

LIGHT MICROSCOPY LABORATORY EXCERCISE

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1. Introduction

The objective of this unit is to understand and learn how to use the light microscope. In the first part of this lab, test samples will be used to characterize the microscope. Then images of both fixed and live cells will be acquired and processed. The following microscopy modes will be used:

- (1) Bright-field microscopy
- (2) Phase contrast microscopy
- (3) Dark field microscopy
- (4) Fluorescence microscopy

You will need to understand basic concepts of microscopy, such as magnification, image formation, numerical aperture (NA) and resolution, illumination and Kohler illumination, etc. The Zeiss-sponsored website from which the above links were taken is just one of many excellent microscopy-education resources.

You will also need to use the Fiji/ImageJ image processing environment. A tutorial lecture delivered at the 2015 ImageJ Developers and Users Conference provides an introduction to the required concepts and vocabulary. The workshop slides (which do not always display well in the video) can be found here.

2. APPARATUS

2.1. Inverted Microscope. The lab uses an Accuscope 3032 inverted microscope, whose manual can be found [here](#). The scope uses [infinity optics](#), and is fitted with $4\times/\text{NA}=0.1$, $10\times/\text{NA}=0.3$, $20\times/\text{NA}=0.4$, and $40\times/\text{NA}=0.6$ phase objectives. These objectives are designed for use with a coverglass thickness of 1.2 mm. This is appropriate for imaging with culture dishes that are usually used in live cell imaging. When imaging fixed cells, you will need to image through the glass slide rather than the coverslip, in order to have the correct coverglass thickness. Essentially, the glass slide will act as the coverglass. Phase rings for the $10\times$, $20\times$, and $40\times$ objectives are included, and the ph2 ring can be combined with the $4\times$ objective to produce darkfield images. A 30 W halogen illuminator is used for brightfield, phase and darkfield imaging. The halogen lamps are powered by external DC power supplies since the original internal supplies have failed.

2.2. Fluorescence Subsystem. A Prior [Lumen 200](#) metal-halide illuminator is coupled to the microscope through a 3 mm liquid light guide. The available filter sets are summarized in Table 1.

TABLE 1. Epifluorescence Filter Sets. The excitation and emission filters are specified as center-wavelength/bandwidth, while the dichroic mirrors are specified at their point of 50% transmission.

Name	Excitation	Dichroic	Emission
DAPI	AT350/50x	AT410DC	AT460/50m
GFP/FITC	480/30x	AT505DC	535/40m
Texas Red	AT560/40x	AT600DC	630/60m

2.3. Digital Image Acquisition. The scopes are equipped with a [Point Gray Chameleon3](#) 3.2MP (megapixel) monochrome camera. Here are two important specifications:

- (1) $3.45\text{ }\mu\text{m}$ pixel size
- (2) 2048×1536 pixels

The camera is coupled to the scope with what was listed by the seller as a $0.5\times$ relay lens. Your spatial calibration measurements will cast some doubt on this value. The full camera specs can be found by clicking on the hyperlink above.

2.4. Test Slides. The lab includes a number of test slides that will be used to characterize the microscope. These include:

- (1) A slide that has a 1 mm scale divided into $10\text{ }\mu\text{m}$ divisions ([Ted Pella 2280-13](#)).

We have a second spatial calibration slide that has four scales on it. The finest scale includes $10\text{ }\mu\text{m}$ divisions. There is a scale with $100\text{ }\mu\text{m}$ divisions, and two isolated dots ($70\text{ }\mu\text{m}$ and $150\text{ }\mu\text{m}$ diameter). The layout of this slide can be found [here](#). Our slides have a clear background. These slides were purchased from Ali Express, are about 5% of the cost of the Ted Pella slide.

- (2) Plastic, uniform fluorescent slides These are used to test uniformity of the illumination, linearity of the camera, and the accuracy of the metal halide intensity adjustment.
- (3) Fluorescent test slides from [Spherotech, Inc.](#) as follows:
 - A mixture of 6 beads sizes: 0.56, 0.9, 3.1, 5.5, 9.9 and $16.2\text{ }\mu\text{m}$ (Cat. No. FPS-M57-6).
 - A mixture of $3.0\text{ }\mu\text{m}$ beads with five intensity levels (Cat. No. FPS-3057-5).
 - A mixture of $3.1\text{ }\mu\text{m}$ beads with five intensity levels, as above, but the different levels are located in separate wells (Cat. No. FPS-3057-5LN).

All of these beads contain a mixture of fluorescent dyes, so they will be visible with all of our filter sets. The specifications of these slides can be found through the hyperlink given above.

- (4) A test slide with fluorescent beads of different sizes and intensities (ie, concentrations of fluorophore). The slide is FocalCheck™ test slide #1, and its specifications can be found [here](#).

2.5. Biological Slides and Plates. A number of biological preparations will be studied. These include:

- (1) Fixed cells

- [FluoCells™Prepared Slide #1](#)

[BPAE](#) cells that have been stained with DAPI to stain the nuclei blue, [MitoTracker®Red CMXRos](#) to label mitochondria, and [Alexa Fluor®488 phalloidin](#) to label [F-actin](#).

- [FluoCells™Prepared Slide #2](#)

[BPAE](#) cells that have been stained with DAPI to stain the nuclei blue, a [BODIPY-FL](#) labeled antibody against [α-tubulin](#), and [Texas Red™-X phalloidin](#) to label [F-actin](#).

There are two prepared slides of fixed cells that are no longer used in the lab, but which appear in this writeup in the appendices. There are:

- (a) [GATTA-Cells 4C](#), purchased from [Gattaquant](#) (Zurich, Switzerland). TOM-20 is a mitochondrial protein, while tubulin and actin are major components of the cytoskeleton.
 - (b) [COS-7](#) cells that have been stained with DAPI, anti-TOM20-OG488, anti-tubulin-TMR (tetramethyl-rhodamine), and actin-SiR. This is also from [Gattaquant](#).
- (2) Live cells that are grown in transparent cell-culture dishes (either 35 mm diameter plates, or 24 well multi-well plates). It is relevant that the objectives in the lab are designed to work with a 1.2 mm coverglass thickness, so they are appropriate for imaging live cells in culture plates, which normally have a thickness of 1-1.2 mm. The cells are grown in cell culture medium, which provides the nutrients and environment that is required for the cells to grow.

2.6. Fluorescein Di-acetate (FDA). [Fluorescein diacetate](#) is non-fluorescent molecule that crosses cell membranes and enters cells. In living cells, esterases convert the molecule into fluorescein, which is highly fluorescent. When fluorescein diacetate is added to a cell culture, the live cells will become fluorescent, and therefore, it is used as a live cell indicator.

The required dilution will depend on the concentration of the stock solution. Our current solution is 0.5 mM in acetone.

2.7. Image Acquisition and Analysis Software. The camera is controlled using open source [Micro-Manager](#) (version 1.4) software. Micro-Manager controls all of the relevant camera parameters, and also is used to acquire time lapse sequences.

The [Fiji](#) distribution of ImageJ is recommended for off-line processing of the images acquired in the lab. Instructions on how to download, install and use Fiji can be found [here](#).

Both Micro-Manager and Fiji are open source applications that run on almost all computer platforms (Linux, Macos, Windows). However, Micro-Manager must interface with hardware (cameras, automated microscopes, motorized stages, light sources, etc). Often, the hardware drivers only run under Windows. For this reason, in this lab, Micro-Manager runs under Windows 10-64bit.

3. EXPERIMENTS

3.1. Spatial Calibration. The image that is acquired by the camera and saved in an image file is a matrix of numbers, each of which corresponds to the intensity of the image at that point. This means that the image is spatially sampled. In addition, the camera digitizes the intensity information into a 12 bit number, which means that there are at most 4096 distinct grey levels.

The goal of this experiment is to spatially calibrate the digital image. That is, we want to know the relationship between the physical length of some feature, and number of pixels that it fills on the camera. We will calibrate the system by imaging an object with known dimensions (in our case, a ruler), and measuring its features (in pixel units) in the image. We should then be able to determine the calibration in units of microns/pixel. The ruler is opaque, so it is best imaged using the bright-field technique. Use tools available in Fiji to make the required measurements.

3.1.1. Tasks.

- (1) Image the ruler using each of the objectives ($4\times$, $10\times$, $20\times$, $40\times$). Acquire images with the ruler in different areas of the field (center, upper left, lower right). This will allow you to determine if the magnification is uniform across the field.
- (2) Save your images. Recommendation – for each objective, add the new images to an album that can be saved as a stack. This is much easier than having many individual image files.

3.1.2. Questions to address in your report.

- (1) List the spatial calibrations (microns/pixel) for each objective. Report on the uniformity of the magnification across the field.
- (2) Is the measured calibration consistent with the theoretically calculated value? What is the error (average and standard deviation)? Is it a random error or a consistent bias? The theoretical spatial calibration is calculated as follows:

$$\frac{\text{Camera pixel size}}{\text{Objective magnification} \times \text{Relay optics magnification}} \quad (1)$$

Based on your measurements, what is the magnification of the relay optics? Give this number to two decimal places. You have measurements for each of the objectives, so provide an average and standard deviation for your estimate of the relay optics magnification.

- (3) Are the relative calibrations as expected? For example, is the $20\times$ objective magnification twice that of the $10\times$.

3.2. Phase Microscopy. In this experiment, you will image live cells using both phase optics and the bright-field method (that is, without the phase ring in the optical system). You will compare the resulting images in order to understand the motivation for using phase optics to image thin, transparent phase objects.

3.2.1. Tasks.

- (1) Acquire phase contrast images and bright-field images of the cells, using the $10\times$, $20\times$ and $40\times$ objectives and the appropriate phase rings. Be sure to acquire phase and bright-field images of identical fields, so that the images can be compared. When switching between phase and bright-field imaging, you probably need to change the camera exposure.
- (2) Acquire an image using the $40\times$ phase ring with the $20\times$ objective. Is this a phase or bright-field image?
- (3) Acquire a phase image at $20\times$, using the correct phase ring, but slide the phase ring slightly right and left, while observing the acquired image. Acquire an image with the phase ring optimally

positioned, and an image with the phase ring slightly shifted, so that you can present them in the report and compare them.

3.2.2. In the report... You should compare phase images to bright-field images. Use tools available in Fiji to demonstrate the increased contrast of the phase images. Show that the phase imaging enhances edges. Show representative images for the cases of a misaligned or mismatched phase ring, and explain why the images change.

3.3. Dark Field Microscopy. Dark field microscopy is done by illuminating the sample with a cone of light whose angle exceeds the numerical aperture of the objective. Therefore, the objective can only collect light that has been scattered from the sample, resulting in light image on a dark background. Dark field microscopes are equipped with a high numerical aperture (NA) condenser, and objective whose NA is lower than the NA of the condenser. Our microscope is not equipped with dark-field optics, but we can still do dark-field imaging by using the $40\times$ phase ring together with the $4\times$ objective, because the NA of the $40\times$ phase ring is greater than the NA of the $4\times$ objective.

3.3.1. Tasks.

- (1) Acquire bright-field images of the ruler using the $4\times$ objective. Now slide the $40\times$ phase ring into position, while still using the $4\times$ objective. Compare the bright-field and dark field images, and save a pair of images that can be used to explain what dark-field imaging does.
 - (2) Image one of the slides with submicron beads using the dark field setup. Try finding bright-field images of these beads, using the $4\times$ objective. You most likely will not succeed in finding bright-field images
 - (3) Acquire bright-field images of the cells using the $4\times$ objective. You may find it easier to use phase imaging at $10\times$ before switching to the $4\times$ objective. Now try to acquire dark field images of the cells. Save a pair of images that can be compared to each other in your report.
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3.3.2. In the report... Show the dark-field the corresponding bright-field images of the ruler, and explain the result. Show also the dark-field and bright-field images of the cells and the beads. Use the intensity-profile tool of Fiji to measure the full-width half-maximum (FWHM) width of the beads, in order to estimate the optical resolution of the $4\times$ objective.

3.4. Fluorescence Microscopy. Fluorescence microscopy is perhaps the most common contrast-enhancement method in microscopy. In this section, you will characterize the fluorescence microscope, and use it to visualize cell that have either been stained with a fluorescence label or have a genetically encoded fluorescent label (that is, some variant of green fluorescent protein – GFP). You will need to learn about [fluorescence](#). In addition to the hyperlink in the previous sentence, this [link](#) and this [link](#) are recommended (the material specific to flow cytometry can be skipped).

3.4.1. Linearity, Field Uniformity and Dark Response. Use a uniform fluorescent plastic slide to assess the linearity of the intensity response and the field uniformity of the system. There are number of parameters that should be tested for linearity:

- (1) Response to changes in camera exposure time.
- (2) Response to changes in illumination intensity.
- (3) Response to changes in strength of the fluorescent label.
- (4) Response when no light is preset (dark response).

3.4.1.1. Tasks. Image one of the plastic uniform fluorescent slides. Find some surface feature, or ask one of the instructors to place a mark on the surface to assist you in focusing only the surface of the slide.

- (1) Acquire an image with the light source set to 100%, and the camera exposure set for the highest signal that is not saturated.
- (2) Select successively, the 75%, 50%, 25%, 10% and 0% settings on the metal-halide light source. At each step , acquire and image (add the images to an album). Save this album for later processing.
- (3) Set the camera exposure to 10 ms, and adjust the light-source intensity control to get an image that is low, but still above zero, Then acquire images with the camera exposure set to 10 ms, 20 ms, 50 ms, 100 ms, 150 ms, 250 ms, and 500 ms. These images should be saved as a stack for further analysis. You may stop acquiring when the imager saturates. For each exposure, you should acquire an image with the light course set to zero. These "dark" images will be used later to characterize the dark noise of the camera.

3.4.1.2. In the report... The above data will be analyzed for linearity. You will need to measure the response of the camera (mean and standard deviation, though in this experiment the mean is most relevant) as a function of either source intensity setting or camera exposure setting, and determine if the response is linear. (Hint: use the histogram function to acquire statistics of the intensity.)

Measure the histogram in five sections of the images (center and four corners), and report on whether the system is spatially uniform.

Finally, measure the dark current that was acquired at the different exposures, and determine the linearity and offset of the dark current.

3.4.2. *Resolution and Linearity.* [FocalCheckTM test slide #1](#) or the [Spherotech, Inc.](#) slides described above will be imaged to test the spatial resolution of the system.

3.4.2.1. Tasks.

- (1) Image the beads, beginning with the largest beads and working towards the smallest beads, until you can no longer resolve the beads. This must be done using the 10 \times , 20 \times and 40 \times objectives. Acquire and save the images. If you are imaging the Spherotech slide with six bead sizes, you do not have to move between wells, but you will need to image a few fields to make sure you have all of the sizes in your images.
- (2) Acquire images of the 1 μm beads in focus (as for all of the images) and also acquire two out-of-focus images. The two out-of focus images should be acquired as one just above the focal plane and one just below the focal plane. We will want to know if the image goes out of focus "symmetrically", or in an asymmetric manner (which is indicative of strong spherical aberration).
- (3) The FocalCheckTM test slide has a row of 6 μm beads, which differ in the strength of their labeling, so that they form an intensity series. The Spherotech FPS-3057-5LN slide has a row of 3 μm beads, which differ in the strength of their labeling. Using one of these slides, image the beads in the different wells, and plot the intensity of the beads against the strength of the labeling. Be sure to include the standard deviation of the measured intensity on plot, and include a linear regression line.

3.4.2.2. In the report... show the results of the linearity and resolution tests. Use the intensity-profile tool of Fiji to measure the full-width half-maximum (FWHM) width of the beads, in order to estimate the optical resolution of the objectives. This is best done with the smallest size bead that could be measured.

The particle analyzer and histogram tools can be used to measure linearity of the response. Use background subtraction and thresholding to locate the beads, and the particle analyzer to measure their average intensity. The histogram tool is very useful for measuring intensities over areas, as with the uniform fluorescent slides.

3.5. Fluorescently Labeled Fixed Cells. In this experiment, you will work with the prepared slides of fixed cells that are described above. You will image the three labels (DAPI, BODIPY-FL™ or Alexa Fluor™488, and Texas Red™ or CMRos. WHEN IMAGING WITH THE DAPI FILTER SET, WHICH USES UV EXCITATION, YOU MUST WEAR THE SAFETY GOGGLES PROVIDED IN THE LAB.

3.5.1. Tasks.

3.5.1.1. FluoCells™ Prepared Slides.

- (1) Use the filter set appropriate for red fluorescent dye to image either the mitochondria or F-actin (depending on which slide you have). Our experience is that these cells are easiest to find when imaging the red dyes on these slides.
- (2) Image the cells using the 20× objective. You will probably find that the focus needs to be adjusted a little between the different color channels.
- (3) Select the appropriate filter set to image the green label (blue excitation, green emission). You should see the the appropriate green-labeled component.
- (4) Image the DAPI using the appropriate filter set (UV excitation, blue emission). You should see the nuclei.
- (5) Save these images as a stack – in your report you will overlay the images, and verify that labels appear where they are expected in the cell.
- (6) Set up a time lapse acquisition for DAPI, in which images are acquired every 10 seconds for 5 minutes. You should observe that the DAPI emission decreases as the dye is photobleached. Save the time sequence, and use it to measure the rate of photobleaching (that is, the time constant of the exponential decay).

3.5.1.2. In the report... show representative DAPI, green channel and red channel images separately, and a pseudo-colored overlay (DAPI should be blue, the green-emitting fluorophore should be green, and the red-emitting fluorophore should be red). Use the background subtraction tool in order to remove the large uniform background from the images. The image can be enhanced by taking advantage of the fact that the labeled structures (mitochondria and tubulin) are punctate or thin lines, while the background is either uniform or slowly changing. Be sure to check that the three channels appear to be correctly aligned. Appendix D.6 shows how this can be done.

Show a photobleaching response curve (intensity vs time) for the DAPI. If the time series is arranged as an image stack, then the Z-profile tool for processing stacks (**Image>Stacks>Plot Z-axis Profile**) can be used to generate the decay curve. Do not forget to account for the dark (offset) signal. Fit the decay curve to an exponential function, and determine the photobleaching time constant.

4. LIVE CELL IMAGING

4.1. Cells that express H2A-RFP. This experiment will be done using live B16 melanoma cells that express a fusion protein histone H2A-mRFP. mRFP is a red-emitting fluorescent protein. Recall that histones are the proteins that form the nucleosomes around which the DNA is coiled. Therefore, H2A-mRPF is expected to be localized to the nucleus.

4.1.1. Tasks. Aquire phase images of cells. Now acquire fluorescence images of the cells using the appropriate filter set. Set up a timelapse series to acquire images every 15 seconds for 10 minutes, and save the timelapse series as a stack.

4.1.2. *In the report...* show representative phase and fluorescence images of the cells. Show the separate images, as well as a fused composite image (as was done for the fixed cells). In the composite image, use grey for the phase image and red for the H2A-mRFP channel. Try to segment the nuclei using background subtraction, thresholding, and the particle analyzer. Use an edge detector to find the outlines of the nuclei, and then project the stack into a single image to see if you find evidence of movement of the cells.

4.2. Timelapse Imaging - Phototoxicity. In this experiment, you will look for evidence of phototoxicity of UV illumination. Time-lapse microscopy will be used to follow the cells during irradiation.

4.2.1. Tasks.

- (1) Set up the microscope to produce phase images of the cells, using the $40\times$ objective. If a clear image cannot be obtained with the $40\times$ objective, then use the $20\times$ objective.
- (2) Set up Micro-Manager to acquire images every minute for 30 minutes. This will be the control experiment, in which we observe cells which are not irradiated with UV. However, they are being imaged in an unheated plate, which means that they are not in an optimal environment (which would be heated to 37°C).
- (3) Repeat the previous step using a different field that is well separated from the first field, but this time irradiate the cells with UV from the DAPI filter set.

4.2.2. *In the report...* show the control and irradiated cells at time $t=0$ and at the endpoint of the timelapse acquisition, and describe the evidence (if any) that the UV irradiated cells have been damaged. Try to find a morphological measurement that correlates with the visible damage that may be seen. Spatial variance, area or roundness are examples of parameters that may work.

4.3. Live cell Indicator. In this experiment, you will observe the conversion of fluorescein diacetate (which is not fluorescent) to fluorescein (which is highly fluorescent) in live cells. The protocol is to add fluorescein diacetate to the culture medium. The fluorescein diacetate will enter the cell, where it will be converted to fluorescein, which cannot cross the cell membrane. After the fluorescein diacetate is added to the solution, the cells will be imaged over time to observe the fluorescence within the cells over time. The cells will be in 6-8 wells of a 24-well multiwell plate, so that the experiment can be repeated conveniently.

4.3.1. Tasks.

- (1) Choose one of the wells, and acquire phase images of the cells using the $20\times$ objective..
- (2) Add fluorescein diacetate to the culture medium, so that the final concentration of fluorescein diacetate in the medium is about $1\text{ }\mu\text{M}$. Wait a couple of minutes to allow the FDA to enter the cells and be hydrolyzed
- (3) Acquire fluorescence images the cells using the appropriate filter set (blue excitation and green emission). Note the exposure and excitation setting required to obtain high quality images. To minimize photobleaching, you may wish to set the exposure to 100 ms and reduce the excitation intensity as required to prevent saturation of the camera.
- (4) Wash the well that you just used, by pipetting out the medium (which now contains FDA) and replacing it with fresh medium. We suggest doing this twice.
- (5) Now observe the fluorescence of the cells. The background should be much darker. Set the excitation to 100% and lower the exposure as necessary to avoid saturation. This should be one quicky to avoid bleaching the cells.
- (6) Acquire a timelapse acquisition for 1 image every 10 s, for 5 min. This timelapse should record the photoleaching of the fluorescein.

- (7) Now move to a different well. Find a appropriate field and again acquire a phase image.
- (8) Set up a time-lapse acquisition, acquiring every 200 ms for 120 s. Use the camera setting that you found for the first well, but double the excitation percent.
- (9) This part is tricky. You will need to start the time lapse, and then immediately add the FDA **without moving the multiwell plate**. The time-lapse should record the increase in intracellular fluorescence when the FDA is added. If the cells go into saturation as the fluorescence increases, reduce the excitaiton to about half (for example, from 50% to 25% or 25% to 10%). You should be able to compensate for this during the image processing.

Note that the cells are being imaged in an unheated plate, which means that they are not in an optimal environment (which would be heated to 37°C). However, in our experience, they remain viable for the duration of the experiment. We again point out that the last part of this experiment is difficult. You will have some spare ells with cells, so that you can repeat the experiment a few times if necessary.

4.3.2. In the report... show three fluorescent images of the cells - one at the start, one in the middle, and one at the end. For the time-lapse images, plot the average fluorescence of each cell vs time. One of the time-lapse stacks should contain the increase in intracellular fluorescence following addition of FDA. Try to measure how fast this occurred. The others will show the photobleaching of the dye.

A typical result is shown in Figure 1

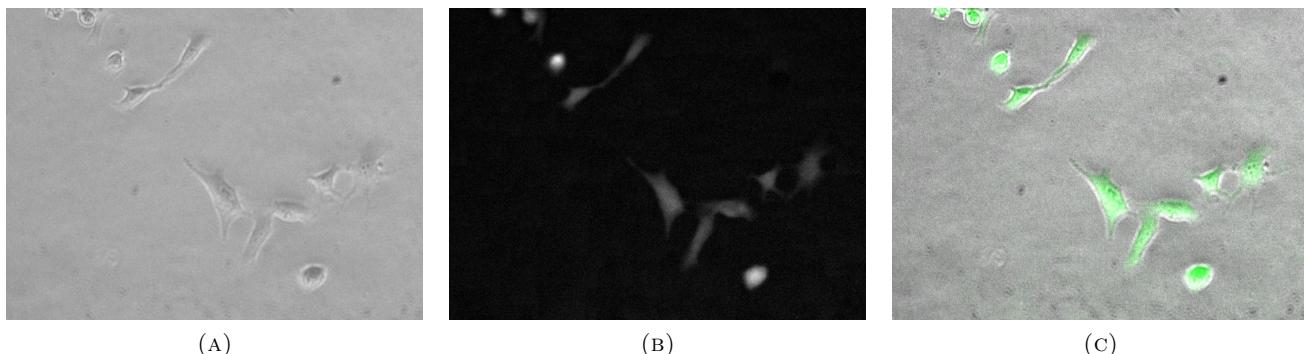


FIGURE 1. (A) Phase image of live B16 cells (B) Fluorescence image of live B16 cells after addition of fluorescein diacetate. The broad background was subtracted from this image. (C) Composite image: phase is grey, FDA is green. Contrast was adjusted to improve visibility in the document.

When you do this experiment, you will find that the fluorescence in the medium increasea with time. Therefore, in order to visualize the fluorescence that is due to the intracellular enzymatic activity, you must estimate and then subtract the background. In your report, you should show a plot of fluorescence vs time for the cells (a least a cell), the background, and for the cells with the background subtracted. Figure 2 shows the result that I obtained. In your report explain these three curves. More about this experiment can be found in Appendix E.

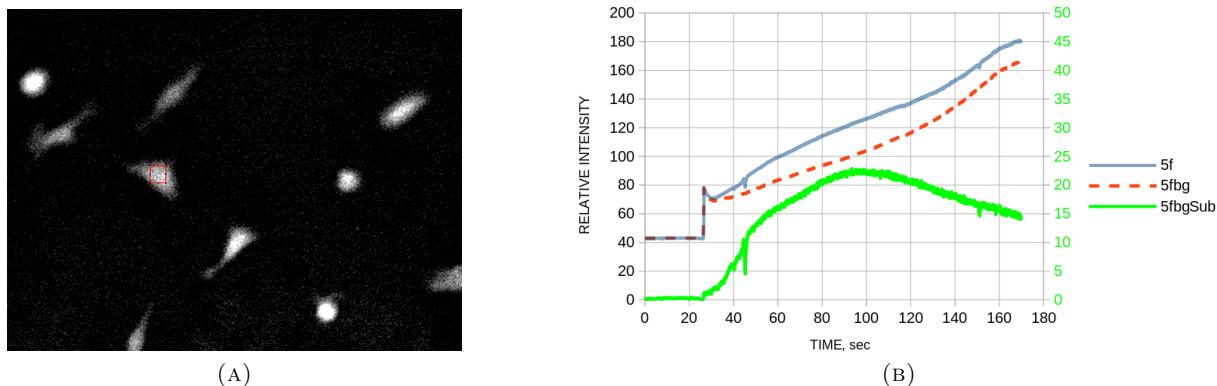


FIGURE 2. (A) One image from a stack of 3398 images acquired every 50 ms (B) Plots of fluorescence vs time. The cell fluorescence was measured within the red square region of interest (ROI). The background was measured in a similar ROI located on the background. $5f$ is the total fluorescence in the marked area. $5fBg$ is the background culture medium fluorescence, and $5fbgSub$ is the fluorescence within the marked area after subtraction of the estimated background. The last curve uses the expanded scale on the right.

APPENDIX A. FLUORESCENT DYES

In this lab, we use fluorescent labels to produce contrast in cells. Here we will add some information about these dyes. Before reading this material, be sure to learn about fluorescence, for example by following [this link](#). There are many on-line viewers that can show absorption and emission spectra of many fluorophores and fluorescent proteins. Here is one [example](#).

A.1. DiI-C18(3). The structure of DiI-C18(3) is shown in Figure 3. The ring structures and methine bridge create a set of conjugates π -bonds that allow electrons move relatively freely between the two ends of the molecule. This is responsible for the optical characteristics (absorption and emission spectra) of the molecule. More about cyanine dyes can be found [here](#). Notice that the cyanine dyes form a family of fluorophores whose excitation and emission spectra can be tuned by the length of the polymethine bridge in their structure. DiI-C18(3) has two very hydrophobic hydrocarbon chains (18 carbons in length) that

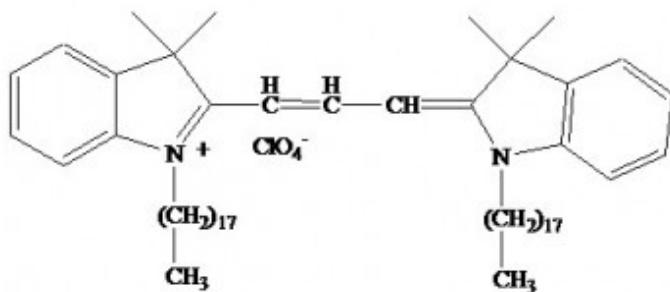


FIGURE 3. Chemical structure of DiI-C18(3), taken from www.abbkine.com/product/dii-diic18-3-bmd00071/.

cause the molecule to be hydrophobic. Therefore, this dye is used to label membranes (which have a very hydrophobic interior).

A.2. DAPI. The structure of DAPI is shown in Figure 4. DAPI is fluoresces most efficiently when it is in contact with DNA, and it is therefore used to visualize nuclei (where most of the cell’s DNA is located) and condensed chromosomes (for example during mitosis). Information about DAPI can be

found [here](#). Notice that the conjugates π -bonds of DAPI are not spread out over as long a length as for

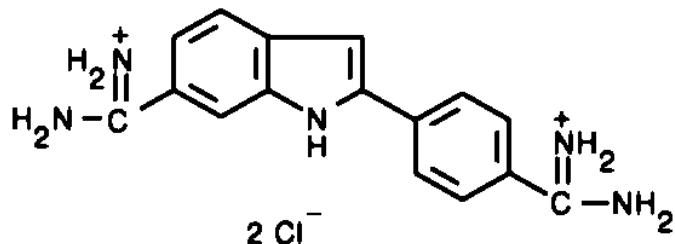


FIGURE 4. Chemical structure of DAPI, taken from www.thermofisher.com/order/catalog/product/D1306

Dil-C18(3), and the excitation and emission spectra are correspondingly shifted to shorter wavelengths. It is often the case that the excitation and emission peaks of a fluorophore move to longer wavelengths as the conjugated π -bond structure increases in length (think of the energy levels of a particle in a box, which you learned about in your quantum physics course).

A.3. OG488 and TMR. The structure of Oregon Green 488 (OG488) is shown in Figure 5(A). Compare it to the structure of fluorescein, which appears in Figure 5(B). Not surprisingly, these have similar absorption and emission spectra. Fluorophores often come in families. Figure 5(C) shows the structure of TMR. This is also similar in structure, but the substitution of amine groups in place of the hydroxyl groups results in a shift of the spectra to longer wavelengths.

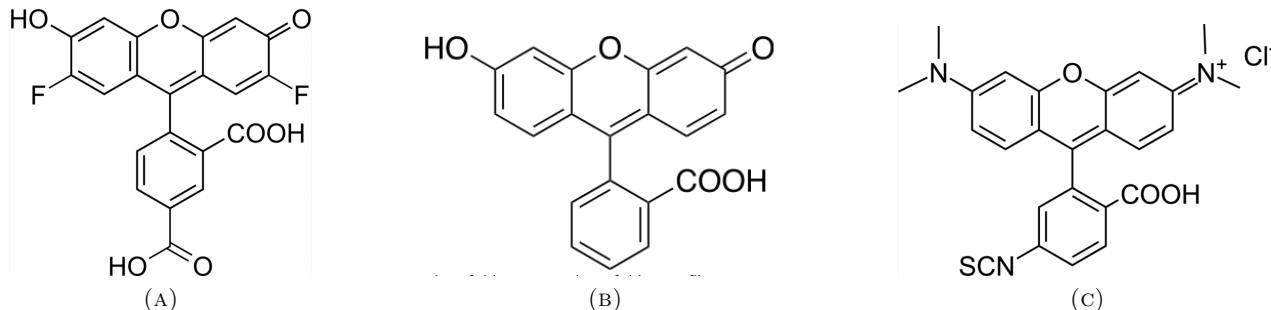


FIGURE 5. (A) Structure of OG488, taken from www.aatbio.com/products/5-og488-acid-oregon-green-488-carboxylic-acid-5-isomer.
 (B) Structure of fluorescein, taken from www.aatbio.com/products/fluorescein-cas-2321-07-5.

(C) Structure of tetramethylrhodamine, taken from www.aatbio.com/products/6-tritc-tetramethylrhodamine-6-iso thiocyanate-cas-80724-20-5

The isothiocyanate group (SCN) at the bottom left allows the dye to be attached to proteins (which are rich in amine groups). This structure is therefore actually TMR-isothiocyanate.

A.4. Fluorescein diacetate. Fluorescein diacetate is shown in Figure 6(A). The ester bonds at both ends of the thre ring cause the molecule to lose its fluorescence. The molecule is also neutral at physiological pH, allowing it to pass through cell membranes.

Once inside a live cell, esterases cleave the ester bonds, converting the molecule to fluorescein, shown in Figure 6(B). This molecule is fluorescent, and is also anionic at physiological pH, so it cannot pass through the cell membrane, and remains trapped inside the cell.

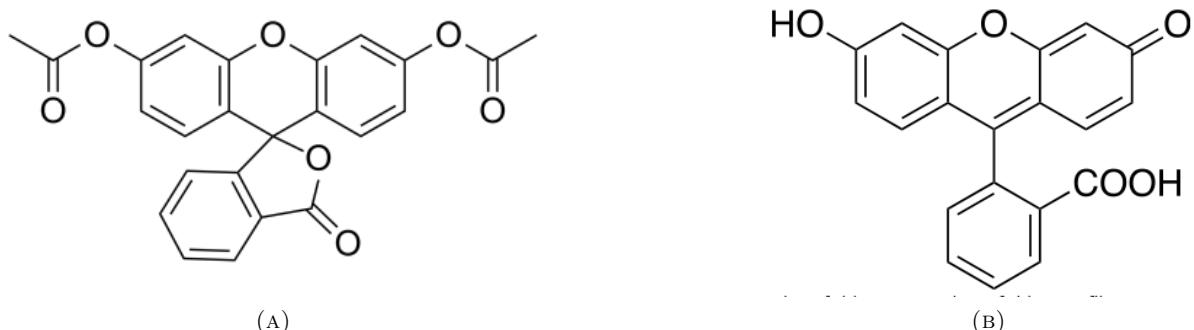


FIGURE 6. (A) Structure of fluorescein diacetate. (B) Fluorescein, to which (A) is converted after cleavage of the ester bonds.

APPENDIX B. FLUORESCENT PROTEINS

Nature has evolved a family of fluorescent proteins, the first of which was discovered in the jellyfish *Aequorea victoria*, and emitted green light, so it is called GFP (green fluorescent protein). Since then many variants of this protein have been developed, resulting in a family of fluorescent proteins (FP) whose emission peaks cover almost the entire visible light spectrum, as well as part of the near infra-red (NIR). The Nobel Prize in Chemistry was awarded to the developers of this technology. (Taken from en.wikipedia.org/wiki/Green_fluorescent_protein).

Plasmids containing genes for these proteins are readily available (for example, through [addgene](#)), and these can be transfected into cells, which causes the cells to synthesize their own fluorescent label. Often, the FP is fused onto one of the ends of some protein of interest, thus allowing researchers to observe that protein in live cells.

In this lab, we observe cells that express a protein which is a fusion of histone H2A and mRFP (monomeric red fluorescent protein). Since histones are localized to the nucleus, and are associated with chromatin, these cells have nuclei that emit red light when excited with green or yellow light. There is another important characteristic of these cells. They have undergone “stable” transfection, which means that the plasmids not only entered the cells and express the H2A-mRFP fusion, but they have been incorporated into the genome (that is, into one of the chromosomes) so that when they undergo mitosis, the new cells will continue to express the fusion protein. Thus, we have a new cell line that can be used to generate fluorescent cells for the course for as long as needed.

APPENDIX C. SOME INSIGHTS ON MICROSCOPY

C.1. Numerical Aperture. The numerical aperture (NA) is related to the widest cone of light that can enter the lens system (or, equivalently, the largest cone of light that a lens system can produce). There are two major effects of NA.

- (1) The spatial resolution of an objective depends on the NA. Larger NA means that finer detail can be resolved. A very good discussion of this, with excellent interactive tutorials, can be found [here](#).
- (2) A higher NA objective will produce a brighter image, all else being equal, because a wider cone of light is captured by the objective.

C.2. Magnification. Consider that the image produced by the microscope is a constant size (that is, the camera as a fixed area detector). Therefore, as the magnification increases, the area of the object that is seen decreases. If the object emits a certain amount of light per unit area, the brightness of the image will decrease with increasing magnification, because as the magnification increases, the detector is filled with light from a smaller area on the object. The effect of magnification and NA on image brightness is explained [here](#).

C.3. Phase microscopy. Phase contrast microscopy is implemented by illuminating the sample through an annular aperture (that is, with a ring of light), and aligning a phase ring in the back focal plane of the objective to be coincident with the image of the illumination ring. The two rings can be observed in the back focal plane of the objective by removing the ocular and looking down the microscope. In practice, small telescope (often called a phase telescope) is usually provided with the microscope, and can be used to view the rings more comfortably. This [link](#) points to a phase microscopy simulator, which can be used to "operate" a phase microscope, and better understand the alignment of the phase illumination, phase plate, and phase telescope.

C.4. Geometric optics simulator. You can build and test a simulation of a compound microscope using an online [geometric optics simulator](#).

APPENDIX D. IMAGE PROCESSING EXAMPLES

As stated above, the [Fiji](#) distribution of ImageJ is recommended for off-line processing of the images acquired in the lab. Here, we provide some examples of how this tool can be used. These examples are provided as macro scripts that run ImageJ commands. This section by no means covers all of what you need to do, but it provides examples to help introduce you to many features that you will use. The scripts are provided together with images or image stacks for which the scripts are known to work. You may load the scripts into the Fiji script editor and run them on the test images provided.

One of the most powerful features of ImageJ is its scripting tools. You can develop a method, and then run it on many images without having to repeat all of the steps manually. The best way to do this is though the API (Application Programming Interface), that allows you to write code in Java or Python. A number of excellent tutorials are available on-line to assist those who wish to program using the API. [Here](#) is an excellent tutorial for scripting in Python.

However, for beginners writing simple scripts, the ImageJ macro language is easiest to learn. The macro language syntax is described [here](#), and the command set is documented [here](#). A rich set of examples can be found [here](#).

The scripts discussed here can be found in the 83411 [github repository](#).

D.1. Analysis of a series of images. In a number of the experiments, a series of images are acquired in which a single parameter is changed. These images are best saved as a stack, so that they can be opened in ImageJ and processed together. [segmentAndZprofile.ijm](#) is a script that can identify objects in one frame, and then measure their intensity in the other frames. The hyperlink to the program includes some images that can be used to verify the operation of the program. The script is extensively commented, and introduces tools such as the Particle Analyzer and the ROI (Region Of Interest) Manager. Figure 7(A) shows a plot of the mean bead intensity versus percent illumination (set on the Prior Lumen 200 illuminator) together with the best fit linear regression line for these data. Figure 7(B) shows one of the images of a field of 4 μm beads. The red outlines show the identification of the beads by the particle analyzer. Note that the Z-axis Profiler can be used on time series to provide a time-axis profile (for example, in order to measure fluorescence photobleaching).

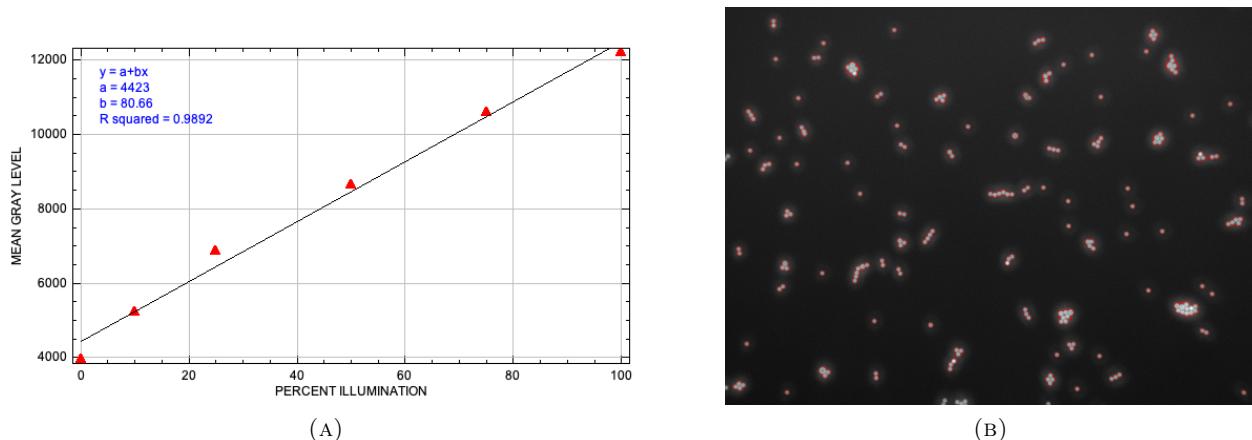


FIGURE 7. (A) Mean bead intensity vs percent illumination. (B) Image of beads (100% illumination intensity). The red outlines were created by the particle analyzer (PA), and show the objects that the PA identified as beads or bead clusters.

D.2. Spatial calibration. The spatial calibration is required to relate the distance in pixels on the image to the true size of the object. [spatialCalibration.ijm](#) is a script that prompts for an image of the ruler, prompts the user to draw a line or rectangular region of interest on the ruler, prompts the user to input the number of pixels that cover a known distance, and then calculates the spatial calibration in microns/pixel. It also set the spatial calibration of the input image. There is a test image provided with the script. It contains a stack of ruler images, taken with the $4\times$, $10\times$, $20\times$ and $40\times$ objectives. One of the test images is not aligned very well with the X-Y axes, but it is good enough for testing. Note that the image brightness changes with the different magnification. Image brightness decreases as magnification squared, and increase as NA squared. You can use the Z-axis profile tool introduced above to see if the image brightness in the test stack follows these relationships.

D.2.1. Automated spatial calibration. [autoScale.ijm](#) is a script that can automatically identify the 100 micron lines on the ruler and determine the number of pixels between them. Therefore, it can automatically find the spatial calibration (in microns/pixel). Note that this macro uses the [Extended Particle Analyzer](#) which is part of the excellent [BioVoxcel Toolbox](#) written and maintained by [Jan Brocher](#).

Figure 8(A) shows an image of the ruler that was acquired with a $10\times$ objective. The green outlines surrounding the 100 μm markings were created by the autoScale macro, and confirm that the macro found those markings. Figure 8(B) shows a line intensity profile along the red line that is drawn on the image in Figure 8(A). The sharp decreases in intensity correspond to the 100 μm markings, and they are seen to be 100 μm apart.

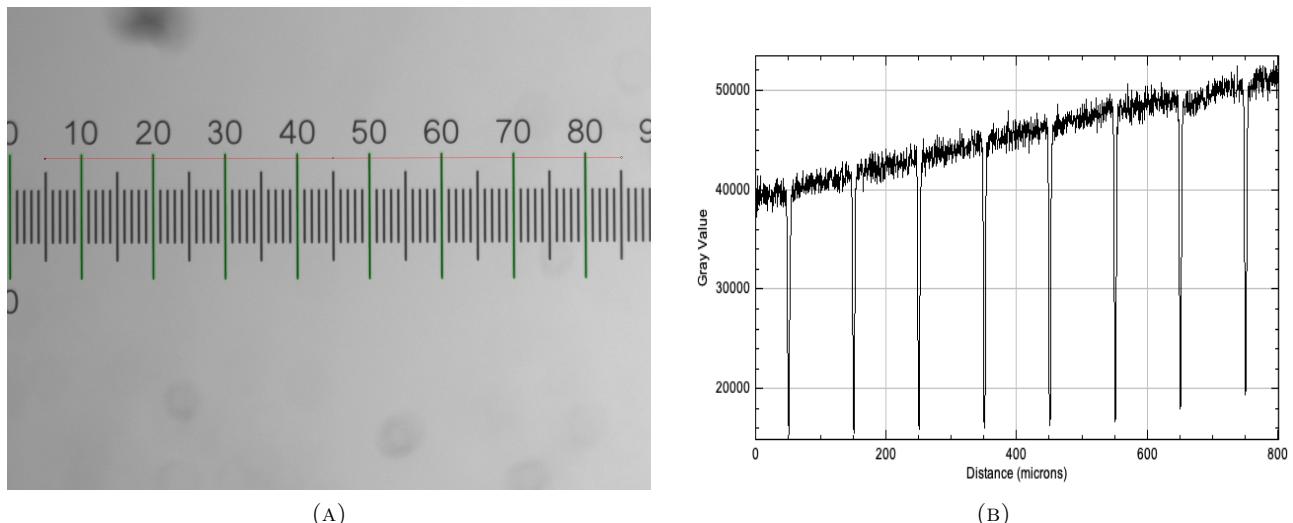


FIGURE 8. (A) Brightfield image of the ruler, acquired with a $10\times$ objective. The green outlines surrounding the $100\text{ }\mu\text{m}$ markings were created by the autoScale macro, and confirm that the macro found those markings. (B) Line intensity profile along the red line in the image, after the image was calibrated using the autoScale macro. The sharp decreases in intensity correspond to the $100\text{ }\mu\text{m}$ markings.

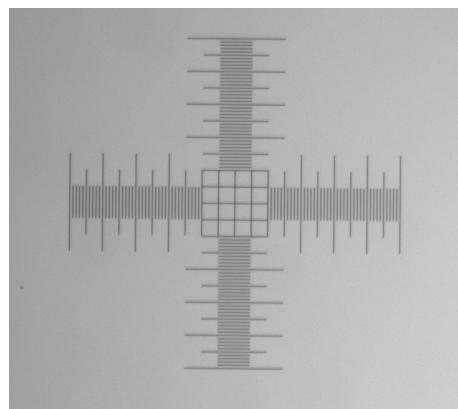


FIGURE 9. Finest scale has vertical and horizontal scales with $10\text{ }\mu\text{m}$ spacing, and a $50\text{ }\mu\text{m}$ grid in the middle.

The log window shows the following output:

```
image path: <long pathname that does not fit on this page>
image name: magSeries190204.tif
Width = 2048 ; Height = 1536 ; Channels = 1 ; Slices = 1 ; Frames = 5
Slice = 2
Objective magnification = 10X ; Minimum size (pixels) = 2500
Number of pixels between extreme 100 micron markings = 1830
Number of 100 micron gaps = 8
Spatial calibration = 0.4372 microns/pixel
```

The Ali Express calibration slide has a different layout for its finest scale (10 µm per division), seen in Figure 9. This layout will confuse the autoScale script. There is an updated version of autoScale in the course code [github repository](#) called autoScaleDev220222.ijm. The description at the top of the script

describes how to use it with the Ali Express slide. A magnification series of the Ali Express slide 10 μm scale can be found in the spatial calibration directory of the repository.

D.3. Intensity ratios within a stack. [zProfile.ijm](#) is a script that prompts for an image stack, and calculates the running ratios (ratio of current slice to previous slice) of the mean intensity of slices in a stack. A plot of the intensities and a table that includes the ratios are produced. The program also prompts for the dark level (offset) of the system.

D.4. Dark noise vs exposure time. [darkNoise.ijm](#) is a macro script that receives as input an image stack that consists of dark images (images acquired with the camera sensor not exposed to light) acquired at different camera exposure times. It then plots the mean gray level of each image against exposure time, and displays these data together with the best fit straight line. The offset and slope of the dark noise vs exposure is also displayed. The script expects the dark images in the stack to be positioned in order of monotonically increasing exposure. Figure 10(A) shows the output of a dark noise measurement with camera gain set to 10dB, while Figure 10(B) shows the output of an identical measurement with camera gain set to 25dB. The thermal component of the dark noise is amplified by the higher gain, and this manifests itself with a much larger slope.

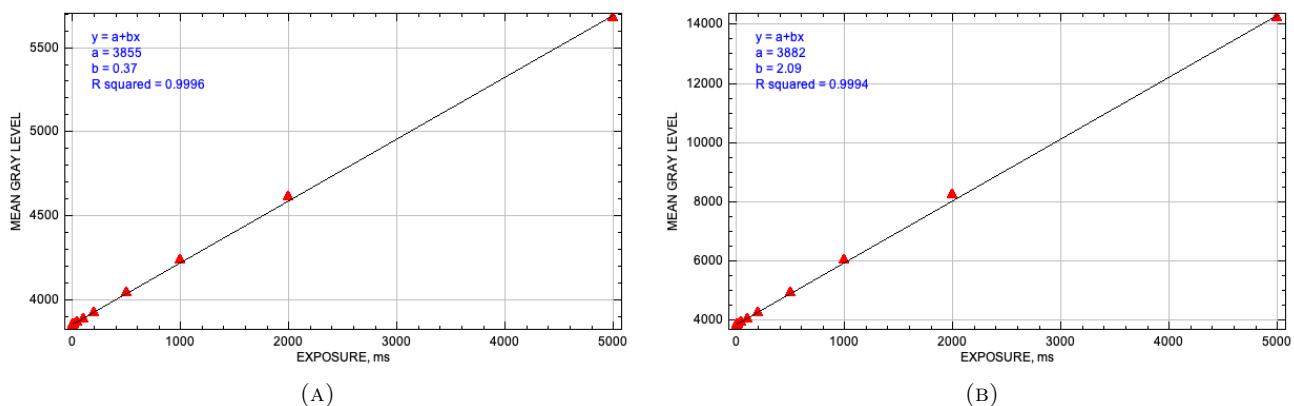


FIGURE 10. Dark response vs exposure time. The red triangles are the measured response, and the line is the best linear fit. The exposure times that were measured are (4,10,20,50,100,200,500,1000,2000,5000)ms. (A) Camera set to 10dB. (B) Camera gain set to 25dB. Notice the much greater slope with the higher camera gain.

D.5. Creating composite images. Fluorescence microscopy is often done using multiple fluorescent labels, as done in this lab. It is often desirable to overlay the images of these labels, so that their relative positions within the cells are easily seen. An example of this is shown in Figure 11A, which shows an image of three of the labels on the GATTA-Cells 4C test slide. Figure 11B is a phase image of the same field. This figure was created as follows:

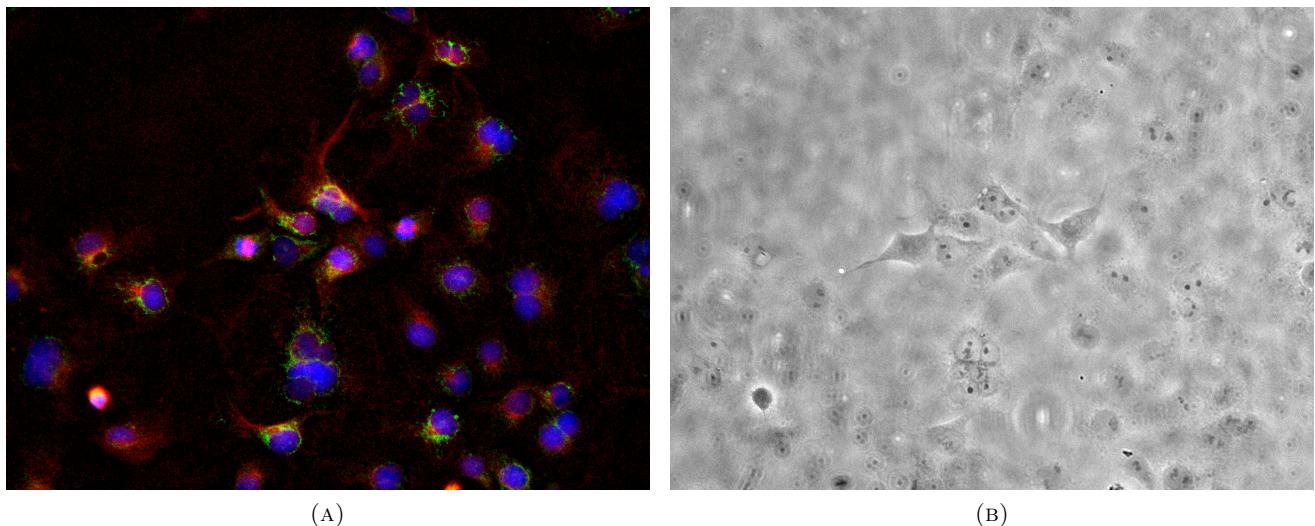


FIGURE 11. (A) 3-channel image of the GATTA-Cells 4C slide. DAPI labels the nuclei and is colored blue, TOM20-OG488 labels mitochondria and is colored green, and tubulin-TMR labels part of the cytoskeleton and is displayed as red. (B) Phase image of the same field. The nucleoli appear as dark spots inside the nuclei of the cells.

- (1) The three fluorescence images and the phase image were acquired within MicroManager, and saved as a stack.
- (2) The images were opened as a stack in ImageJ, and then separated into individual images using `Image>Stacks>Stacks to images`
- (3) The three fluorescence images were combined into a single composite image using `Image>Color>Merge Channels....`
- (4) The composite images was converted to an RGB images using `Image>Color>Channels Tool... ,` then selecting `More...` from within the Channels Tool, and then selecting the Convert to RGB option.

The RGB color image could have been created directly from `Image>Color>Merge Channels....` However, it is convenient to make the composite, and to use the Channels Tool to view the channels individually or together. The phase image could also be merged into the composite image, and sometimes it is useful to overlay a color image onto the phase image. However, in this case, I felt the resulting combination made the fluorescent labels difficult to see.

D.6. Correcting misaligned channels. The three fluorescence channels are acquired by manually moving the filter slider prior to each acquisition. If this is not done very carefully, the slide may move when the filter slider is moved, and the color channels will not be properly aligned to each other. A rather extreme example of this appears in Figure 12, where the red image is extremely shifted relative to the other two channels. The image was corrected using [Gabriel Landini's Align_RGB_planes plugin](#). To use this plugin, you must add it to your Fiji installation by downloading the class file to the Fiji plugins folder.

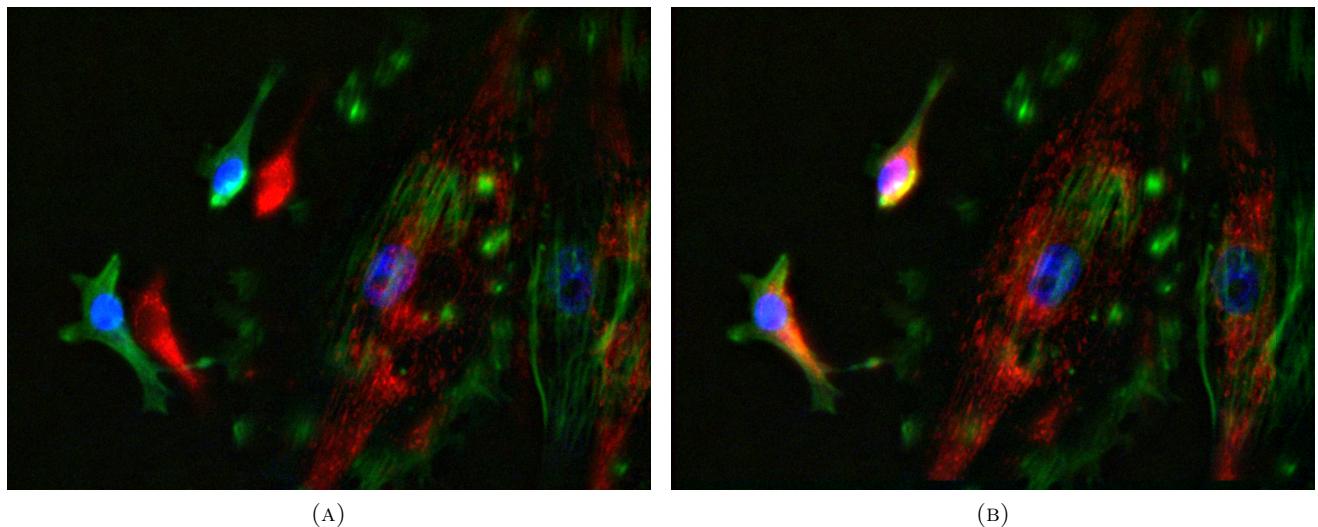


FIGURE 12. (A) 3-channel image of the FluoCells™#1 slide, with shifted channels (B) Image (A) after alignment. The red channel was contrast-stretched to make it more visible.

Alternatively, you can split the three channel stack into separate images as described above, then each image can be shifted as desired using **Image>Transform>Translate...**, and then the images can be merged as described above.

While large shifts such as that in Figure 12 are unusual, it is common for small shifts to occur even in systems with automated filter selection. There is a rich variety of tools, both manual and automated, for correction of such shifts, as well as for registration of time-lapse sequences.

APPENDIX E. MORE ABOUT THE FLUORESCEIN DIACETATE EXPERIMENT

As noted above, measuring the kinetics of the increase in intracellular fluorescence following addition of FDA to the medium is tricky. This is because the increase takes place within about a minute. After that, the photobleaching of the fluorescein will dominate, the fluorescence will decrease. Therefore, you and your lab partner should coordinate your actions, so that a few seconds after beginning the acquisition, the dye is injected into the medium.

If an image is acquired every 200 ms for two minutes, 600 images will be acquired. Each image is about 3 million pixels, and each pixel is 2 bytes (16bits/pixel), the resulting stack will be very large. To make this more manageable, we suggest that when you process the image, you first steps should be to convert the image to 8-bits/pixel (**Image>Type>8-bit**), and to scale it by 0.5 in each spatial direction (**Image>Scale...**). You should also set the properties (**Image>Properties...**) so that stack the is defined as a time series with the appropriate time interval and spatial scale.

There are many ways to estimate the background. The method that was used in Figure 1 was:

- (1) Copy the entire image stack (**Image>Duplicate...**).
- (2) Gaussian blur on the copy (radius=100, entire stack) (**Process>Filters>Gaussian Blur...**)
- (3) Subtract the blurred copy from the original, creating a new image (**Process>Image Calculator...**)

You will probably need to stretch the contrast (**Image>Adjust>Brightness/Contrast...**) in order to properly see the result.

The plots in Figure 2 were created as follows:

- (1) The Z-profile tool (`Image>Stacks>Plot Z-axis Profile`) was used to created a plot of intensity vs slice number in a selected ROI. The plot was then saved as a list, and LibreOffice was used to import it to a spreadsheet.
- (2) The slice number was converted to a time scale by multiplying with the interval between frames.
- (3) The usual spreadsheet tools were used to create the plot.