjustments (Fig 3C). For the beam expander, the infinity space between the 2 lenses must increase to equal the sum of the 2 focal lengths between the lenses ($f_1 + f_2$). The collimated light, perhaps from a laser or a distant star, focuses at the focal point of lens 1, which is coincident with the focal point of lens 2. The light is recollimated by lens 2, with a larger width. Simple trigonometry of similar triangles can show that the change in the beam width is equivalent to the ratio of the 2 lenses: f_2/f_1 . Because light can move through optical systems in either direction, the same system can also reduce the size of a collimated beam, if light goes through lens 2 and then lens 1. The students can check that the beam expander can also still be used as a 4 $\times$ magnifier, with a larger infinity space in the middle. We recommend that these changes be explored or commented on while reminding students that these different terms (magnifier, beam expander, and telescope) can be used for the same basic optical system, depending on the application. This will help them to draw connections throughout the course.

2. Laboratory module 2: aperture stops, field stops, focal planes, and numerical aperture

The second hands-on lab module focuses on the manipulation of light through field stops and aperture stops. The concepts of this lab module are important for students to be able to design and build the condenser, so it should

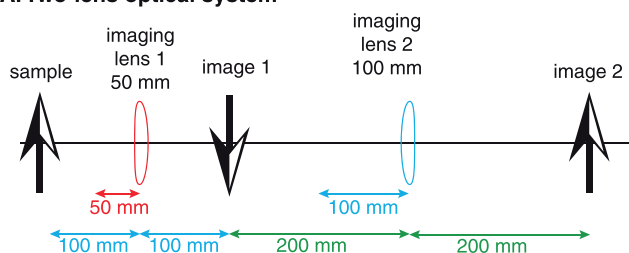
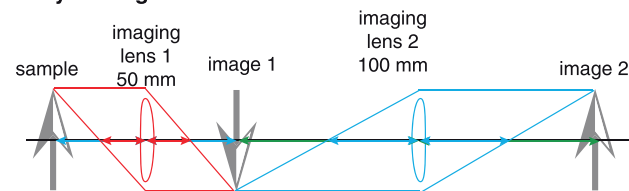
A. Two-lens optical system**B. Ray Tracing**

Fig 4. Two-lens system used for field stop and aperture lab module. (A) In this example, the system makes 2 images of the same sample by placing the sample 2 focal lengths from each lens. Any combination of lenses should work for this, but we show a 50- and a 100-mm lens. The image formed between the 2 lenses will be inverted, compared with the sample and the final image. (B) Ray tracing and analytical techniques can be used to demonstrate why there is not magnification of the images and why the images invert.

be done before the microscope building project is started (Table 2).

In the background section for the worksheet, students are reminded that they can manipulate light with the material properties of an object, such as in a lens, but that they can also manipulate the light by blocking parts of the light path by using irises. Further, the location, shape, and size of the iris or other blockage changes the way light propagates through the optical train. The goals for this activity are the following: (a) how to manipulate light by using irises; (b) acquaint students with field stops and aperture stops to give some physical intuition about the wave nature and Fourier optics that can be used in optics and microscope design; (c) physically demonstrate the differences between field stops and aperture stops; (d) build and tinker with a multilens system with a field stop and aperture stop and discuss different focal planes within that system; and (e) discuss the connection between the aperture size and the numerical aperture of a lens system.

The hands-on activity about aperture stops and field stops has 3 parts: (a) building and

tinkering with field stops, (b) building and tinkering with aperture stops, and (c) understanding numerical aperture. For these sections, it is helpful if a commercial microscope stand is nearby for pointing out the same features within the black box of the microscope body.

(a) Field stops. A field stop is an iris that controls the location of the light. Much like the curtain on a stage, it physically blocks the light from parts of the sample or the detection region. Managing light by using field stops is increasingly important for modern applications that need to control the location of light on a sample, such as for fluorescence recovery after photobleaching, fluorophore or chemical photoactivation, or other new optogenetic switches. Most of the time, we do not want to put the physical barrier to block the light on the sample. Instead, it is easier to put the light blocking barrier in a plane that is conjugate to the sample. If a commercial microscope is nearby, you can show the students how the field stop in the condenser works to block the light by either following the light path or by using the eyepieces while opening and closing the field stop. Note that the microscope condenser should be in Köhler illumination to observe the field stop in focus.

To demonstrate these concepts in a hands-on way, we have students build a new 2-lens system, with alternating focal planes between the lenses. The easiest way to do this is to place the source (ruler or phone) 2 focal lengths from a first lens to make a real image 2 focal lengths away from that lens. A second lens is placed so that the image from the first lens becomes the source for the second lens, which again is placed 2 focal lengths away (Fig 4). The images should not change size (no magnification) and are translocated and inverted after each lens. Students are encouraged to use an index card to trace the rays through the system to find the locations of the images, draw the system, and trace the rays through the system to understand the image formation.

To use the idea of the field stop, students are instructed to place an iris between the lenses at any location. They make the iris and open and

close, attempting to find the best location for the field stop. We stress that the image of the iris will be focused when the iris is placed at the correct location. When properly placed to act as a field stop, the iris should be at the image location between the lenses (Fig 4).

When the best location is found, closing the iris results in the final image being blocked on the edges with a focused edge. At this location, the iris acts as a field stop. When they find the best location, students are directed to draw the images with the iris open or closed and trace the rays through the system from the field stop to show that the edge of the iris will be imaged on the image plane.

(b) Aperture stops. Using the same 2-lens system (Fig 4), students are prompted to add a second iris to determine the best locations for an aperture (leaving the field stop iris in place). Students are reminded that aperture stops cut out the angles of the light and act as a dimmer switch at the image plane. If students are having a hard time, we recommend using a white board to draw the expected ray diagram for the system (Fig 4B). Have the students identify locations where the rays that make the image are diverging or converging. Then, have the students place the iris in these locations to see if they can act as an aperture stop, dimming the light at the image planes.

Using an index card, students can find locations along the optical path where the aperture acts as a field stop and the field stop acts as an aperture stop. When using very short focal length lenses, this can often happen in close proximity. We encourage instructors to enable this sort of discovery or play and not be frustrated if the aperture stops and field stops tend to mix. By demonstrating the attitude of curiosity without the stress of needing the right answer, the students will be more open to learn from tinkering with the optical systems.

Using the commercial microscope stand, students can find and demonstrate the working of the aperture stop within the condenser. Using the eyepieces or index cards, the aperture stop works to dim the amount of light on the sample. As with the field stop, the

condenser's aperture stop needs to be placed in Köhler illumination for this activity.

(c) Numerical aperture. The numerical aperture of a multilens system is the quantification of the smallest aperture stop size within the system. It depends on the smallest angle light rays made between the centerline and propagated through the entire system. The numerical aperture can be set by an iris or by the physical limitation of the lens holders. All optical systems have a finite numerical aperture, which is part of the reason why resolution is limited: we literally lose information when light rays are unable to propagate to form the image.

Because the irises in the 2-lens system are variable, students are asked to change the settings of the irises from the smallest radius to the largest radius and determine the numerical aperture of the system for each setting. When the irises are open, the system often has the numerical aperture set by the lens holders instead of the irises. Comparisons can be made with microscope objectives, which are described by their numerical aperture. Often in objectives, the numerical aperture is limited by the size of the initial opening that allows light into the system.

3. Laboratory module 3: detectors (cameras)

Modern light microscopes use cameras, which are arrays of light detectors, to digitize images and record spatial structure over time. Modern light detectors can be incredibly sensitive and fast, able to see a single photon at a 30 frame per second frame rate or faster. Modern cameras can also detect a wide range of wavelengths. All these properties make even inexpensive cameras better than the human eye. For this lab module, it is often best to incorporate it into the curriculum just before the students finish building the microscope by adding the objective and the camera.

For the hands-on activity in which students can learn about the camera detector, we give some background about detectors, detector arrays, how camera arrays move the data from the detector chip to the computer, and the meaning of the gray scale on the screen. The learning goals of the section are to (a) help students understand what cameras detect; (b)

show students how to make an image on a camera; (c) have students become acquainted with the camera software and use FIJI–ImageJ to quantify images; (d) give students training on the meaning of the gray scale and increase perception of how the gray scale changes with exposure time, averaging, binning, gain and offset, and other settings; and (e) discuss measurement and quantification of light using a detector with sources of noise, measuring dark counts, discussing saturation, and quantifying the linear range of the detector.

The hands-on activity about cameras is broken into 4 parts: (a) making the camera work and making an image on the camera, (b) using the camera as a photodetector (quantifying intensity measurements and photon count), (c) using additional camera features. Some of these activities can be done after the microscope is built or even by using a commercial microscope stand.

(a) Making the camera work and making an image on the camera. Prior to capturing and analyzing microscopy data, students need to understand how to run the camera to take images. We typically have students spend some time loading and running the software on either provided computers or his or her own laptop. Once the software is loaded and working, students connect to the camera to try to see something. As students are accustomed to working with cameras on phones and computers regularly, we first suggest that students not mount the camera, but rather just point it at something and try to take a picture. If the camera is working, the student should see black and white fluctuating images on the screen. When the student waves his or her hands in front of the camera, the student can often make a shadow of his or her hand on the camera but not make a true image, because scientific cameras are simply a bare chip of photodetectors. There is no lens to create an image. Scientific cameras are unlike phone cameras with a built-in lens to make an image.

Before a student becomes frustrated, we suggest the student try to mount the camera and mount a lens in front of the camera. Students are asked to make an image of an

object that is far away, such as across the room. The worksheets ask leading questions about the placement of the single lens compared with the camera. If the object is sufficiently far away, the optimal location is to place the lens 1 focal length away from the camera detector. This helps to drive home the lesson that the camera is a detector, but the spatial structure of the image comes from the lens.

(b) Using the camera as a photodetector. The next guided activity helps students understand that the camera is a photodetector that can be used to quantify light intensity. Like all detection and measurement devices, there is a linear range of the measurement, and there are upper and lower bounds that the detector cannot measure. Students are guided through changing the intensity of light being detected through changing the intensity of the object illuminated, while maintaining a fixed exposure time of the camera. If the object is a part of the room far away, this can be changed by adjusting the room lights or adding neutral density filters. If the object is a sample in the microscope, illuminated by the condenser, they can use the condenser to change the intensity. Doing both activities enables students to appreciate the control the condenser affords the microscope system compared with relying on ambient lighting.

Students should see that, at low light, they cannot observe the object on the camera and can only see background. Students can quantify the intensity to find that it is not zero. The nonzero intensity is often called the *background intensity*, which depends on ambient lighting, light blocking, temperature, and the detector itself. Students are encouraged to block all light to the camera to measure the background level or *dark counts* due to the intrinsic camera noise (typically thermal noise).

As the light level increases, students should be able to not only observe the object but also measure the intensity of the light from the sample. If using an LED that can vary intensity, the intensity set point of the LED can be directly compared with the measured intensity on the camera. Plotting this detector measurement versus the LED setting intensity should

demonstrate that there is a linear region of the detector. Specifically, when the light intensity from the LED doubles, the measured intensity by using the camera should also double. As the LED intensity is increased, students will begin to saturate the detector. At saturation, the screen will look completely white, and the object is not observed anymore. This is the end of the linear regime of the camera detector. If a photometer is available, the camera detector range can be quantified. The photometer can be placed directly in front of the camera to collect the same light as the camera. This allows the intensity in gray scale to be converted to the intensity of light in watts.

(c) Using additional camera features. Modern scientific cameras have a number of ways to control the intensity. Unlike our eyes that can only control the light with the iris (aperture stop), cameras can electronically control the amount of time they collect light (exposure time) or control the electronic gain on the electrical systems of the detector. Students need to be aware of these additional controls, because they may not always be able to control how bright their sample is by using the incident light. To demonstrate these other capabilities, students are guided through activities in which they hold the light intensity onto the sample constant and instead alter the exposure time or electronic gain of the system. The same regimes of background noise dominated (light limited), linearly increasing, and saturating can be observed when altering each of these other controls. The more time students have to manipulate these various camera features, the more accustomed they will become to understanding the different ways they can control the images they collect.

B. Project: building a microscope

Interspersed with the scaffolding lab modules, students are given time to design and build a light microscope. For this curriculum, the microscope project is broken into parts: (a) building the condenser, which is done after the first 2 lab modules on lenses, images, and apertures; (b) using an objective to make an image on the camera, which is done by using

the camera that was introduced in the detector lab module prior to this part of the building project; and (c) testing the magnification and resolution of the microscope. On successful completion of the microscopy building project, students will be able to design and build an independent system onto the microscope to address scientific questions as an advanced project. These parts are assessed through students' presenting to instructors and each other at a poster session. The expectations for the presentation are set by using a rubric that students are given in advance. Here, we describe the different parts of the microscope project and give examples of how students could make designs. The best part is that the options and pathways to success are infinite!

(a) Building the condenser. The microscope condenser is an optical system that controls the brightness and the location of the illumination on the sample. The condenser can influence the resolution of the image obtained. Control of the incident light is created by using lenses to collect the light and irises as aperture stops and field stops to control brightness and location. It is best to have students work on this part of the microscope after they have completed the first 2 laboratory modules. After completing the iterative work on those modules, students find creating a condenser fairly easy. The most important aspect to emphasize is that students need to build the condenser in Köhler illumination. Specifically, they need the sample plane to be evenly illuminated and the light controlled by 2 irises: a field stop and an aperture stop. Instructors should emphasize that students do not want an image of the LED lamp on the sample.

To design a good condenser, the students must understand and use the concepts of image formation, focal planes, collimation, apertures, and field stops. The project of designing the condenser can be as simple or as complex as you need for the class. We stress that there are an infinite number of ways to place the optics to make a condenser and that students are allowed to do anything they want. Students are allowed to use any resources to help them make the condenser design, but

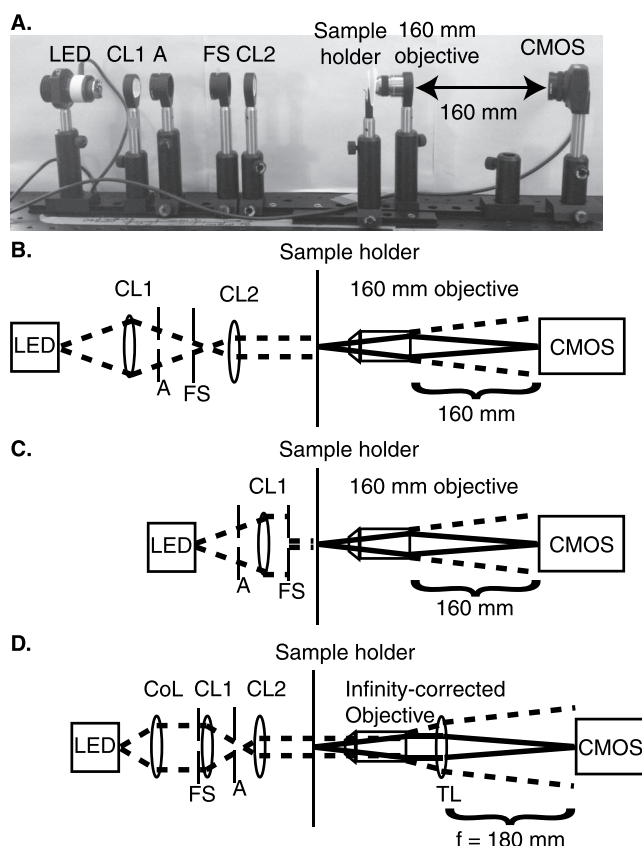


Fig 5. Student-designed optical systems for transmitted light microscope. (A) Photograph of a working transmitted light microscope created by students. The left side shows a light-emitting diode (LED) as the light source, an iris to act as an aperture (A), 2 lenses act as a condenser lens (CL1 and CL2) to collect and collimate the light at the sample, a second iris to act as a field stop (FS), and then the sample holder. After the sample, a 20 $\times$, 160-mm objective is used to create an image on the CMOS camera 160 mm away from the back of the objective. (B) Schematic diagram of the optical train of the microscope constructed and shown in panel (A). (C) Schematic diagram of a different design for a microscope condenser with 1 lens, and the aperture and field stop irises are in alternate locations. (D) A schematic diagram of condenser with 3 lenses, the aperture and field stop irises are in alternate locations, and the imaging path uses an infinity-corrected objective that requires a tube lens (TL) with a focal length of 180 mm.

they need to test the design to make sure the sample plane is evenly illuminated and controlled by the field stop and aperture.

When building the condenser, students will often realize that they do not know where the sample plane is located. The student might ask you where it should be placed. Remind the student that it is his or her design, and the student can place it anywhere, but the student has limited space on the table or breadboard!

In the semester-long optics course, the students are assessed by using poster presentations. Students are given a rubric, so they know what needs to be on the poster to demonstrate that the condenser works as expected. Specific parts of the poster include (a) exact ray diagrams with all components and distances labeled, (b) analytical ray tracing with matrix methods of the designed condensers to demonstrate that the condenser will illuminate the sample and that the apertures and field stops work, as expected, and (c) a demonstration that the condensers control the light location with the field stop and the intensity with the aperture at the sample plane. Students use photographs of the light path and the final illumination region on the sample plane. Students are also asked to measure the numerical aperture of the optical system with the aperture stop open and closed, which is performed most easily by using analytical ray tracing.

As described previously, a good condenser does not create an image of the LED at the sample plane. The condenser makes an image of the field stop iris in Köhler alignment, allowing the students to assess if the field stop was correctly placed. Students from the life science field said that applying knowledge of Köhler alignment to create the condenser is one of the most challenging parts, but it is also the most exciting when a configuration is found that works.

The condenser designs of students are the most variable and interesting part of the microscope design. Students in our courses have used 1, 2, or 3 lenses to create evenly illuminated light on the sample plane (Fig 5). All designs have 2 irises: one for a field stop and one for an aperture stop (Fig 5). Any of these designs are correct and functional, as long as they control the field of illumination, the brightness of illumination, and do not create an image of the LED on the sample plane. This is a beautiful part of optics—if it works, it is correct, and there are many ways to solve the same problems. Other aspects that could be considered include the locations of the entrance and exit pupils and the efficiency of the

system (how much light is able to pass through the system).

(b) Using an objective to make an image on the camera. After creating the condenser, students next have to use an objective to create an image on the CMOS camera detector. The lab module on the camera helps students understand how to make an image and quantify the intensity of the system and comes just before this part of the microscope project to facilitate the usage and placement of the camera in the microscope optical train.

One concept that stumped students in early offerings of the course was that when designing the imaging path, the LED is not the object we are interested in imaging. Instead, we are interested in making an image of the sample that we place in the sample plane. This can be confusing because the students must use the illumination light of the LED, but we do not want to image the LED itself. The inclusion of lab modules on lenses and apertures helped to clear these misconceptions early, before building the microscope.

To build the imaging path, it is helpful if the students have a sample to image. We found that it is easiest if the students use a glass slide, written on with permanent ink, as a sample to start. This slide is mounted in a fixed, pressure mount (Table 1). The slide is placed in the sample plane of the condenser exactly where the field stops, and apertures are aligned to control the location and brightness of the light in Köhler illumination.

(1) The 160-mm objective: Students start their iterations by using an objective with low magnification ($4\times$ or $5\times$) and 160-mm focal length to create an image on a screen and ultimately onto the CMOS camera detector. The objective is mounted into a holder by using an adapter ring to adapt the Royal Microscopical Society threads to the threads of the lens holders (Table 1). Movement of the position of the objective along the rail works for gross focus. A small, one-dimensional translation stage under the objective holder allows for fine focus (Table 1). With the 160-mm objective in place, students can change the sample from the slide with the permanent marker to a

micrometer scale. They can use the micrometer scale to check the magnification and resolution (see the “Testing the magnification and resolution” section). Examples of designs with 160-mm objectives are shown in Figure 5.

(2) Infinity-corrected objective: Depending on the purpose of the project for the course, we also encourage students to replace the 160-mm objective with an infinity-corrected objective with low magnification ($4\times$). Infinity-corrected objectives are used on modern microscopes because they have an infinity space of collimated rays at the back of the objective between the objective and the tube lens. This region has a space to place various optical components, such as phase rings or prisms, which can enhance contrast. It also allows more flexible space in the microscope design. It is good to remind students that they have already seen and tinkered with infinity spaces in the lab modules.

Infinity-corrected objectives require a tube lens of a specific focal length to achieve the correct resolution and magnification. Each manufacturer’s design requires a specific tube lens (Fig 5D). We encourage students to determine how infinity-corrected lenses work through trial and error. They often are missing the tube lens, have trouble creating an image, or choose an incorrect tube lens and obtain the wrong magnification (see the “Testing the magnification and resolution” section). If they are missing the tube lens, they can usually still create an image far from the objective, effectively at infinity.

Designing and building the imaging path reaffirms the concepts of image formation and ray tracing. In the following, we describe how the students can test these concepts with their microscope. Because we are imaging with a camera, the image formation is straightforward. For instance, students can create an image on an index card and then move the camera detector to that position, reinforcing the concept of real image formation. For students who are familiar with microscopes in which they use an eyepiece, it is stressed that the optical train to the camera is different than that going to the eyepieces and ultimately to the

eye. In particular, the eyepieces on a commercial microscope act to collimate the light again because the eye has a built-in lens in front of its detector, the retina.

(c) Testing the magnification and resolution. Once the students are able to create an image on the CMOS camera, focus it, and take a picture with the camera software, they need to determine the magnification of the optical system and compare it with the magnification listed on the objective. Individual objectives are made with specific microscope optical systems in mind. In particular, each microscope manufacturer has slightly different optical requirements for the objective, including the distance from the objective to the sample (working distance), distance from the back of the objective to the camera (focal distance), and the specific focal length of the tube lens, if it is needed (for infinity-corrected objectives) or not (for fixed focal length objectives). All these differences can alter the magnification achieved by an objective; thus, the students need to carefully calibrate the microscope they build. Ultimately, the objectives are built for specific magnifications to achieve a certain numerical aperture and resolution (see the following); thus, the students should first attempt to establish the correct magnification for their objective.

Magnification calibration can be achieved by imaging a micrometer, graticule, or diffraction grating with a known spacing between the rulings (Fig 6). Once the image is in focus and saved, the student needs to open the image by using FIJI–ImageJ. The students can open the image, regardless of the format of the image, adjust the brightness and contrast, zoom in or zoom out on the image, crop the image, and save in a variety of file formats with FIJI–ImageJ.

To measure the distance between 2 lines, most students will use the Measure tool in the Analyze menu. The types of measurements that are recorded can be set in the Set Measurements menus of the Analyze menu so that the length can be saved as a text file. The students need to know the units of the measurements, which will usually be pixels. They can draw a line region of

interest between 2 lines and measure the distance in pixels between these 2 lines. Using the known distance between the 2 lines and the measured number of pixels, they can find the size of a single pixel in micrometers.

When performing the magnification measurement, students often become confused because they do not know how to equate the pixel size to a magnification. To determine if the size of the pixel they measure is correct, they must know the actual physical size of a pixel of the camera. The camera's physical pixel size is given in the specifications for the camera. For the CMOS cameras we use (Table 1), the physical pixel size is $5.2 \times 5.2 \mu\text{m}$. We do not tell the student the pixel size but insist that the student search the documentation for the camera to find it. It is an important experimental skill to know how to find the information for your own equipment, whether online or from the paper documentation that comes with the equipment.

Once the students know the physical size of the pixel, they can compare the known pixel size to the measured pixel size. Because the image is being magnified, and the physical pixel size is staying the same, we expect the new pixel size to be smaller by a factor equal to the magnification. The magnification can be calculated as

$$M = \frac{P_{\text{known}}}{P_{\text{measured}}}, \quad (2)$$

where M is the magnification, P_{known} is the known pixel size of the camera ($5.2 \mu\text{m}$), and P_{measured} is the measured pixel size from FIJI–ImageJ in micrometers.

Students often find that the magnification is not what they expect, given the power of the objective they have chosen (Fig 6). There are several reasons why this can occur. First, if they use a 160-mm focal length objective, they often do not put the CMOS chip at the required 160 mm from the objective on the first try. If the CMOS is at a different distance from the detector, the students will still be able to focus the image, but the magnification will be incorrect. Even a small deviation in the placement of the CMOS detector will result in differences in the magnification (Fig 6).

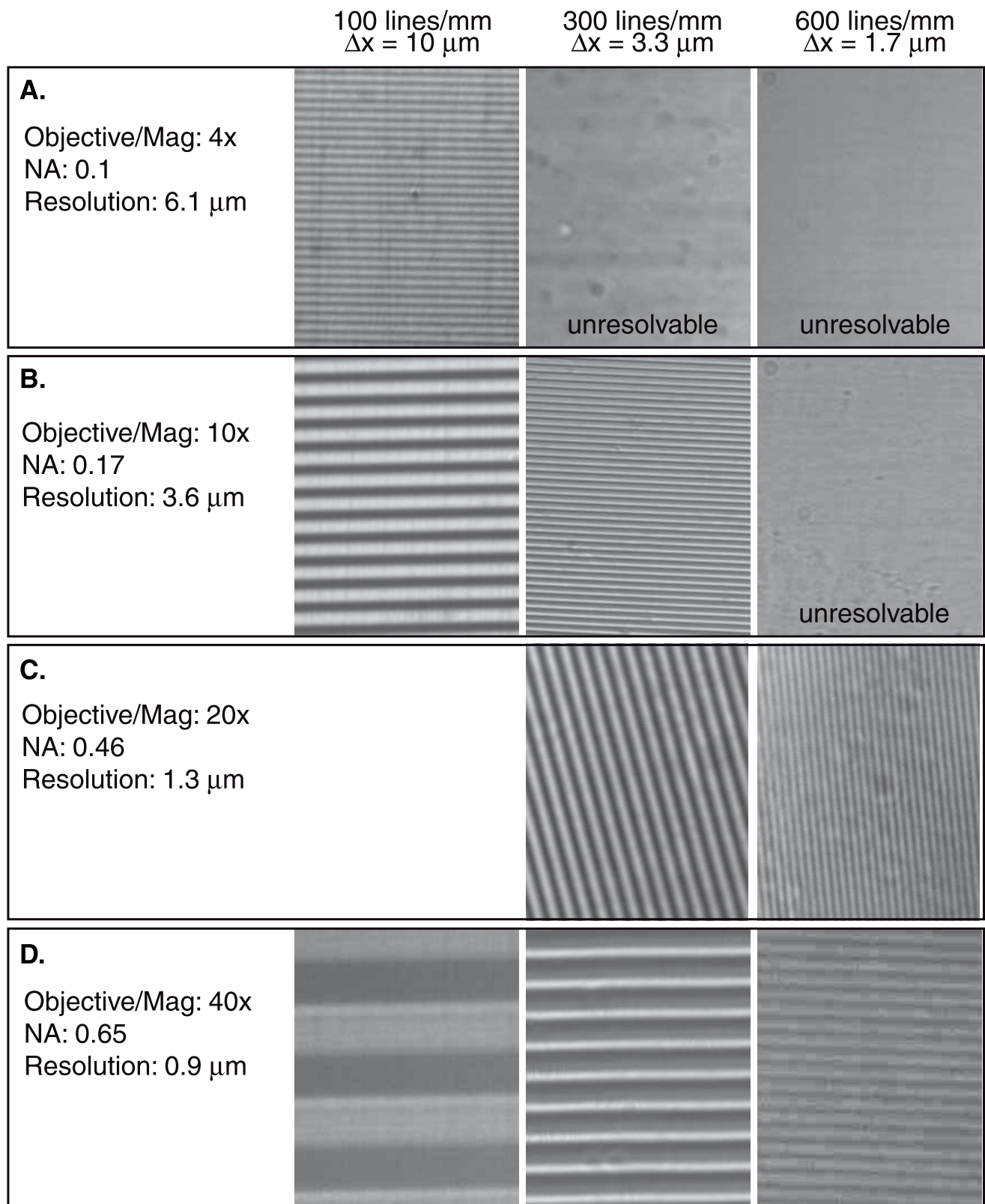


Fig 6. Examples of correct and incorrect imaging of a 1-mm scale bar with 100 of the $10\text{-}\mu\text{m}$ markings by using 3 different objectives. Incorrect placement of the objective or incorrect tube lens focal lengths will result in incorrect magnifications. (A) For the 4 $\times$, 160-mm objective, the correct magnification should be 4 $\times$, and the correct pixel size after magnification of a $5.2\text{-}\mu\text{m}$ pixel is $1.3 \mu\text{m}$. Incorrect placement of the CMOS camera from the back of the objective results in an incorrect pixel size of $2.2 \mu\text{m}$ and an incorrect magnification of

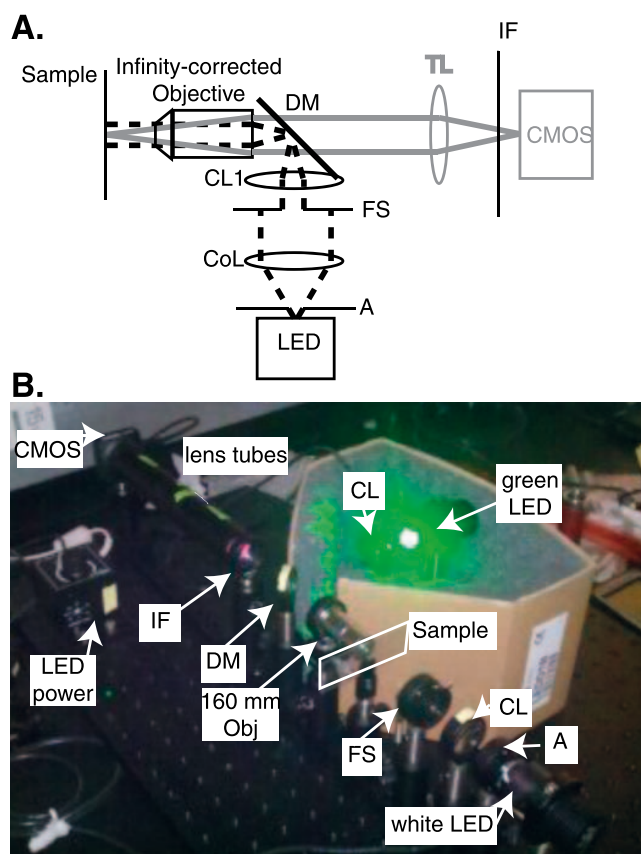


Fig 7. Example data taken by students of diffraction gratings with spacing of 100, 300, and 600 lines per millimeter taken with objectives with magnifications of 4×, 10×, 20×, and 40×. The magnification (mag), numerical aperture (NA), and theoretical minimum spacing (resolution) expected for each objective is given in the first column. (A) The 4× objective is able to resolve the 100 lines per millimeter because the distance between lines is 10 μm but not the 300 or 600 lines per millimeter with spacing of 3.3 and 1.7 μm . (B) The 10× objective can resolve the 100 lines per millimeter, and just barely resolve the 300 lines per millimeter, but not the 600 lines per millimeter. (C) The 20× and (D) 40× can resolve the lines from all diffraction gratings.

Second, if students use an infinity-corrected objective, the magnification will depend on the specific tube lens that is forming the image on the camera (Fig 5). The tube lens length is based on the manufacturer. We suggest that the students go online and learn about tube

lenses and determine the needed tube lens length. Students will measure the wrong magnification because they often choose a random lens, or the tube length with the correct focal length is not available. They will still be able to focus the image and make a measurement, but the magnification will be incorrect (Fig 6). We find that the trial and error of measuring and failure allows them to tinker further to correct mistakes and enhances learning and memory about these concepts. These missteps and corrections often occur in a real research setting, thus replicating a more realistic research environment.

After creating an imaging path with the correct magnification, we instruct the students to test the resolution limits of their microscope. We provide several diffraction gratings with decreasing line spacing, including 100, 300, and 600 lines per millimeter. We do not list these gratings in the kit (Table 1) because there is one set for the class that students share. We use old, scratched gratings that the department was going to discard, but new ones with 1,200 lines per millimeter are available at Thorlabs for less than \$100. For the semester-long optics course, we insist that students image the 3 diffraction gratings by using 2 different magnification objectives. Typically, lower magnification objectives have lower numerical apertures and thus lower resolution (Fig 7). The lines of finer gratings cannot be distinguished by low-power, low numerical aperture objectives. Students learn from texts or via online resources how to determine the theoretical resolution of their objectives by using the following equation:

$$d_{\min} = 1.22 \frac{\lambda}{\text{NA}}, \quad (3)$$

where $d_{\min}$ is the minimum distance that is resolvable, λ is the wavelength of light (500

Fig 6 (continued) 2.3×. (B) For the 10× objective, the correct pixel size is 520 nm. The incorrect setting for the 10× is due to incorrect camera placement with respect to the 160-mm objective to give a pixel size of 700 nm and a magnification of 7.4×. (C) The 20× objective is an infinity-corrected objective, and the correct pixel size is 260 nm. In the incorrect image, a tube lens with focal length of 100 mm is used instead of the correct 180-mm tube lens to give a pixel size of 450 nm and a magnification of 11.5×. Although the correct focal length tube lens is 180 mm, we did not have the correct lens, so a 175-mm lens was used. This lens achieves a pixel size of 290 nm and a magnification of 18.2×.

nm), and NA is the numerical aperture of the objective (11, 12). Using the gratings, the students must calculate the spacing between the lines and determine if they expect the objective to be able to resolve the lines. For instance, a 4 $\times$ objective with a numerical aperture of 0.15 should be able to resolve gratings with spacing of 4 μm or larger. The grating with 100 lines per millimeter has a spacing of 10 μm , and a grating with 300 lines per millimeter has a spacing of 3.3 μm . Thus, we would expect for the 100 lines per millimeter grating to be resolvable but not the 300 or 600 lines per millimeter (Fig 7).

As is often true, the experiment rarely perfectly matches the theory. The students find that when the theoretical resolution is near the spacing of the grating, they may or may not be able to resolve the spacing (Fig 7). Often, it depends on the numerical aperture of the condenser and the actual wavelength of light the students are using. We suggest for the students to match the numerical aperture of the condenser to the numerical aperture of the objective to obtain the highest resolution. Further, if the students make the condenser aperture too large or too small compared with the objective, the image becomes too dim or too washed out to resolve. Such examples allow instructors to bring up advanced topics and discuss the microscope system as a whole.

We encourage students to use different objectives with different numerical apertures to test the resolution and the accuracy of Eq. 3. This is easiest to test if the student has access to different objectives with the same magnification but varying numerical apertures. Some objectives have irises within them, allowing direct comparison of resolution with the numerical aperture, but these objectives can be expensive. These additional tests allow students to delve deeper into the concept of measurement and how accurately they can measure distance with a light microscope. They can also speculate on other parts that could be altering the resolution of the system they built, such as the condenser or the intensity of light propagating through the system.

C. Discovery: open-ended advanced project

There are several advanced projects or topics that can be driven by student interest to introduce more advanced imaging and analysis techniques. In this section, we describe one additional building project, the epifluorescence path, and one analysis technique, super-resolution fitting to gain higher accuracy in distance measurements.

1. Discovery: epifluorescence

In the optics course in which the microscope building was a semester-long project, the final part of the course (~ 4 wk) is devoted to students building an advanced optical system onto the basic microscope. One particularly popular system to build was the epifluorescence path. Epifluorescence is a heavily used, modern technique in the life sciences. Most modern microscopes have an epifluorescence imaging path (Fig 2). Epifluorescence imaging is achieved through *epi-illumination*, in which the excitation light shines onto the sample from the same direction that the viewer will image the sample. In other words, the epi-illumination path must pass through the objective. This can be observed in the schematic in Figure 2, where the epifluorescence lamp and dichroic mirror are under the objective. The reason for this configuration is that the majority of the background photons from the illumination will pass up and away from the sample and camera, which will reduce the background fluorescence significantly.

Additional equipment for epifluorescence includes an additional LED illumination source, color filters, additional lenses and irises, and some method of blocking extra light from the system. We used a green LED for red fluorescence. If a white light LED is used, the students need to be sure that the desired excitation wavelength is part of the LED's spectrum. Further, the system will require an excitation color filter to allow only the desired wavelengths to hit the sample. The wavelengths of the filters for excitation depend on the fluorophore being used. There are a number of resources online to help students find the

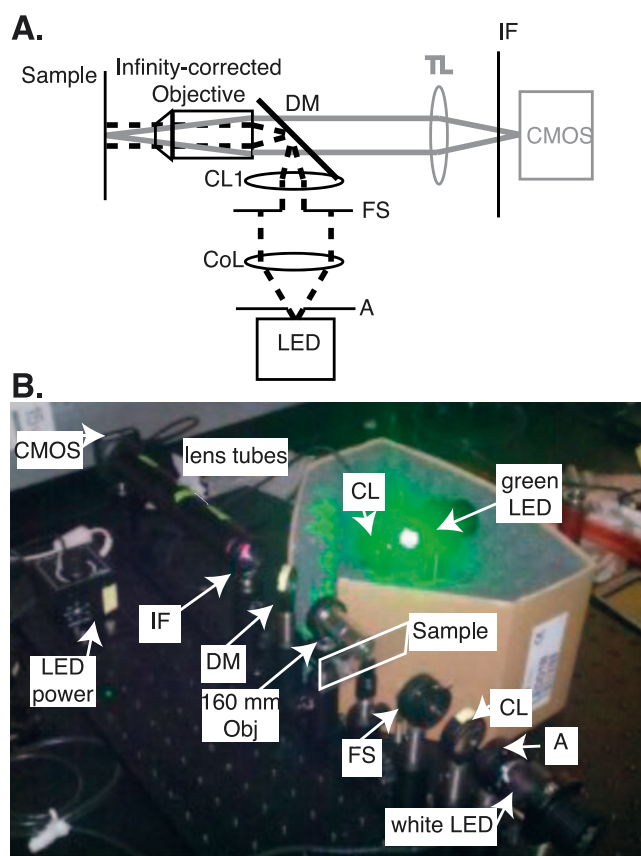


Fig 8. Example epifluorescence microscope design created by students. (A) Schematic diagram of epifluorescence path (the transmitted light condenser is not shown). The dashed lines represent the excitation light rays that are emitted by the green light-emitting diode (LED). A = aperture, CoL = collecting lens, FS = field stop, CL1 = condenser lens 1, and DM = dichroic mirror. Solid lines represent the emission light rays of fluorescence that come from the sample and are imaged onto the CMOS camera by using a tube lens (TL). Before the fluorescence light goes to the camera, other wavelengths are blocked by using an interference filter (IF) that selects the wavelength of the emission light of fluorescence. (B) Photograph of an epifluorescence microscope created by students that uses a 160-mm focal length objective and a single CL in front of the green LED. They also use a DM that reflects green light into the objective onto the sample. After the dichroic mirror, an interference filter that picks the correct fluorescence wavelength is used before the CMOS camera, 160 mm away from the objective. This group used a metal lens tube and cardboard to block stray room light.

best filters for each fluorophore (13). Having students learn how to find and use online resources is a research-level skill they will use in any position in the future.

In the epi-illumination scheme, students need to reflect the excitation wavelength into the objective and ultimately the sample but still

allow the emitted fluorescence light to go to the camera. To achieve the spectral separation, a special dichroic beamsplitter is used, called a dichroic mirror. The dichroic mirror is positioned at a 45° angle to reflect the excitation light into the objective (Fig 8). The optimal dichroic mirror depends on the excitation and emission wavelengths of the fluorophore you choose (13).

Once the excitation light hits the sample, the sample will fluoresce, provided the correct fluorophore is present. We recommend using a very bright sample to begin. For green fluorescence using blue excitation light, a cover glass with highlighter marker ink on it will work. Highlighter marker ink has a high concentration of fluorescein, a nontoxic, organic dye that is bright but photobleaches quickly. For red fluorescence using green excitation light, students can use rhodamine dissolved in water or rhodamine-labeled beads. Rhodamine is an inexpensive organic fluorophore that is carcinogenic, so students should be careful handling it. The first sample should have a significant amount of fluorescent dye so that the fluorescence can be observed by eye when the excitation light hits the sample. Some of the fluorescent light will shine back through the objective and be directed to the camera.

To image the emitted fluorescence light on the camera and block other wavelengths, students need to use an emission filter. The optimal wavelength for emission for the chosen fluorophore can be found by using online resources (13). The emission filter should be positioned in front of the camera. Students found that positioning the filter directly in front of the detector or using black metal tubes helped to reduce stray light going into the camera.

By using samples with a high density of fluorescent molecules, students should be able to see bright light on the camera even with the room lights off or the sample completely covered by light-blocking fabric. The fluorescence intensity depends on the amount of excitation light hitting the sample. Students can raise or lower the intensity of the fluorescence LED to make sure that the light

on the camera is coming from fluorescence and not stray room light or scattered excitation light.

Once the students verify that the image on the camera is coming from the high-density fluorophore sample, they can perform several tests. For example, the students can change the excitation and emission filters to determine which are best for the sample. It is often easy to find inexpensive discontinued or used filters on sale from filter manufacturers, such as Omega, Chroma, or Semrock. Others can be found on online auctions.

Another test is to make fluorescent samples with a spatial structure to image on the microscope. Two examples include fluorescent beads or oil–water emulsions with surfactant and fluorophores in one phase. Note into which phase the fluorophores will separate. It is useful for the number of objects (beads or droplets) to be relatively low so that individuals can be visualized. Commercially available fluorescent beads diluted about 1:100 into water or buffer are typically at a good concentration. Students can create a flow chamber from a slide, cover glass, and double-stick tape. The chamber can be sealed on the sides by using wax or epoxy, if needed. Individual fluorescent beads should be visible, but they are much dimmer than the high-density sample.

If beads are not visible at first, more light blocking may be necessary. Students found it best to block stray room lights with cardboard because stray light contributed to background noise and made it difficult to image (Fig 8). Students also tried a variety of dichroic and emission filters to optimize the wavelengths for the supplied fluorescent beads. Using the images of fluorescent beads over time, students performed experiments to track motion, measure diffusion, and obtain a sense of the Boltzmann distribution in a gravitational field, such as those described previously by (14).

2. Discovery: super-resolution

Recently, super-resolution techniques have been used to improve the imaging capabilities of fluorescence microscopy. Some techniques, such as stimulated emission depletion (15) and structured illumination microscopy (16) imag-

ing, use optics to create patterns of light that are smaller than the diffraction limit. Other methods, such as photoactivation localization microscopy (PALM) (17) or stochastic optical reconstruction microscopy (STORM) (18) image individual fluorescent molecules one at a time and fit each one with high accuracy to find the center of the molecule. Individual molecule locations are overlaid together to create an image, such as pointillism.

The second type of super-resolution, PALM and STORM, uses a priori knowledge of the intensity profile expected to obtain a higher accuracy of localization of individual fluorophores (19). For instance, we know beforehand, that the linear optical system of the microscope takes pointlike sources and convolves them with the point-spread function of the objective to create a two-dimensional (2D) Bessel function (12). The Bessel function appears strikingly like a 2D Gaussian function, with the majority of the intensity coming from the center and decaying exponentially at the edges (20). By fitting the intensity profile of the convolved image of a single point source to a 2D Gaussian, the center of the intensity pattern can be localized well, assuming there are enough photons and the pixel size is neither too small or too large (21).

Similarly, if the students have a priori knowledge about the sample they are imaging, they will be able to determine the distances between objects with better accuracy than allowed by traditional resolution limits. Here, we present a similar analysis method, such as PALM or STORM, to find the spacing between the lines of a diffraction grating with accuracy far higher than the diffraction-limited uncertainty would allow. The a priori knowledge is that the students are imaging a grating with a repeating structure at a constant spacing. Before we describe the technique, note that this technique was developed by undergraduate students in an advanced physics laboratory.

Students developed a super-resolution method to find the spacing of a grating by using ImageJ–FIJI. Instead of measuring the spacing between the lines by drawing a line by hand,

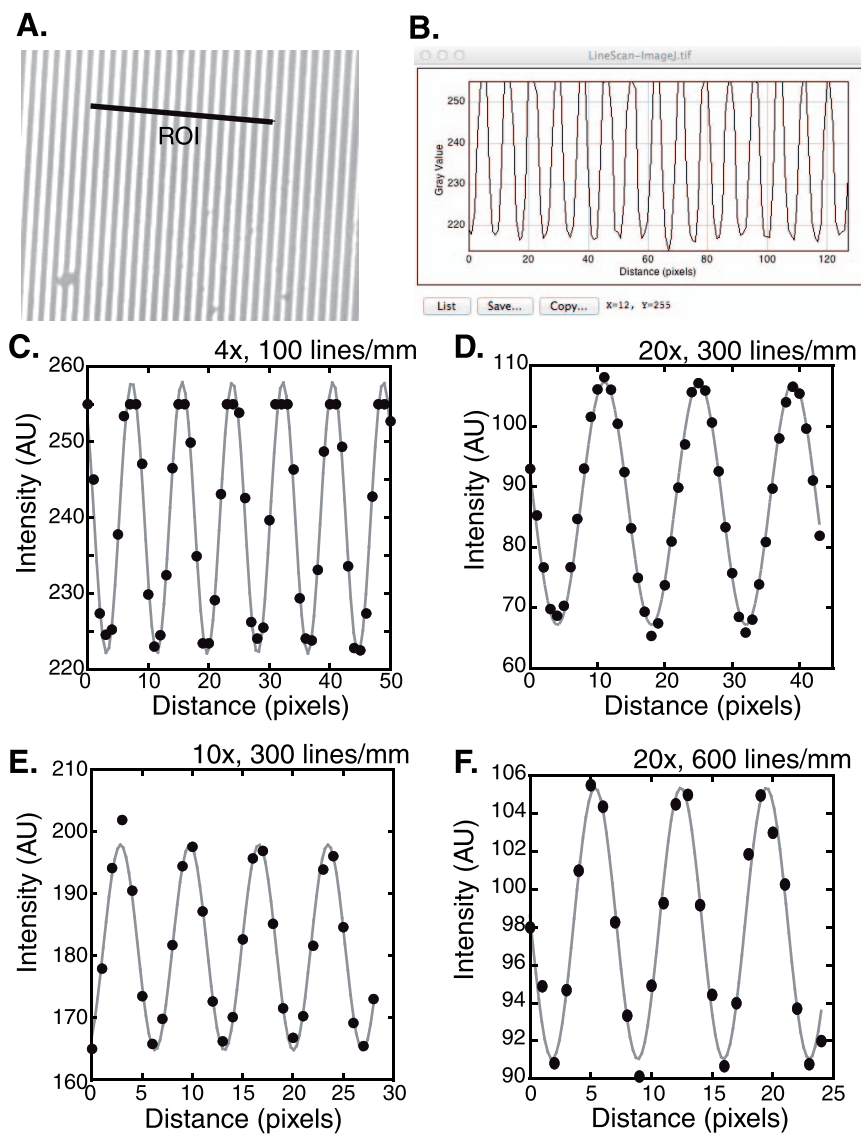


Fig 9. Super-resolution fitting of diffraction grating image to achieve accuracy better than the resolution limit. (A) Image of a diffraction grating with a line region of interest (ROI) drawn perpendicular to the grating lines. (B) Screenshot from ImageJ showing the line scan of the intensity along the ROI. (C) Intensity as a function of distance with given sinusoidal function to the 100 lines per millimeter grating imaged with the 4 $\times$ objective. Fit parameters are $I_0 = 240.0 \pm 0.1$, $A = 17.9 \pm 0.3$, $x_0 = -3.07 \pm 0.04$, and $\lambda = 2.076 \pm 0.002$, the goodness of fit was $R^2 = 0.98$. (D) Intensity as a function of distance with sine wave fit to the 300 lines per millimeter grating imaged with the 20 $\times$ objective. Fit parameters are $I_0 = 87.2 \pm 0.2$, $A = -20.1 \pm 0.3$, $x_0 = -13.474 \pm 0.08$, and $\lambda = 3.505 \pm 0.008$, and the goodness of fit was $R^2 = 0.99$. (E) Intensity as a function of distance with sine wave fit to the 300 lines per millimeter grating imaged with the 10 $\times$ objective. Fit parameters are $I_0 = 181.3 \pm 0.3$, $A = 16.5 \pm 0.4$, $x_0 = 1.14 \pm 0.05$, and $\lambda = 1.72 \pm 0.06$, and the goodness of fit was $R^2 = 0.99$. (F) Intensity as a function of distance with sine wave fit to the 600 lines per millimeter grating imaged with the 20 $\times$ objective. Fit parameters are $I_0 = 98.2 \pm 0.2$, $A = -7.12 \pm 0.3$, $x_0 = 0.06 \pm 0.07$, and $\lambda = 1.77 \pm 0.01$, and the goodness of fit was $R^2 = 0.97$.

students used the intensity profile across the grating (Fig 9A). The intensity profile was found by drawing a line across several of the grating lines and then using the Plot Profile tool in the Analyze menu of ImageJ-FIJI. The plot profile function will create a new window of a plot that shows the intensity as a function of the

distance along the line that was drawn (Fig 9B). Performing these functions demonstrate to the students the concept that the gray scale observed on the image corresponds to a numeric value. If the image is an 8-bit image, absolute black is a numeric value of 0, and absolute white has a numeric value of 255.

Depending on the settings of the intensity and exposure, the students may also be able to observe the linear dynamic range of the camera, the noise-limited regions, and the saturated regions. These are important concepts for quantification of data, especially image data.

To save the numeric array of intensity as a function of distance, the student will need to list the values and save the data as a text file. This file can be opened in Origin, Igor, Kaleida-Graph, MATLAB, Python, or similar programs to be plotted and fit with a sine wave (Figs 9C–F). Students next fit the data to a sine wave of the form:

$$I(x) = I_0 + A \sin\left(\pi \frac{x - x_0}{2\lambda}\right), \quad (4)$$

where $I(x)$ is the intensity as a function of the distance, x , I_0 is the background intensity, A is the amplitude, λ is the wavelength, and x_0 is the phase offset in the x direction.

The distance between the grating lines is equal to the measured λ from the fit. The error of this fit is much smaller than the resolution limit; thus, the measurement is more accurate. The high accuracy is thanks to extra knowledge of the repeating pattern to fit multiple lines. The magnification can be determined by using this same method and can be found with higher accuracy. The uncertainty for finding the distance between lines of a grating can be significantly smaller (one to tens of nanometers) than the expected resolution of the system, which is approximately half the wavelength of the light (hundreds of nanometers).

V. CONCLUSION

We have described a laboratory experimental curriculum and equipment for students to build a working light microscope that duplicates the light path of a modern microscope. The equipment can be used as a hands-on project for learning geometric optics and the principles of measurement and uncertainty. We have used the equipment and tasks described here for a semester-long optics course, an advanced laboratory course for physics majors, and an interdisciplinary graduate lab module. The

equipment we describe is inexpensive; the cost for a complete system in which one begins with nothing costs less than \$3,000. Parts can often be purchased used, borrowed, or 3D printed, making this system easy to acquire. For instance, many optics labs already have screws and other small optomechanic parts, and many microscopists have older model objectives that they simply do not use anymore. The basic system we describe can be further modified to include more advanced systems, such as an optical tweezer (22–24).

Teaching the fundamental skills of optics and microscopy is essential to continue to push the boundaries of resolution and imaging modalities, such as those celebrated by the Nobel Prize. Equally important is the ability to bring low-cost image diagnostic abilities to the masses. Recent advances in making small, compact, and accessible microscopes for a wide audience include the paper-based foldscope (3), a community microscope kit using your cell phone (25), and FlyPi microscope, an open source, higher-end microscope system based around a Raspberry Pi (26). The goals of many of these systems are either education on a massive scale or detection of harmful microbes in drinking water. These systems are often easy to use, inexpensive, and have important causes, but they are kits with explicit directions on how to assemble the microscope. They do not give students the experience of tinkering or failing and learning from mistakes. The modular optics laboratory curriculum described here is needed to train the students who will become tomorrow's innovators. They could develop the next best microscope, whether it is a boundary-pushing, Nobel Prize-winning system or a low-tech, easy-to-use model for a massive market. The fundamental physics of optics is at the core, and the skills to invent must be nurtured.

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AUTHOR CONTRIBUTIONS

RK was a student in the Optics for Biophysics course BIO577/578 in spring of 2013; AC, MH, JS, AT, and SZ were students in the advanced laboratory course for physics majors PHYS440 in fall of 2013. JLR was the instructor for BIO577/578, PHYS553, and PHYS440, where the students were performing the experiments resulting in the data shown. JLR is also responsible for drafting and editing figures and manuscript for publication. Each of these students contributed data, images, and edited the manuscript.

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