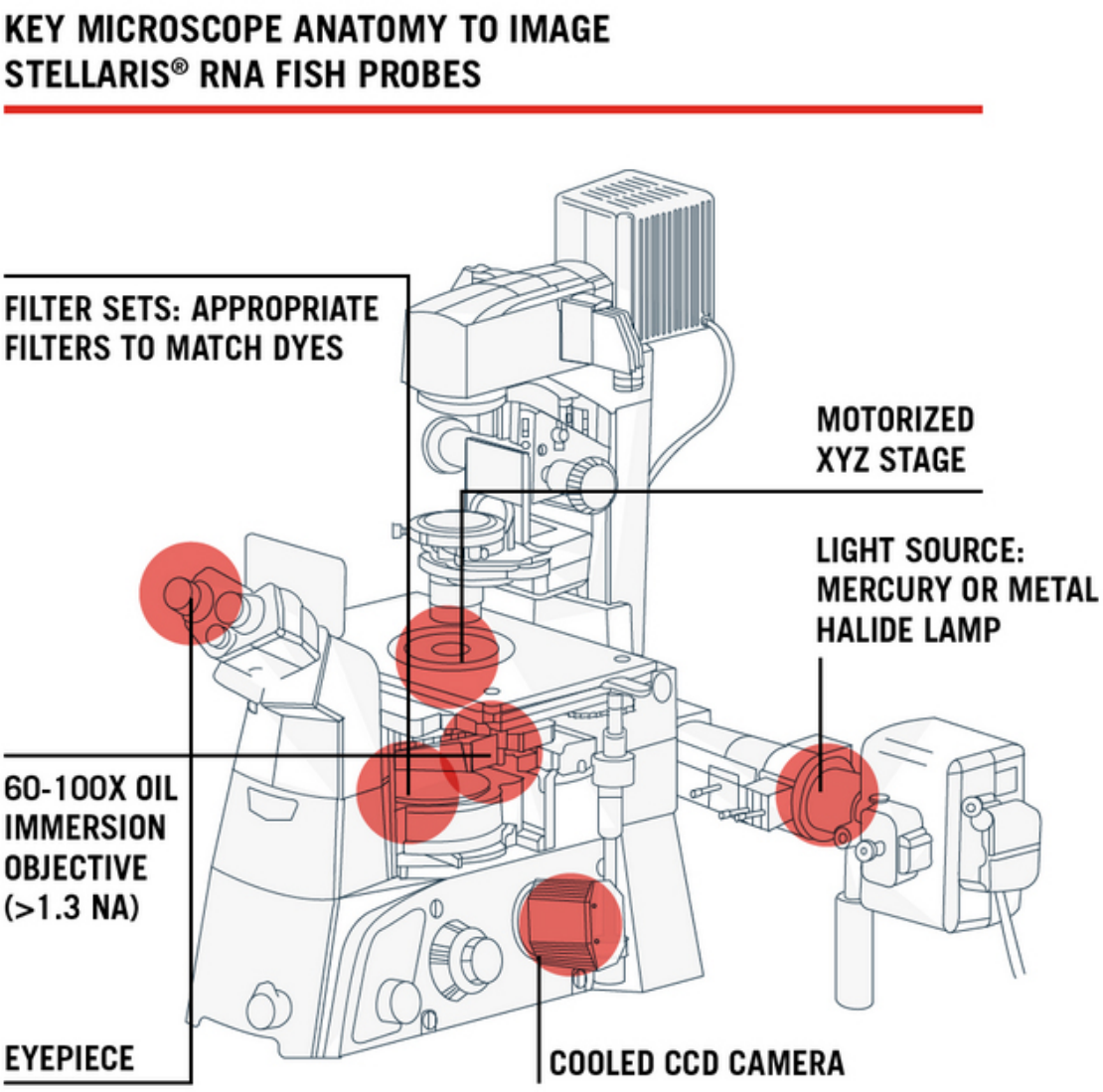
Imaging Stellaris® Assays Part 1: *Get to Know Your Microscope*

Stellaris® FISH is an RNA detection technology performed by means of in situ hybridization. This technology and all of the incredible applications it supports would not exist without a detection tool. The detector in this scenario is a fluorescence microscope.

The microscope is the single major limitation for scientists who are new to the Stellaris technology. The reason being – well, this is a new technology and fluorescence microscopes are not ordinary instruments found in every lab across the country. These are highly intricate, sensitive, and costly pieces of real estate that reside primarily in core laboratories to provide additional interrogation of biological systems through optics.

Every microscope system is unique. In other words, it's not a packaged deal, but rather entirely customized. From the nuts and bolts of the hardware to the software that coordinates all your movements, every piece is different from one lab to the next. Streamlining a protocol for each and every combination of system imaginable would be impractical. So whether your lab uses Nikon, Leica, Olympus, Zeiss, or something else, this blog article is intended to share fundamental microscope requirements to help you obtain a great Stellaris image.



We understand, once your probes have finished hybridizing, you're eager to image your Stellaris assay right away. But before we jump into imaging, which will be covered in a Part II portion of this blog series, here are 5 things you should know about your microscope hardware.

1. Use a Fluorescence Microscope

Although confocal microscopes are all the rage right now, we recommend novice microscopists to experiment with a wide-field fluorescence microscope first. In-house, we use a Nikon Eclipse Ti-E inverted microscope.

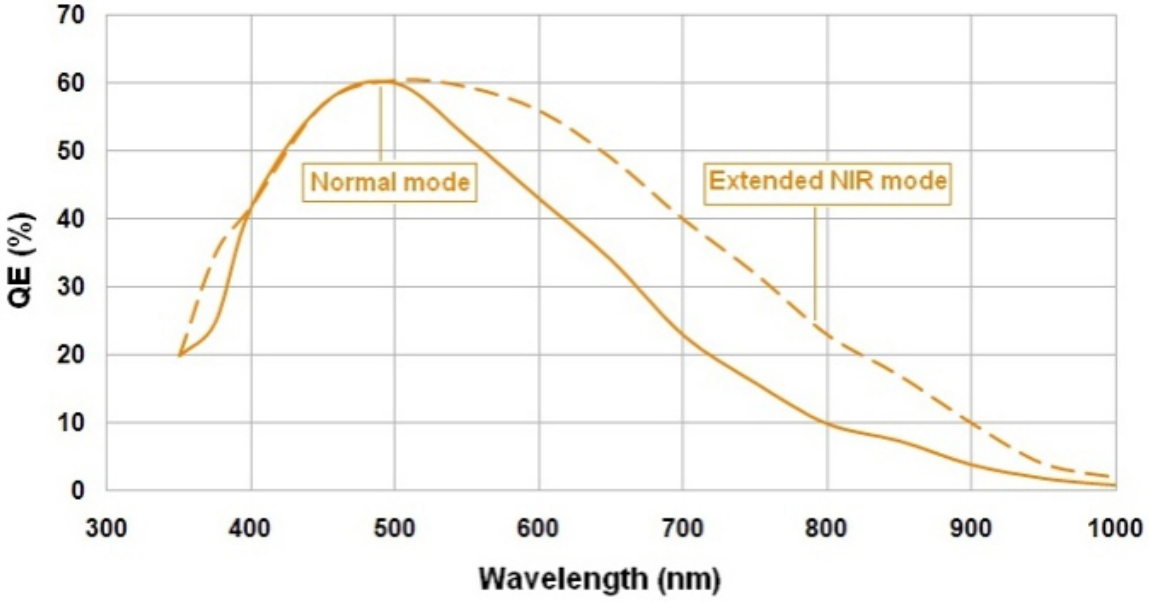
2. Be Mindful of your Light Source

You need a strong light source - such as a mercury or metal-halide lamp. We use a Prior Lumen 200 metal halide. LEDs have come a long way in the past five years and some newer LED-based light sources can also work. We had the pleasure of demoing two LED light sources, CoolLED's pE-4000 and the Lumencor SOLA light engine. Both LED light sources were successful for the detection of single molecules of RNA.

3. Secure a Cooled CCD Camera, Low-Light is Key

We recommend a standard cooled CCD camera – ideally optimized for low-light imaging rather than speed (13 µm pixel size or less is ideal). In-house we use an Andor Clara. An essential feature to know about your camera is its Quantum Efficiency (QE) (Fig. 1). QE is the measure of the effectiveness of an image sensor to produce a measurable electron signal from incoming photons1. The greater the number of photons converted into electrons by the camera for a given photon signal means a higher QE, which is better for imaging Stellaris. QE is an important feature to understand because it is wavelength-dependent.

Figure 1. Quantum Efficiency



Take for example this QE curve that came from the specifications sheet of our Andor Clara camera. Quantum efficiency starts to drop as you move farther up the visible spectrum, past 600 nm. So say for instance, your camera has a QE of 33% near 650 nm. This means that for every 3 photons coming in, one electron is produced.

This Andor Clara has an extended NIR mode that boosts the QE closer to 50%, which is advantageous for imaging our Quasar® 670 dye. It is worth checking if your camera has a similar capability. The take-home message is if you label your RNA target with a longer wavelength fluorophore and your camera has a low QE, the signal to noise value of your fluorescent RNA molecules are likely to be lower and harder to discern.

4. Acquire High Resolution Objectives...RNA is tiny!

You need a 60-100x oil-immersion objective with a high Numerical Aperture (NA) of 1.3 or greater. The quality of your Stellaris image depends on your NA and resolution. The resolution is your ability to distinguish fine details within your specimen, like the diffraction limited single molecule RNA spots. The numerical aperture of your microscope is a measure of its ability to gather light2.

Higher values of numerical aperture allow for incidental rays of light to enter the front lens of the objective, which allows for a more resolved image so smaller structures can be visualized with clarity.

Our Nikon is equipped with a CFI Plan Apochromat λ 60x Oil NA 1.4. The Plan (planar) objectives are flat-field corrected, which means that the image you see in your field of view looks flat as opposed to curved.

Each wavelength of light passing through the lens of a microscope refracts or “bends” at different angles such that the common focus point is slightly different for each color. Apochromatic lenses are corrected for chromatic and spherical aberration3.

So, when you shine various wavelengths of light upon a specimen, the plane of focus remains just about the same for each. In general, most 60- 100x oil immersion objectives have a high NA, but that is not to say they don’t exist! This is worth double checking.

5. Find the Correct Filter Sets

After you’ve designed a Stellaris probe set capable of detecting your RNA of interest, you need to pick a dye (fluorophore) to serve as your label so you can find those RNA molecules under the microscope. Your fluorophore of choice must be compatible with the filter sets already equipped on your microscope.

A simple way to check compatibility is to visit the company’s website whose filters you are using. An alternative is to purchase filter sets for the fluorophore you want to use.

Take a look at our Quasar 570 dye shown in Figure 2A. A filter capable of detecting this dye must have a similar absorption and emission spectra as seen in Figure 2B.

Figure 2A. Quasar 570 Absorption and Emission Spectra

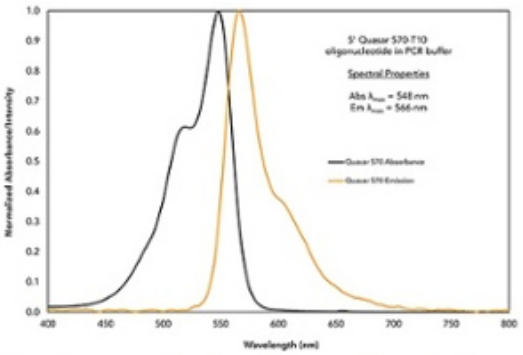
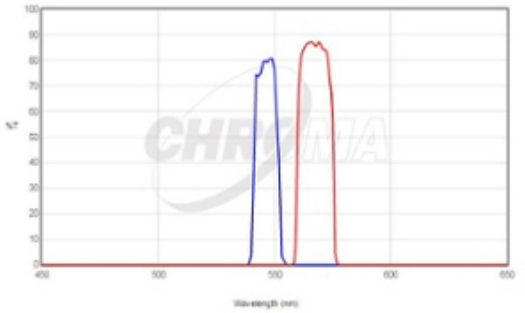
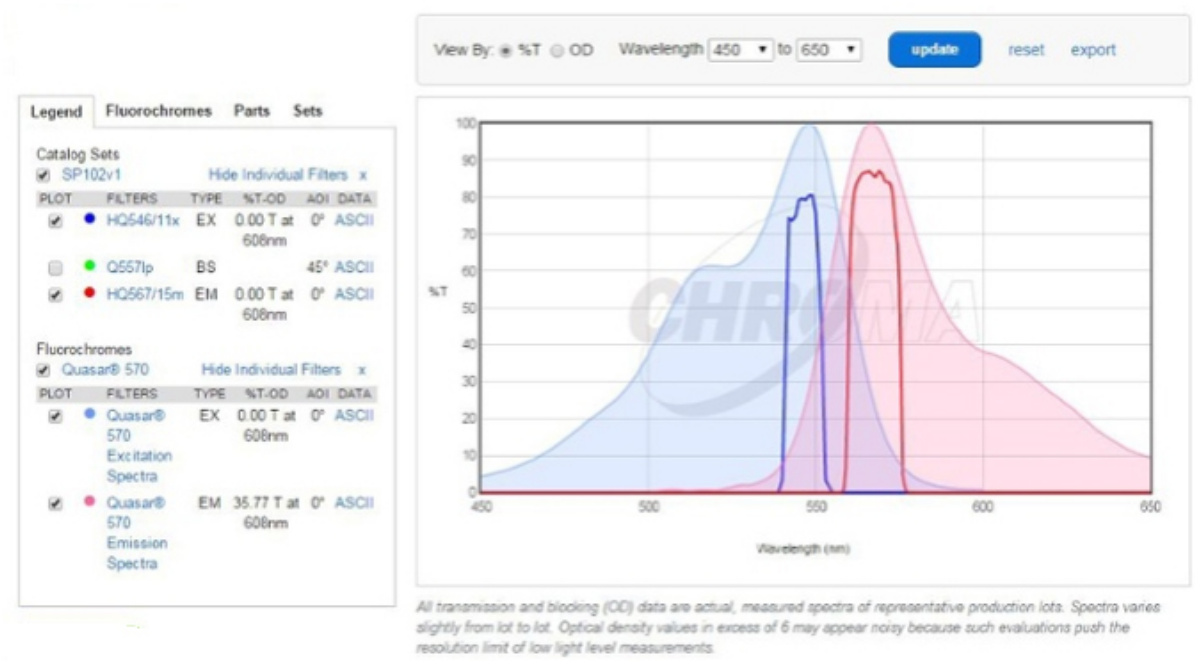


Figure 2B. Excitation and Emission Spectra of Chroma's SP102v1 Filter



Our dyes are listed on **Chroma's website** so overlaying these two graphs to make sure the spectral properties align with one another becomes rather easy! On the Chroma website, click the fluorochromes tab (to the right of the Legends tab) and search for your dye of interest, in this case Quasar 570. Once selected, click Reload Plot, and voilà!

Figure 2C. Overlay of Dye Spectra and the Filter Spectra



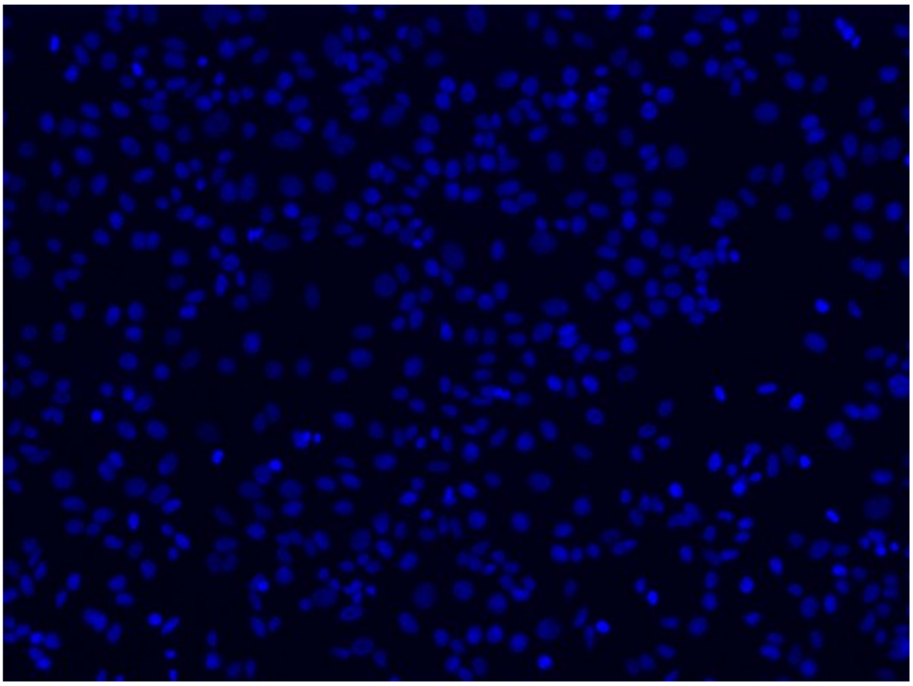
Quasar 570 has an absorption maximum at a wavelength of 548 nm and an emission maximum at 566 nm. As you can see, it aligns quite well with this filter from Chroma, and the absorption and emission wavelengths are well separated, preventing bleed-through into a neighboring channel.

Well there you have it! We hope this article helps you become more familiar with the microscope components needed to successfully image Stellaris RNA FISH probes. Keep an eye out for Part II of this series where we will go over tips on using software to capture and optimize your Stellaris images.

Imaging Stellaris® Assays Part 2: *How to Acquire Stunning Images*

Here we continue our "Imaging Stellaris Assays" blog series. We last left off talking about the most important components: the microscope and the hardware features necessary to detect your Stellaris signal. In this blog article, we will walk you through the technique of finding your cells and acquiring a sharp and stunning image.

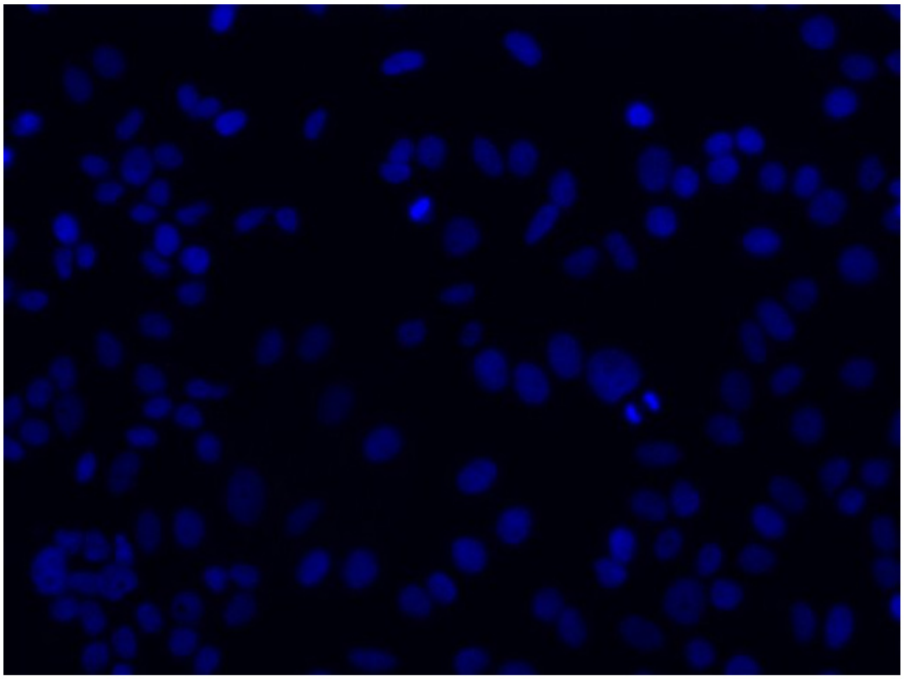
With the assumption that the microscope is on and all necessary components are synced and connected to the software, place the microscope slide into the slide holder, move a 10x objective directly under (if using an inverted scope) or over (if using an upright scope) the center of your coverslip, and begin to find your plane of focus. This is best done by using the DAPI channel to focus on the nuclei of your cells, which were stained according to the adherent cell protocol. DAPI (4', 6'-diamidino-2-phenylindole) binds strongly to A-T rich regions of double-stranded DNA. Bound to DNA, this dye absorbs light with a maximum of 358 nm and brightly fluoresces with a maximum at 461 nm. Once directly under or over the centre of your coverslip, select the DAPI filter and start to search for the nuclei of your cells.



10x FOV of cell nuclei through DAPI filter

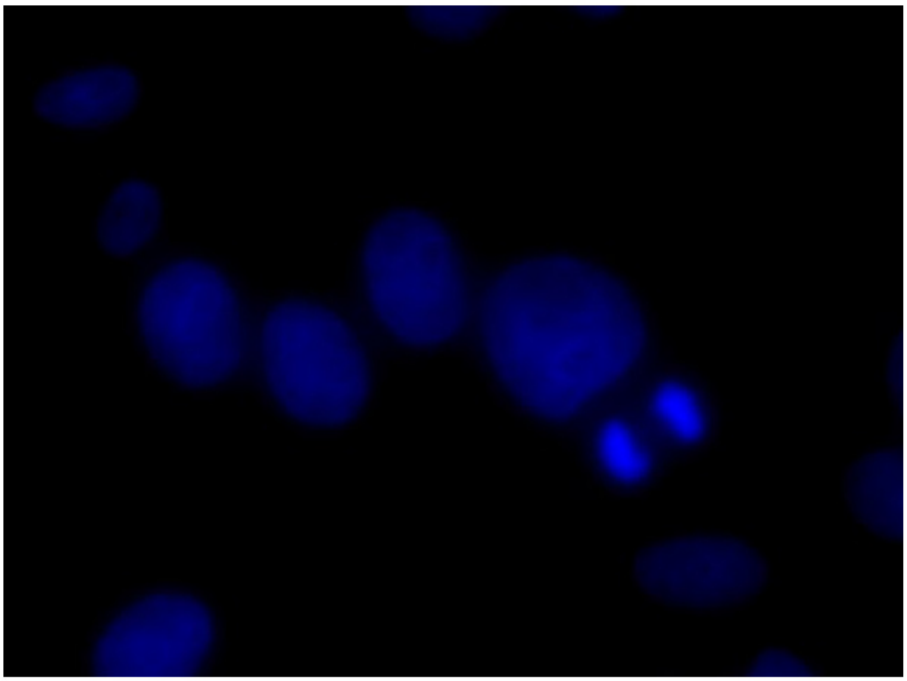
Once you have succeeded in producing a comparable image to the one above, move your objective away from the slide and rotate to the next higher objective. The slide should remain fixed so that you

are only moving the objective. Most microscopy platforms today have an encoded stage that retains the z-value of your last position when rotating objectives. The idea is to transition towards higher magnification optics (20x, 40x, 60x) until you reach an objective with a 1.3 or greater Numerical Aperture (NA). As you may remember, the NA is directly related to your ability to resolve the single molecules of your target RNA.



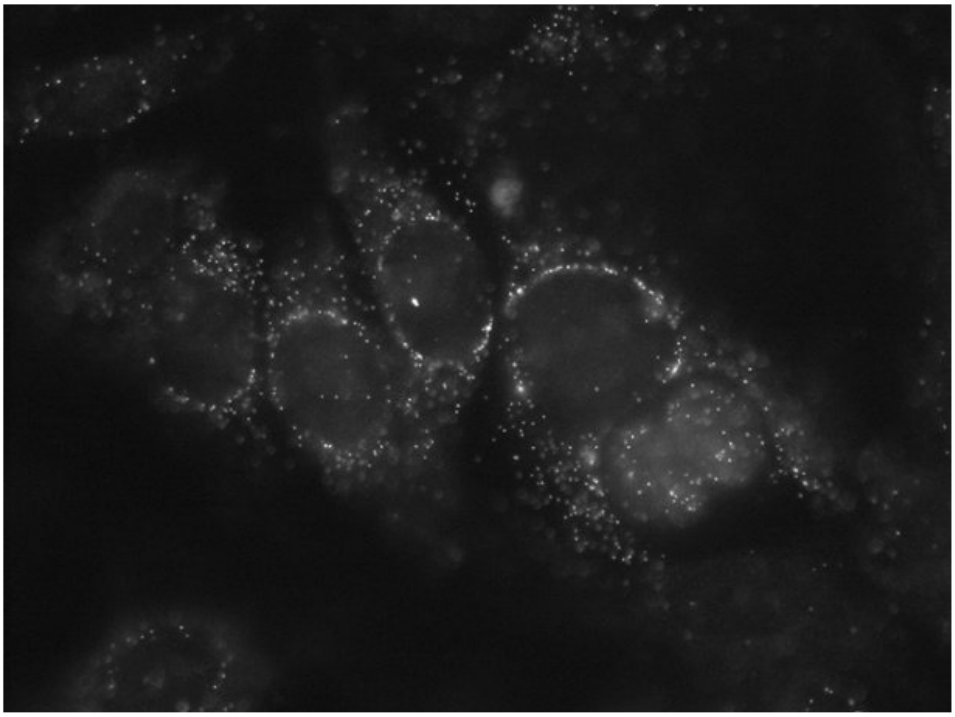
20x FOV of cell nuclei through DAPI filter

After producing an image comparable to the one given above, it is time to move on to a 60x oil-immersion objective. An oil-immersion objective means a small drop of oil must be in between the objective lens and the microscope slide, as opposed to just air. It is good practice to place that oil on the slide instead of the surface of the objective as excess oil can drip down into the barrel of the objective, if your microscope is inverted. An oil-immersion objective is used to increase the resolving power of your microscope because oil has a higher refractive index than air. The light propagating through this oil immersion lens creates a larger angle of light to be collected, thereby creating a higher NA value and the opportunity for single molecules of RNA to be resolved.



60x FOV of cell nuclei through DAPI filter

A powerful feature of Stellaris FISH is the ability to visualize different RNA variants from one or multiple genes through simultaneous detection of distinctly colored fluorescent probes (multiplexing). It's important to remember that in order to image these distinct fluorescent labels, you must have the means to spectrally separate the fluorescent dyes using narrow band filters. After finding your plane of focus using DAPI, your next move is to select the filter suitable to detect and excite the dye molecule labeled to your target RNA. Adjust your exposure setting to 1 second and you're ready to go Live and shine the appropriate wavelength of light on your sample.

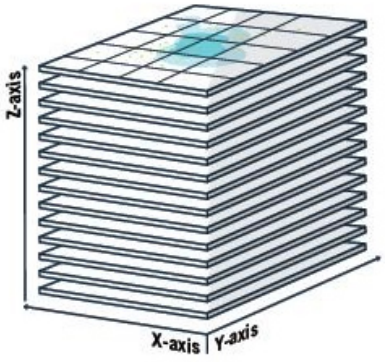


Here we are looking at a single z-slice illustrating single molecules of RNA from the cellular proliferation biomarker MKI67 in MCF7 cells.

This image is a single snapshot in time. Or said another way, this is a single z-plane of focus. A z-plane is commonly referred to as a z-slice, and this particular z-slice has a thickness of 0.30 microns (µm). This single z-plane image is insufficient for publication. The reason is that the thickness of this single focal plane is smaller than that of an MCF7 cell and therefore most of the cell's RNA remains out of focus.

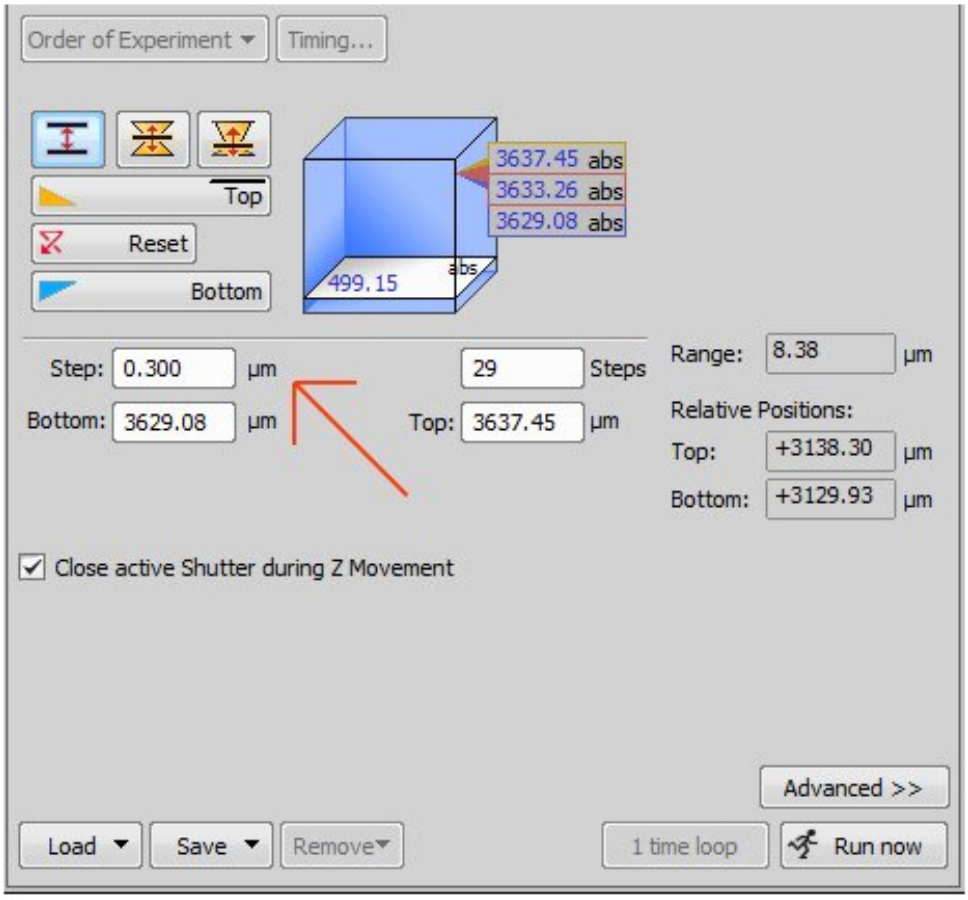
Let's take a step back, way back, to further explain this idea. In the late 1800's, Ernst Abbe, research director of the Carl Zeiss Optical Works, wrote down the rules for a lens to form a sharp image. He wrote that four fundamental improvements are possible - working distance, field of view, depth of field, and resolution. As a scientist who has undergone rigorous years of education and training, you are keenly aware of the concept of trade-offs, an underlying mechanism of evolution and the existence of life on this planet. Well in optics, it's no different! The higher the resolution that is achieved means you will notably sacrifice your field of view, depth of field, and working distance.

Your ability to resolve single molecules of RNA relies on having a high numerical aperture objective, which gives a shallow depth of field. Let's say for example, your MCF7 cell is, on average, 9 µm thick. Capturing a single z-plane of focus that is 1 µm-thick means you are missing out on any RNA expression that is found in the other 8 microns of the cell. Understanding the total RNA content of a cell is analogous to understanding the architecture of a building; a blueprint of any single floor is simply not sufficient, instead a compilation of blueprints for every floor is needed. You can avoid missing any relevant RNA



expression by focusing and imaging up and down through the cell you are observing. This is a focus stacking technique known as z-stacking. A z-stack is the capture of multiple z-planes through your cell of interest.

Try to imagine you are incrementally "stepping" through your cell. The size of your step, or stride, through the cell correlates to the thickness of each z-slice. Z-stacking allows you to capture the entire 3-D sample volume. Downstream processing will render the 3-D data and compress it into a 2-D image.

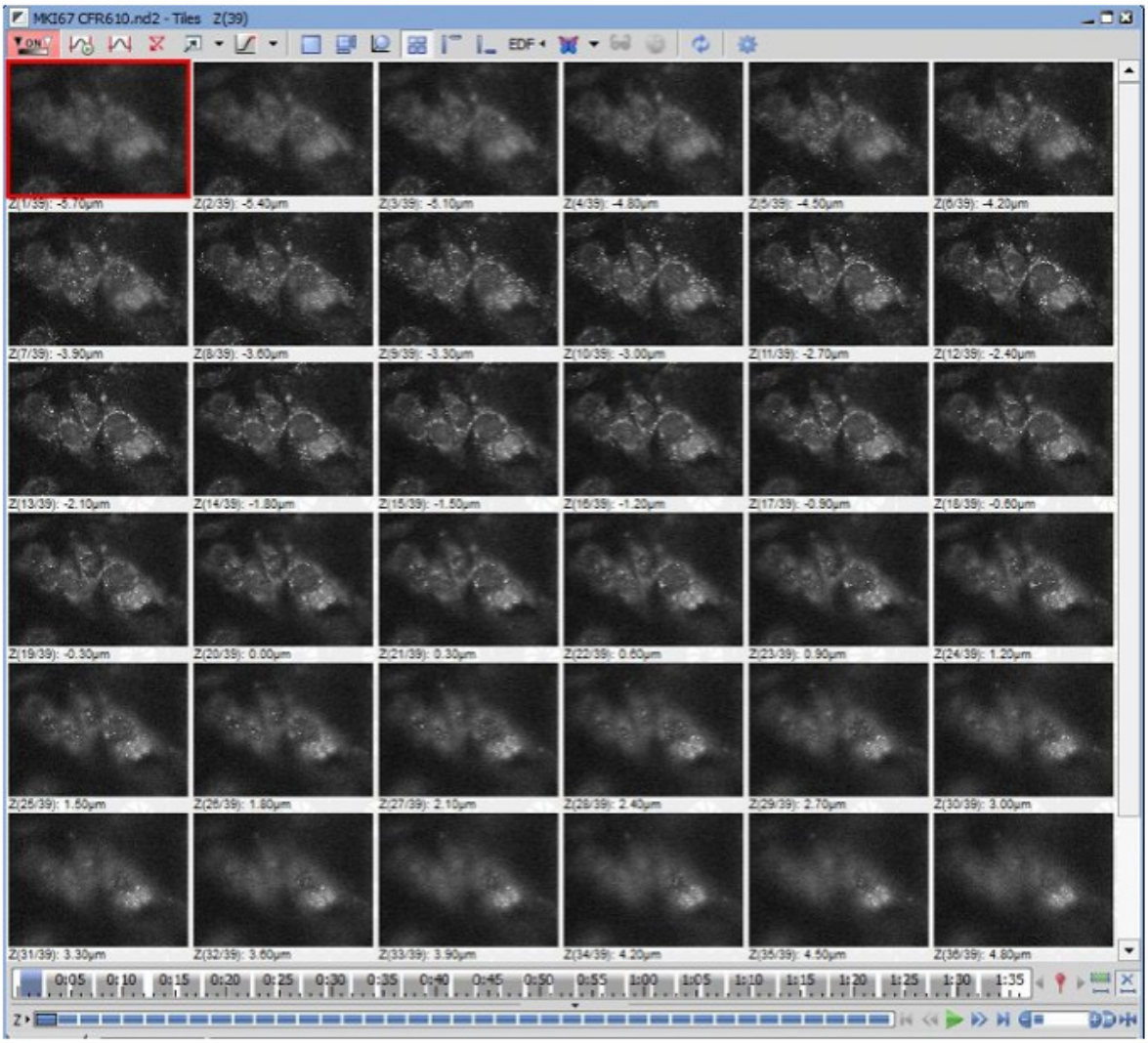


This is an acquisition box that lets you adjust the parameters of z-stacking and step size.

Here you will see how to change the step size. We recommend acquiring z-stacks with a step size of 0.25-0.30 µm. Experienced microscopists may employ a larger step-size to increase the rate of acquisition or decrease the amount of light exposure, however, it is always important that the tradeoff is understood.

Today, many wide-field fluorescence microscope systems are equipped with a motorized stage that enables the automatic capture of a z-stack. It is possible, albeit time consuming, to collect a z-stack by manually stepping through each focal plane and acquiring each z-slice individually.

Either way, the end message remains the same - it is crucial that you as a researcher are acquiring all the data relevant to your experiment through z-stacking. It is through this focus-stacking technique that the depth of focus can be reconstructed to create a single image entirely in focus.



These are the 36 individual z-slices that form the complete z-stack for capturing total RNA expression of MKI67. Each z-slice is 0.3 microns thick. Various RNAs come in and out of focus as you step through the cell.

After you acquire a z-stack of your target RNA, you can now switch back to your DAPI filter. You can maintain the same XY coordinates and z-stack parameters and run the acquisition again, but this time capturing all relevant focal planes of the DAPI stained nuclei within your field of view. It is important the field of view remains identical because downstream image processing will allow you to overlay your RNA of interest within the corresponding cell nuclei.

Your microscope has been properly set up and you have successfully imaged your target RNA of interest! We hope this series has not only helped you acquire a great image, but also provided further insight and understanding into the fundamentals of microscopy. Microscopy is tricky, and it can get frustrating at times, but these are the basic to-do's you want to follow first. Then, we promise, it gets really exciting. Stay tuned for the last article of this blog series that will go over downstream image processing.

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Imaging Stellaris® Assays Part 3: *Creating Professional, Publication-ready Images*

Here is the final instalment of our "Imaging Stellaris Assays" blog series. In our two previous posts, we've gone over microscope hardware and image acquisition. In this article, we will walk you through some simple downstream image processing that will turn your raw Z-stack images into spectacular-looking, publication-ready images.

Step-by-Step Overview:

1. Opening your Raw Image Files
2. Pre-processing Checks
3. Creating a Maximum Intensity Projection
4. Generating a Composite Image
5. Adjusting Brightness and Contrast
6. Adding a Scale Bar
7. Saving your Final Image

Let's Get Started

We will be using the "Fiji Is Just ImageJ" (Fiji) image processing package, which is an open source free distribution package of ImageJ. Fiji expands on the standalone ImageJ program to include many useful functions and plugins contributed by the community downloadable at http://fiji.sc/Downloads. The steps in this article should also be compatible with most other image processing software.

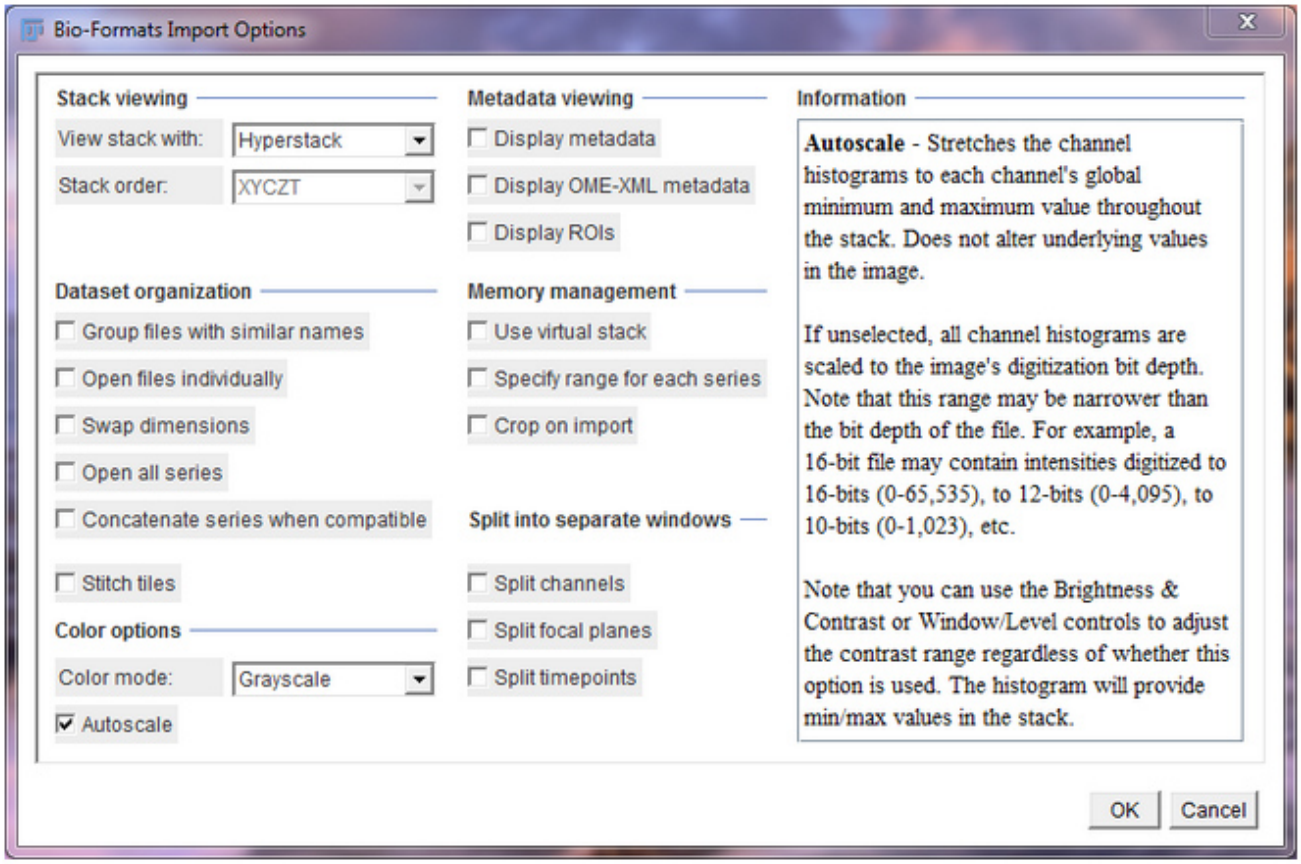
For an in depth introduction to ImageJ and Fiji, please read the Getting Started page (http://fiji.sc/Getting_started). There are also additional resources on how to use ImageJ on the ImageJ Tutorials page (http://fiji.sc/Category:Tutorials).

Opening your Raw Image Files

After starting up ImageJ, you can easily open your raw image files by dragging the file into the ImageJ window. Begin by dragging the 60x Z-stack file of the cellular proliferation biomarker MKI67 in MCF7 cells we took in the last blog article into the ImageJ window.

If you haven't read our previous blog article, or forgot how to create the 60X image stack, click here for a refresher. After dragging the file into Fiji, you may see the Bio-Formats Import Options window appear.

Note: Some file types do not require the Bio-Formats Importer. If the Bio-Formats Importer window doesn't open, don't worry. Skip the Bio-Formats instructions and jump to the Pre-processing checks section.



Bio-Formats Import Options Window

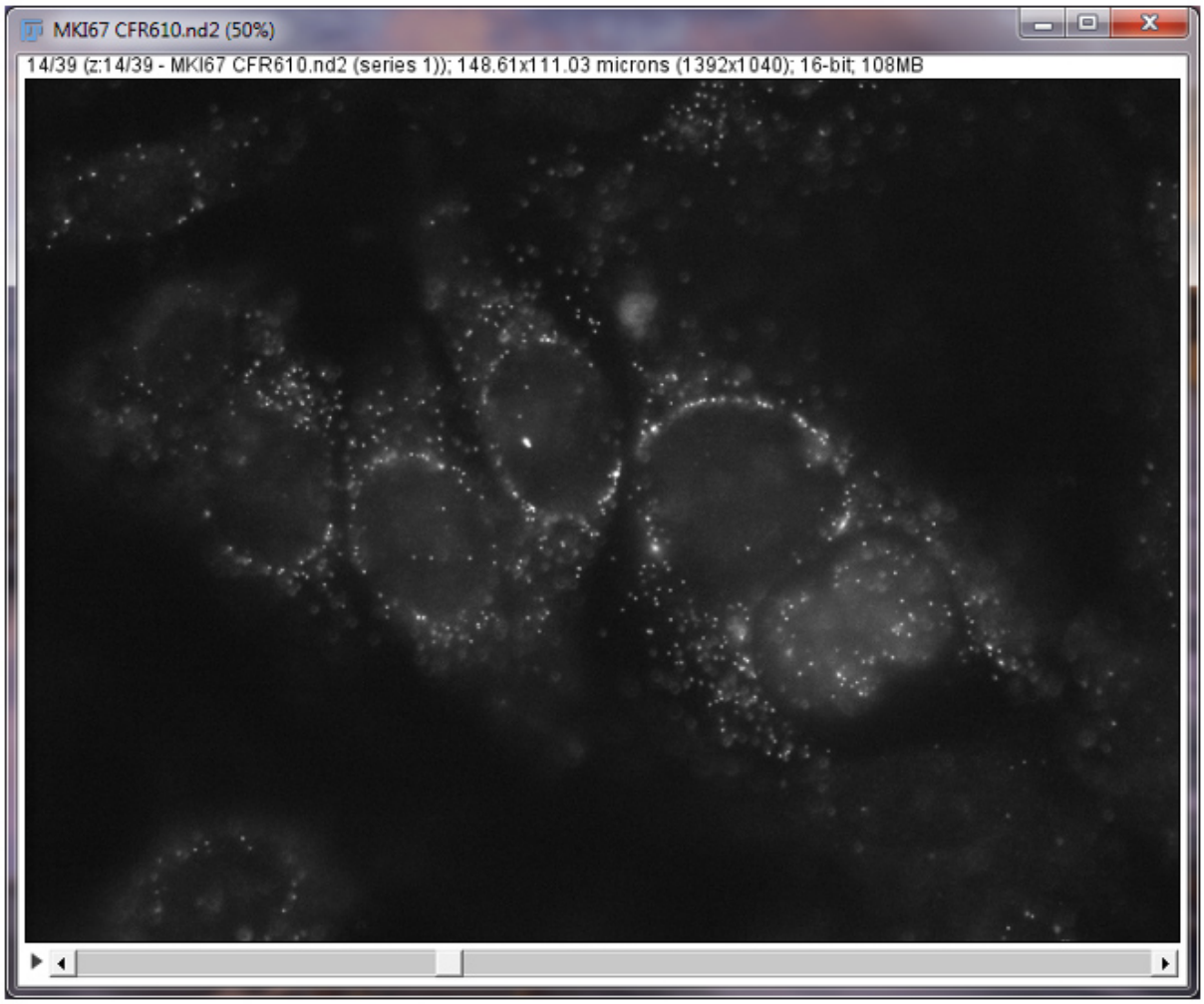
Be sure to select **Hyperstack** in the "View stack with" drop down and **Grayscale** in the "Color mode" drop down menu. Check the **"Autoscale"** check box to make viewing images easier. Press "OK" to continue.

Depending on your computer's processing speed, it may take a few seconds for the file to open. ImageJ is loading your image into its working memory, which can take a while for larger images.

> **Tip:** If your computer is frequently taking a long time to open these files, you may want to consider checking the "Use virtual stack" checkbox especially if you just want to quickly check a few individual images in your Z-stack. ImageJ will only load one slice of your image at a time, significantly speeding up the opening of the image file. The only downside is switching between slices takes much longer.

Pre-processing checks

Once your image is open you can use the scroll wheel or the left and right arrow keys to move through the Z-stack. You can see your position in the Z-stack in the top left corner or with the bar on the bottom.



Single z-slice illustrating single molecules of RNA from the cellular proliferation biomarker MKI67 in MCF7

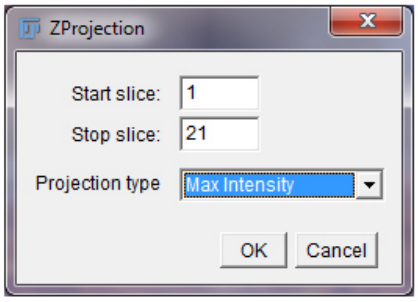
It is good practice at this point to briefly scroll through your Z-stack to look for any errors such as missing ends of cells, false (artifactual) signal, significant drift in the XY plane, or free floating autofluorescent particles. These objects or errors can end up in your final picture after processing and should be accounted for in the final analysis. We will cover how to work with these errors in a future article.

> **Tip:** If you don't see anything in your Z-Stack, your display ranges might not be set correctly. Press Shift + C to open the Brightness and Contrast window. Click on "Auto" to automatically adjust your image to the correct display values. The "Auto" button will adjust to the current slice, so it's usually a good idea to scroll to the middle of the Z-stack where there is signal. Manual adjustment may be required.

Creating a Maximum Intensity Projection

After you've checked out the stack, we can go ahead and create a Maximum Intensity Projection (MaxIP) of our Z-stack. A MaxIP is a rendering method that compresses a 3D Z-stack into a 2D image. Each XY position in the resulting image is the brightest pixel of the entire stack. This is great for Stellaris images since signal molecules should be the brightest spots in the stack.

To do this, we are going to use the built-in Z Project tool. To get there, click on “Image” > “Stacks” > “Z Project…”

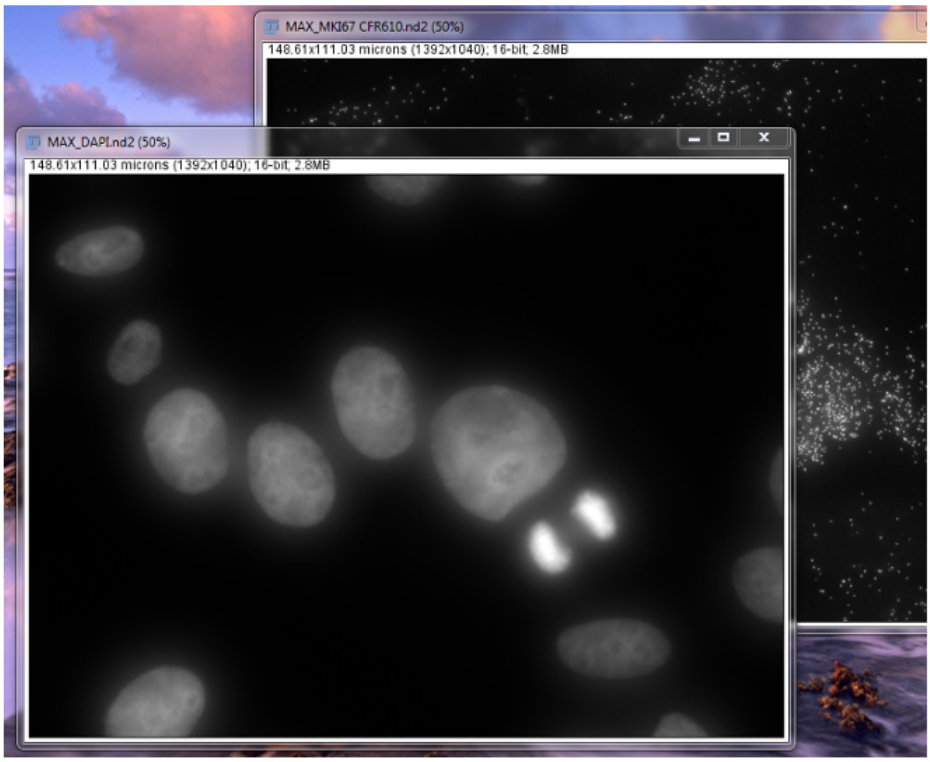


Z Projection Options Window

The Start and Stop slice boxes should be set to the maximum and minimum slice numbers by default. Set the “Projection type” to “Max Intensity” and click “OK.”

Tip: If there are many extra slices on either end of your stack, you may want to consider changing the start and/or stop slice numbers to truncate the MaxIP to the correct area. Congratulations! You just created your first MaxIP! We are one step closer to producing a publication quality image.

Next, we should open up the DAPI Z-Stack that we took previously. Follow the same steps as above to open an image and create a MaxIP of the DAPI Z-Stack. Be sure to click on the newly-opened DAPI window so that ImageJ knows on which window to perform the MaxIP.

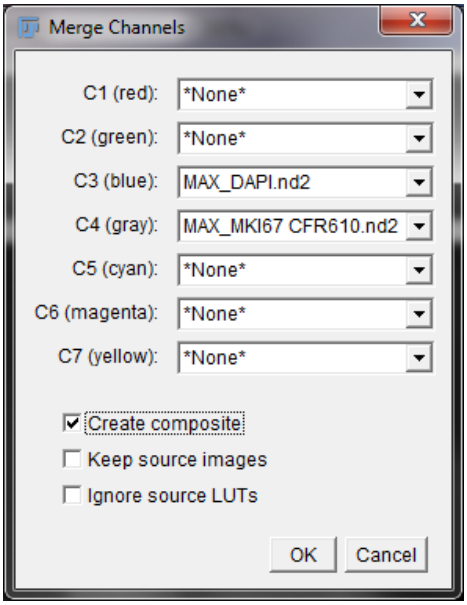


MaxIP of DAPI and MKI67 in MCF7 cells

Generating a Composite Image

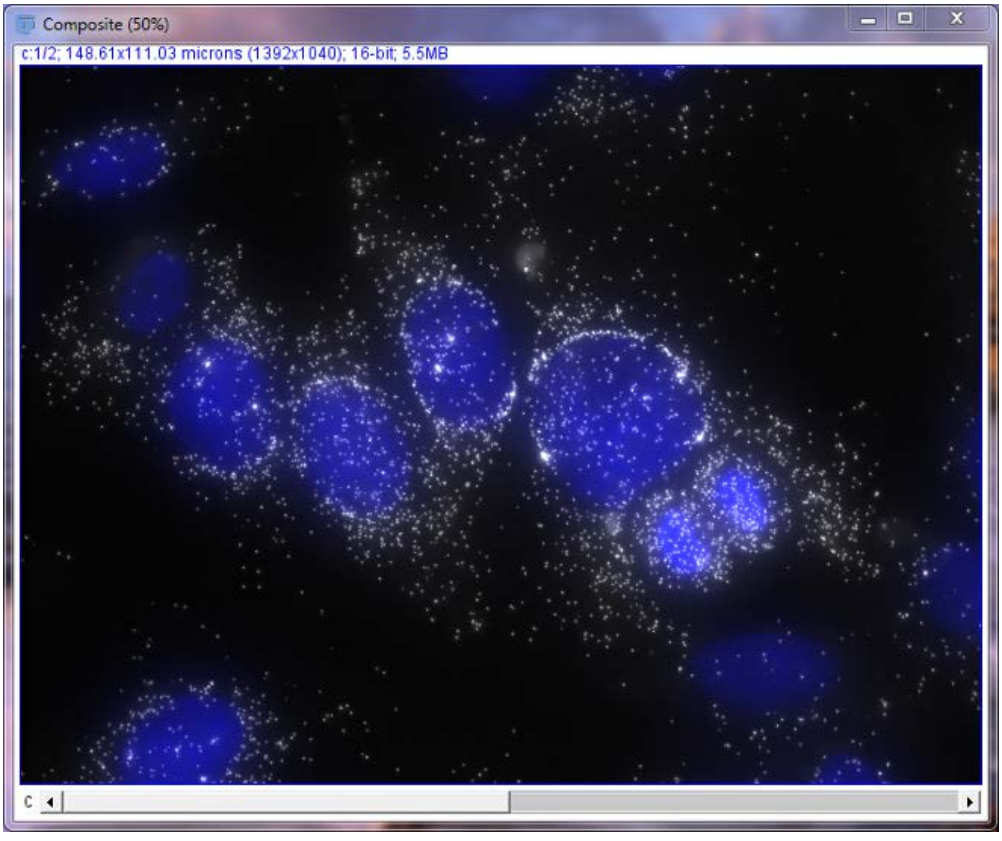
Once we have both MaxIP images open, we’re going to generate a composite image combining the two. We will be pseudocoloring the DAPI channel blue to help distinguish the nuclear stain from the Stellaris

signal. To do this, in the menu, we are going to click on “Image” > “Color” > “Merge Channels…” You will be prompted with the window below.



Merge Channels Options Window

In the drop down menu for C3 (blue) select the title of your DAPI image. Then for C4 (gray), select the title of your Stellaris image and press “OK”. The titles of the MaxIP images should start with “MAX_”. This will create a composite image where the DAPI channel looks blue and your Stellaris signal looks white/gray. You can use any color combination that you would like, but we prefer the blue and white look when working with only one Stellaris Probe Set. After clicking “OK” you should have an image the looks like the one below.



Composite image of DAPI and MKI67 in MCF7 cells

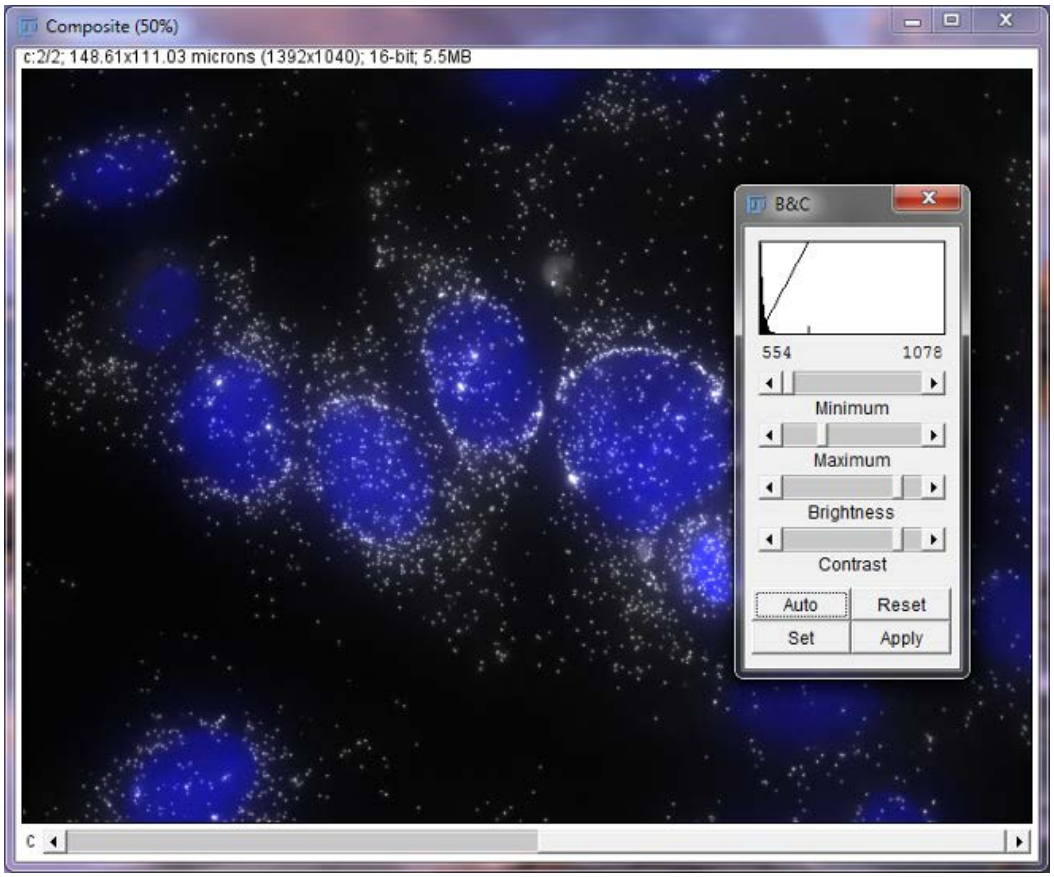
While this image may already look great, most images are going to need additional brightness and contrast adjustments to be publication-ready. Now we're going to perform some basic adjustments.

Please note that the scroll bar on the bottom of the image has returned, but in a different form. While the scroll wheel in the Z-stack allowed us to scroll through the Z-plane, this scroll wheel allows us to select one color in our composite image. This allows us to make changes to one of the original images without affecting the other.

You can switch between these images using the left and right arrow keys or the scroll wheel, just like with the Z-stack we had earlier. Also note, that when you switch between images, the color of the border and header text changes color. Blue is the blue channel (DAPI) and black is the gray channel (MKI67). We are going to use this to independently change the brightness and contrast of the DAPI and MKI67 images.

Adjusting Brightness and Contrast

Press Shift + C to open the Brightness and Contrast window.



Composite image of DAPI and MKI67 in MCF7 cells with Brightness and Contrast Window

The brightness and contrast window has three main parts: histogram, brightness sliders, and other function buttons.

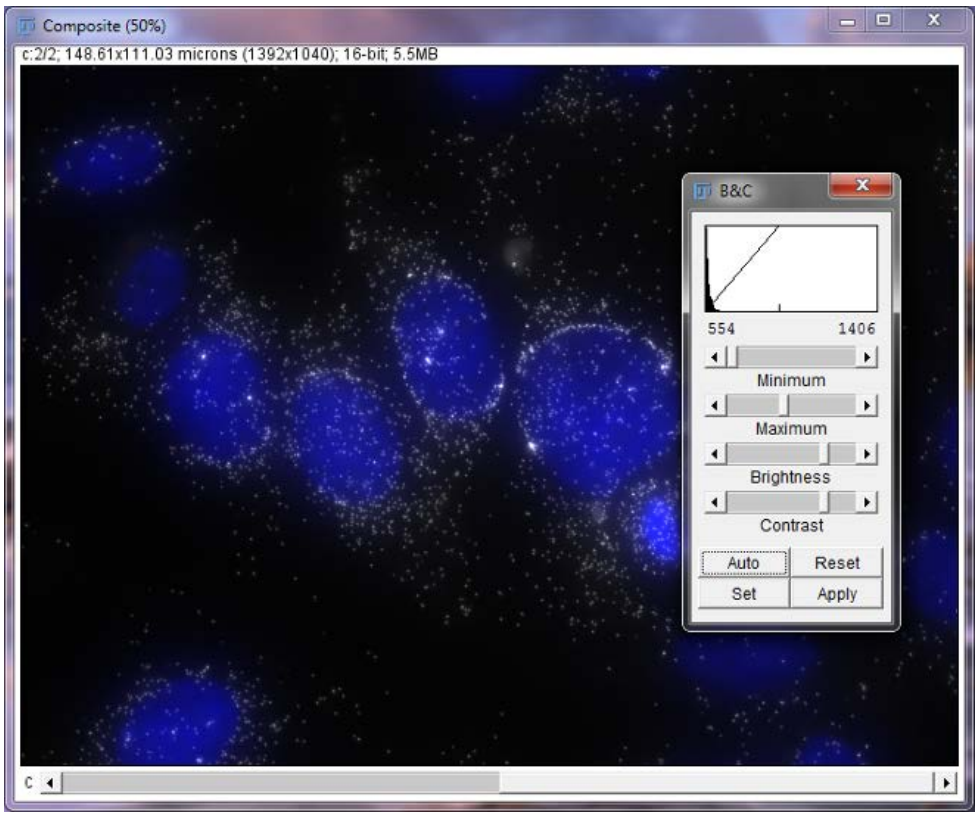
The histogram is a bar graph showing how many pixels are in the image and is sorted by brightness. The left represents darker pixel values and the right represents brighter pixel values. While not both visible in this screenshot, there are two vertical tick marks along the bottom of the screen that represent the minimum and maximum values in the display image. Those values are also written just below the histogram.

The brightness sliders control how the image is displayed. By sliding the minimum and maximum bar, you control what pixel value will be displayed as black and what value will be considered white. Values in between the min and max values will be proportional shades of gray. The brightness slider will move both the minimum and maximum values to the left or right together, while the contrast slider will move the min and max values further apart or closer together. We will be using the Minimum and Maximum sliders to adjust this image.

Use the scroll wheel or arrow keys to select the MKI67 portion of the image. The header text, border, and histogram should be black.

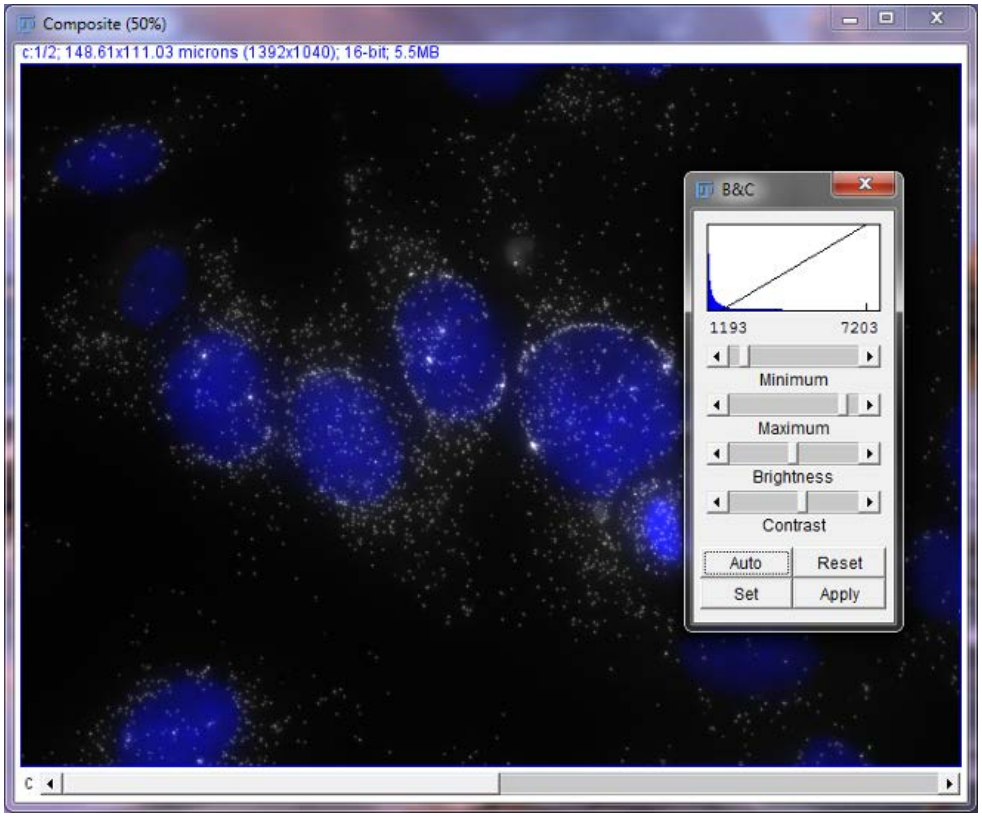
First, we want to make sure the darkest part of the image looks black on our monitor. The darkest part of most images should be the space between cells. Since this area appears black on our monitor, there is no need to adjust the minimum value. If in your image the space between cells appears gray, slide and drag the minimum slider up (to the right) until it looks black.

Next, we want to make sure that the signal in the image appears bright and clear. The signal in this image looks too bright and appears like it is glowing. We will increase the maximum value by sliding the bar to the right to darken the signal in this image. If you cannot see any signal in your image, try sliding the maximum bar to the left to increase the brightness of your signal.



Composite image of DAPI and MKI67 in MCF7 cells with Brightness and Contrast Window, Post-MKI67 Adjustment

Now, use the scroll wheel or arrow keys to switch to the DAPI image. Be sure to click on the Composite window to do this or you will be altering the brightness and contrast settings. The header text, border, and histogram color should change to blue. Switch back to the Brightness and Contrast window. We will adjust the DAPI channel the same way we did with the MKI67 image. Most of the time, DAPI images will look like the nuclei is glowing. We will increase the minimum value to reduce the glow.

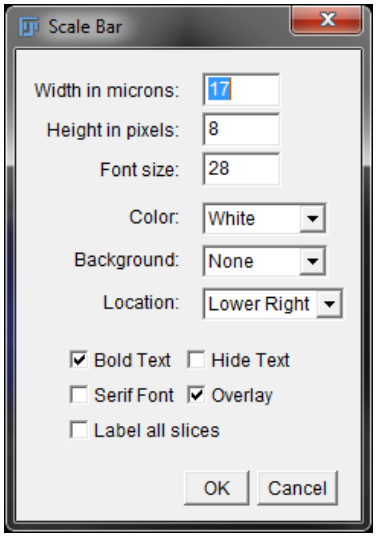


Composite image of DAPI and MKI67 in MCF7 cells with Brightness and Contrast Window, Post-DAPI Adjustment

Alternatively, if you would prefer to automatically adjust the display values, we recommend that you use the "Enhance Contrast" tool. It is a simple-to-use tool that produces well adjusted images. You can find the Enhance Contrast tool under the "Process" menu. Set your Saturated Pixels value to somewhere between 0 and 5. For a Stellaris image, a saturated pixel value between 0 and 1 works well for most images. Make sure "Normalize" and "Equalize Histogram" are unchecked.

Adding a Scale Bar

To add a scale bar, we are going to use the built in Scale Bar tool. Click on "Analyze" > "Tools" > "Scale Bar". You should be prompted with the window below.



Scale Bar Options Window

Set your width in microns to the appropriate number. ImageJ picks a default number based on scaling of the image and the size. In this case, we are going to change the width to 10 microns. We are going to leave the rest of the settings as default. Your image may need some adjustments to background or location if needed. Click “OK” to confirm your scale bar settings.

Tip: If you closed the scale bar options window but need to make changes to the scale bar, you can easily do so by re-opening the scale bar options window and making changes there. This only works when Overlay is checked.

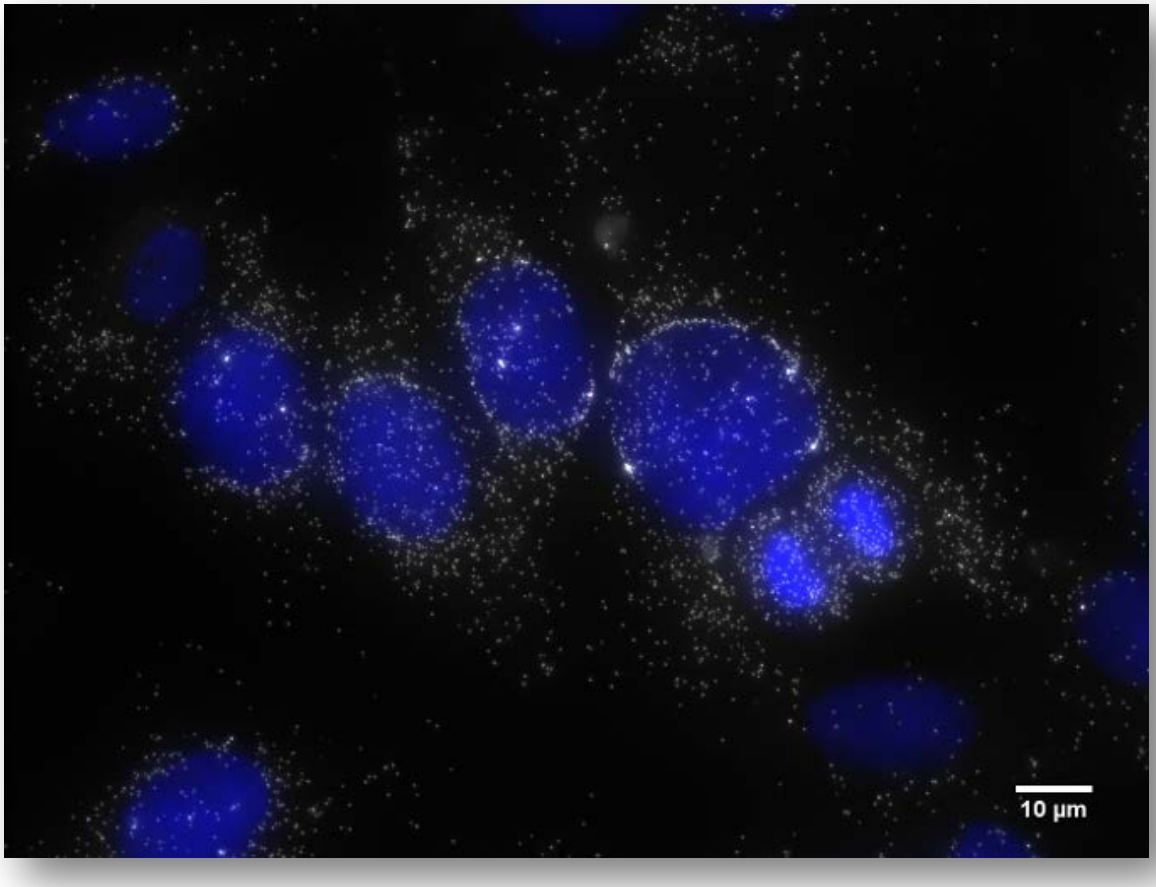
Saving your Final Image

After adjusting your image to your liking, you are now ready to save your image. We recommend saving your file in two formats: raw 16-bit Tiff and 8-bit PNG. Tiff is an image format that will preserve individual pixel values and retain the ability to independently change brightness and contrast in the future.

Open the “File” menu and mouse over “Save As”. Select “Tiff”. Specify the location and filename to save. The image we used is already in the 16-bit file format, so there is nothing to change. If your image is not a 16-bit image, do not change your image to the 16-bit format.

We will then save our image as an 8-bit PNG. 8-bit images are easily viewable by most computers and the PNG file format is a much easier file to view and share with others. We will first change the image to an 8-bit image by going to “Image” > “Type” > “8-bit”. Then we will save our file as a PNG by going to “File” > “Save As” > “PNG”. Select your desired file location, type your file name and click “Save”.

Final words…



Final Composite Image of DAPI and MKI67 with Scale Bar

You should now be more familiar with the basics of producing publication-ready images from your raw image files. In this complete blog series, we introduced the fundamentals of microscopy where we hope you can confidently verify that

- Your microscope has been properly set up
- You have successfully imaged your sample
- You have processed your raw image files into images ready for publication.

We sincerely hope you found the Imaging Stellaris Assays series useful.