

Configuring Your Microscope for NeuroPAL

Dr. Eviatar Yemini
Hobert Lab – Columbia University

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Introduction

A Word About Colors & Pseudocolors ...

***** PLEASE READ THIS!**

- Nearly **ALL** microscopy images are **PSEUDOCOLORED**. Yes, you read that right!
- **GFP** begins emitting in **cyan** with a tail that trails into **red** **BUT** most of the pictures you've seen probably have a **flat green appearance**. Why is this?
- Nearly all **scientific-grade cameras** & photosensors **ONLY detect intensity, NOT color** (wavelength/frequency). Microscopy images are **pseudocolored by software in post processing**.
- Consumer-grade cameras achieve color by employing 3 pixels (with red, green, & blue filters respectively) for every 1 pixel you actually see. The intensity information from each of these filtered pixels is combined to determine the actual color you see in your pictures. Basically, we spend 3 sensors to achieve 1 pixel of color.
- **By combining the primary colors red, green, & blue (RGB) you can create the infinite spectrum of colors we see.**
- NeuroPAL uses 4 fluorophores to achieve its palette: **mTagBFP2 (emits blue)** **CyOFP, TagRFP-T, & mNeptune2.5 all emit red.** **How does NeuroPAL achieve a rich palette of colors from 1 blue fluorophore & 3 red fluorophores?**
- Each of the NeuroPAL fluorophores can be imaged separately by restricting the excitation & emission bands.
- Therefore, we image each NeuroPAL fluorophore independently & **PSEUDOCOLOR** them with software.
- We pseudocolor the panneuronal marker **WHITE** (TagRFP-T) so all neurons can be visualized as white nuclei.
- We pseudocolor the remaining 3 fluorophores as **RED** (mNeptune2.5), **GREEN** (CyOFP), & **BLUE** (mTagBFP2).
- In NeuroPAL, each neuron expresses a specific combination of these landmark fluorophores. When their pseudocolors are combined, we end up with the rich RGB palette you see in the NeuroPAL images.

Which Microscopes Work With The NeuroPAL?

- **Short answer, all of them 😊**
- The next few sections will outline what you need if you have a:
 1. Laser scanning confocal with adjustable spectral bands (e.g. Zeiss LSM).
 2. Laser-based scope with a filter wheel (e.g., spinning disk, lightsheet, ...).
 3. Widefield scope with Zeiss Colibri 7 LEDs.
 4. Widefield scope with broadband excitation (e.g., X-Cite).

How Does NeuroPAL Work With 5 Colors?

(Skip This Page if You Don't Care)

- The NeuroPAL employs 4 fluorophores & leaves a thin emission band, completely clean of their emission, in order to image a 5th GFP(GCaMP)/YFP(mNeonGreen)/CFP reporter's expression.
- NeuroPAL employs the following 4 fluorophores:
 1. TagRFP-T – a panneuronal marker, pseudo-colored white.
 2. mTagBFP2 – a landmark for a subset of neurons, pseudocolored blue.
 3. CyOFP – a landmark for a subset of neurons, pseudocolored green.
 4. mNeptune2.5 – a landmark for a subset of neurons, pseudocolored red.
- If you visualized all the fluorophores at once, they would bleed into each other.
- Instead we selectively use restricted excitation & emission bands to visualize each fluorophore individually.
- The panneuronal TagRFP-T does receive bleed through from some of the other neural landmarks. But, since these landmarks are restricted to neural subsets & TagRFP-T is in every neuron, this bleed through is perfectly ok & only results in some neurons appearing brighter than others in the panneuronal channel.
- This scheme, involving differential excitation & restricted emission, can be visualized here:

<https://searchlight.semrock.com/?sid=4ee16194-a5b1-48ff-92ae-407229f8b3b1>

Avoiding Bleed Through in NeuroPAL (Skip This Page if You Don't Care)

- If you're not careful, the NeuroPAL fluorophores & your reporter will bleed into each other & confuse you.
- The setup pages that follow, configure your scope to avoid any problems.
- If you want to go your own way, please understand & beware of the following potential for bleed through:

1. GFP(GCaMP)/YFP(mNeonGreen)/CFP – your reporter, pseudocolored white.

Boundaries: Excitation=460-500nm, Emission=460-520nm
mTagBFP2 can bleed in if you excite these < 450nm
CyOFP can bleed in if you capture emission > 520nm

2. TagRFP-T – a panneuronal marker, pseudocolored white.

Boundaries: Excitation=530-580nm, Emission>550nm
GFP/YFP can bleed in if you excite it < 530/545nm
CyOFP & mNeptune2.5 will bleed in no matter what (don't worry, TagRFP marks all neurons & these landmarks only mark neural subsets)

3. mTagBFP2 – a landmark for a subset of neurons, pseudocolored blue.

Boundaries: Excitation=300-430nm, Emission=420-470nm(upper bound for GFP/YFP reporters)/445nm(upper bound for CFP reporters)
GFP/CFP can bleed in if you capture emission < 470/445nm

4. CyOFP – a landmark for a subset of neurons, pseudocolored green.

Boundaries: Excitation=460-500nm, Emission>550nm
TagRFP can bleed in if you excite it > 500nm
GFP/YFP can bleed in if you capture emission < 620/650nm (in practice, 600nm is a sufficient lower bound)

5. mNeptune2.5 – a landmark for a subset of neurons, pseudocolored red.

Boundaries: Excitation=590-640nm, Emission>590nm
TagRFP can bleed in if you excite it < 590nm (but you can work with this, by exploiting their differential emission, if you capture emission > 630nm)

Configuring Your Microscope

Configuring Your Microscope:

Laser Scanning Confocal Microscopes with
Adjustable Spectral Emission Bands (e.g. Zeiss LSM)

Laser Scanning Confocal Microscopes with Adjustable Spectral Emission Bands (e.g. Zeiss LSM)

- If you have a Zeiss LSM, I've included several setups for your convenience. Simply open these in Zen & "Reuse" their settings.

You will still have to adjust your laser power, gain, & perhaps even the bands, then save these new settings.

- Otherwise, configure your tracks as follows (you will need 3-5 tracks, depending on your system's capabilities):

Track 1 (panneuronal TagRFP-T):

TagRFP-T (panneuronal): pseudocolored **WHITE**. Excitation=561nm, Emission=565nm-600(or > 600, if necessary)nm

Track 2 (reporter GFP/YFP/CFP & neural landmark CyOFP):

GFP(GCaMP)/YFP(mNeonGreen)/CFP (reporter): pseudocolored **WHITE**. Excitation=488nm, Emission=490nm-520nm

Note: you can increase the band for GCaMP to 490-550nm since you're using the differential. The panneuronal & landmark differentials = 0, over short timescales.

CyOFP (landmark): pseudocolored **GREEN**. Excitation=488nm, Emission>550nm

Track 3 (neural landmarks mTagBFP2 & mNeptune2.5):

mTagBFP2 (landmark): pseudocolored **BLUE**. Excitation=405nm, Emission=410nm-470(or 445 for CFP reporters)nm

mNeptune2.5 (landmark): Pseudocolored **RED**. Excitation=590-640nm (we use 594 or 630nm), Emission>600nm

Note: if you don't own a 590+nm laser, you can excite with 561nm, by only capturing emission > 630nm, but the color balance may appear off due to bleed through.

***** NOTE: If your confocal can't handle 2 fluorophores on a single track, you will have to split them onto separate tracks (i.e., give each of the 5 fluorophores its own track).**

Zeiss LSM: Glossary for Reusable NeuroPAL Settings

- **GFP/CFP/YFP(mNeonGreen)** = the reporter you're imaging (YFP=mNeonGreen)
- **Best** = try this setting first (I optimized it for both the landmarks & panneuronal)
- **Fast** = fast imaging with no filter changes (the "Best" settings are already fast mode)
- **Slow** = slow imaging for weak reporters (more than twice as slow because the filter changes once per z-stack)
- **Underexpose** = your reporter is too bright for the "Best" setting, so you need to underexpose it
- **Overexpose** = your reporter is too weak for the "Best" setting, so you need to overexpose it
- **Fast Overexpose** = *NO PANNEURONAL CHANNEL!!!* speed comes at a price but, how often do you really need to see the panneuronal landmark anyhow?
- **Unadjustable GFP/YFP** = you need great signal from the panneuronal channel (TagRFP-T) & are willing to sacrifice your ability to adjust the GFP/YFP gain (GFP/YFP gain cannot be adjusted since they're now linked to the CyOFP landmark channel). This setting improves panneuronal clarity & is ideal for experiments like mutant analysis.

Configuring Your Microscope:

Laser-based Microscopes with a
Filter Wheel (e.g., Spinning Disk, Lightsheet, ...)

Parts List for Laser-based Microscopes with a Filter Wheel (e.g., Spinning Disk, Lightsheet, ...)

- You will need the following 4 lasers:
 - A. 405nm
 - B. 488nm
 - C. 561nm
 - D. 594nm
- Buy these Semrock parts in the quantities shown:
 - A. 3 Filter Cubes
 - B. 1 LL01-405 (405 MaxLine® Laser-line Filter) * **unless you already have this laser notch.**
 - C. 1 LL01-488 (488 MaxLine® Laser-line Filter) * **unless you already have this laser notch.**
 - D. 1 LL02-561 (561 nm MaxLine® Laser-line Filter) * **unless you already have this laser notch.**
 - E. 1 FF01-591/6 (591/6 BrightLine® Bandpass Filter) * **unless you already have this laser notch.**
 - F. 1 Di02-R488 (488 nm BrightLine® Laser Dichroic Beamsplitter)
 - G. 1 Di01-R405/488/594 (405/488/594 BrightLine® Laser Dichroic Beamsplitter)
 - H. 1 Di02-R594 (594 nm BrightLine® Laser Dichroic Beamsplitter)
 - I. 1 FF02-510/10 (510/10 BrightLine® Bandpass Filter)
 - J. 1 FF01-520/35 (520/35 BrightLine® Bandpass Filter) * **if you want to image GCaMP.**
 - K. 1 FF01-450/660 (450/660 BrightLine® Dual-band Bandpass Filter)
 - L. 1 BLP01-594R (594 nm EdgeBasic™ LWP Filter)
- The configuration can be seen here:

<https://searchlight.semrock.com/?sid=5f274d5d-8c0f-4f06-99fe-f4b2afe1370a>

Configuring Laser-based Microscopes with a Filter Wheel (e.g., Spinning Disk, Lightsheet, ...)

***** NOTE: you MUST turn on ONLY 1 laser at a time, then image the fluorophore. Each track/cube represents one spot on the filter wheel & 1-2 fluorophores by independently exciting each with ONLY 1 fluorophore-specific laser. If you turn on more than 1 laser at a time, you will combine your fluorophores into a single color (a bad idea).**

Configure 3 tracks (filter cubes) as follows:

Track 1 (panneuronal TagRFP-T & neural landmark CyOFP):

Cube 1 = Di02-R594 dichroic + BLP01-594R emission filter

TagRFP-T (panneuronal): pseudocolored WHITE. Laser=561nm + LL02-561 laser notch

CyOFP (landmark): pseudocolored GREEN. Laser=488nm + LL01-488 laser notch

Track 2 (neural landmarks mTagBFP2 & mNeptune2.5):

Cube 2 = Di01-R405/488/594 dichroic + FF01-450/660 emission filter

mTagBFP2 (landmark): pseudocolored BLUE. Laser=405nm + LL01-405 laser notch

mNeptune2.5 (landmark): Pseudocolored RED. Laser=594nm + FF01-591/6 laser notch

Track 3 (reporter GFP/YFP/CFP):

Cube 3A = Di02-488 dichroic + FF02-510/10 emission filter

OR for GCaMP6s ONLY Cube 3B = Di02-488 dichroic + FF01-520/35 emission filter

GFP(GCaMP)/YFP(mNeonGreen)/CFP (reporter): pseudocolored WHITE. Laser=488nm + LL01-488 laser notch

Configuring Your Microscope:

Widefield Microscopes with Zeiss Colibri 7 LEDs

Parts List for Widefield Microscopes with Zeiss Colibri 7 LEDs

***** Note: the Zeiss Colibri 7 LEDs approximate broad lasers. They even include their own excitation filters.**

- You will need the following 4 LEDs (these are standard for the Colibri 7):

- A. UV (385/30nm)
- B. B (469/38nm)
- C. G (555/30nm)
- D. Y (590/27nm)

- Buy these Semrock parts in the quantities shown:

- A. 3 Filter Cubes
- B. 1 Di02-R488 (488 nm BrightLine® Laser Dichroic Beamsplitter)
- C. 1 Di01-R405/488/594 (405/488/594 BrightLine® Laser Dichroic Beamsplitter)
- D. 1 Di02-R594 (594 nm BrightLine® Laser Dichroic Beamsplitter)
- E. 1 FF02-510/10 (510/10 BrightLine® Bandpass Filter)
- F. 1 FF01-520/35 (520/35 BrightLine® Bandpass Filter) *** if you want to image GCaMP.**
- G. 1 FF01-450/660 (450/660 BrightLine® Dual-band Bandpass Filter)
- H. 1 BLP01-594R (594 nm EdgeBasic™ LWP Filter)

- The configuration can be seen here:

<https://searchlight.semrock.com/?sid=084fb884-e755-4551-8f48-e443d8a01cf2>

Configuring Widefield Microscopes with Zeiss Colibri 7 LEDs

***** NOTE: you MUST turn on ONLY 1 LED at a time, then image the fluorophore. Each track/cube represents one spot on the filter wheel & 1-2 fluorophores by independently exciting each with ONLY 1 fluorophore-specific LED. If you turn on more than 1 LED at a time, you will combine your fluorophores into a single color (a bad idea).**

Configure 3 tracks (filter cubes) as follows:

Track 1 (panneuronal TagRFP-T & neural landmark CyOFP):

Cube 1 = Di02-R594 dichroic + BLP01-594R emission filter
TagRFP-T (panneuronal): pseudocolored WHITE. LED=G(555/30nm)
CyOFP (landmark): pseudocolored GREEN. LED=B (469/38nm)

Track 2 (neural landmarks mTagBFP2 & mNeptune2.5):

Cube 2 = Di01-R405/488/594 dichroic + FF01-450/660 emission filter
mTagBFP2 (landmark): pseudocolored BLUE. LED=UV(385/30nm)
mNeptune2.5 (landmark): Pseudocolored RED. LED=Y(590/27nm)

Track 3 (reporter GFP/YFP/CFP):

Cube 3A = Di02-488 dichroic + FF02-510/10 emission filter
OR for GCaMP6s ONLY Cube 3B = Di02-488 dichroic + FF01-520/35 emission filter
GFP(GCaMP)/YFP(mNeonGreen)/CFP (reporter): pseudocolored WHITE. LED=B(469/38nm)

Configuring Your Microscope:

Widefield Microscopes with
Broadband Excitation (e.g., X-Cite)

Parts List for Widefield Microscopes with Broadband Excitation (e.g., X-Cite)

- You will need broadband excitation via an arc lamp or LED-based lighting system.

- Buy these Semrock parts in the quantities shown:

- A. 5 Filter Cubes
- B. 1 FF01-385/26 (385/26 BrightLine® Bandpass Filter)
- C. 2 FF02-472/30 (472/30 BrightLine® Bandpass Filter)
- D. 1 FF01-554/23 (554/23 BrightLine® Bandpass Filter)
- E. 1 FF01-590/20 (590/20 BrightLine® Bandpass Filter)
- F. 1 Di02-R488 (488 nm BrightLine® Laser Dichroic Beamsplitter)
- G. 2 Di01-R405/488/594 (405/488/594 BrightLine® Laser Dichroic Beamsplitter)
- H. 2 Di02-R594 (594 nm BrightLine® Laser Dichroic Beamsplitter)
- I. 1 FF02-510/10 (510/10 BrightLine® Bandpass Filter)
- J. 1 FF01-520/35 (520/35 BrightLine® Bandpass Filter) *** if you want to image GCaMP.**
- K. 2 FF01-450/660 (450/660 BrightLine® Dual-band Bandpass Filter)
- L. 2 BLP01-594R (594 nm EdgeBasic™ LWP Filter)

- The configuration can be seen here:

<https://searchlight.semrock.com/?sid=df967598-bf41-4e7d-8f8c-84babe759655>

Configuring Widefield Microscopes with Broadband Excitation (e.g., X-Cite)

Configure 5 tracks (filter cubes) as follows:

Track 1 (panneuronal TagRFP-T):

Cube 1 = FF01-554/23 excitation filter + Di02-R594 dichroic + BLP01-594R emission filter
TagRFP-T (panneuronal): pseudocolored WHITE.

Track 2 (neural landmarks mTagBFP2):

Cube 2 = FF01-385/26 excitation filter + Di01-R405/488/594 dichroic + FF01-450/660 emission filter
mTagBFP2 (landmark): pseudocolored BLUE.

Track 3 (neural landmark CyOFP):

Cube 3 = FF02-472/30 excitation filter + Di02-R594 dichroic + BLP01-594R emission filter
CyOFP (landmark): pseudocolored GREEN.

Track 4 (neural landmarks mNeptune2.5):

Cube 2 = FF01-590/20 excitation filter + Di01-R405/488/594 dichroic + FF01-450/660 emission filter
mNeptune2.5 (landmark): Pseudocolored RED.

Track 5 (reporter GFP/YFP/CFP):

Cube 3A = FF02-472/30 excitation filter + Di02-488 dichroic + FF02-510/10 emission filter

OR for GCaMP6s ONLY Cube 3B = FF02-472/30 excitation filter + Di02-488 dichroic + FF01-520/35 emission filter
GFP(GCaMP)/YFP(mNeonGreen)/CFP (reporter): pseudocolored WHITE.

Adjusting Your Microscope Settings for NeuroPAL

Adjusting Your Microscope Settings for NeuroPAL

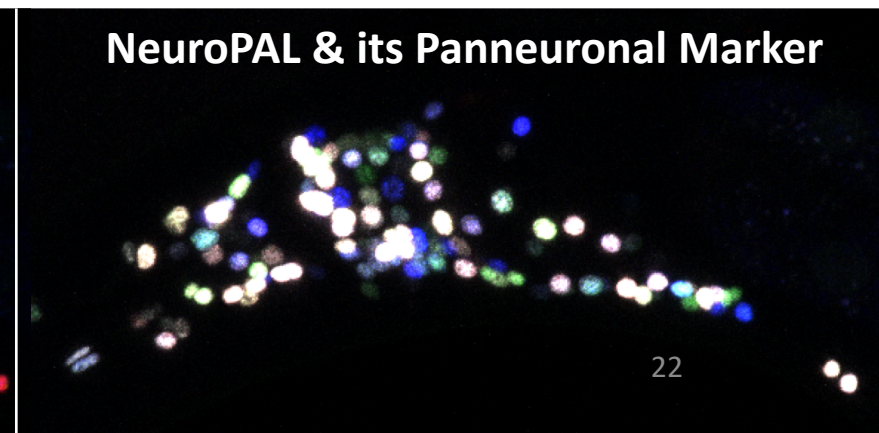
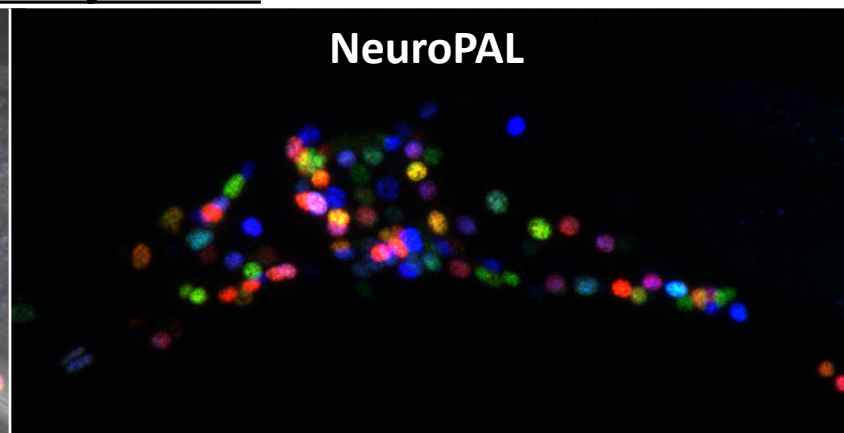
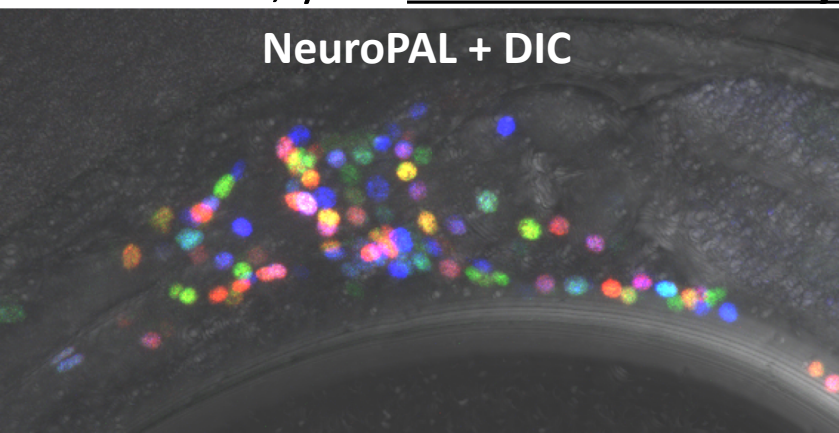
- The next few pages detail how to adjust your scope for imaging NeuroPAL.

Note: I'm using Zeiss's Zen software as an example, your software will likely look different.

- We will go through each one the fluorophore channels and do the following:
 - For laser illumination, you will adjust the laser strength & the photosensor gain to achieve the correct brightness, per fluorophore.
Note: To avoid damaging your equipment, please check with your manufacturer to ensure safe ranges for these 2 variables.
 - For LED illumination, you will adjust the LED strength & exposure time (shutter speed) to achieve the correct brightness, per fluorophore.
 - For arc lamp illumination, you will adjust the exposure time (shutter speed) to achieve the correct brightness, per fluorophore.

***** Note: lasers & arc lamps grow dimmer as they age. If the NeuroPAL colors look like they're dimming, please check your equipment, you may need to replace it or readjust for the aging of your illumination components.**

When done, your **Maximum Intensity Projections** of the NeuroPAL will look similar to these ones:

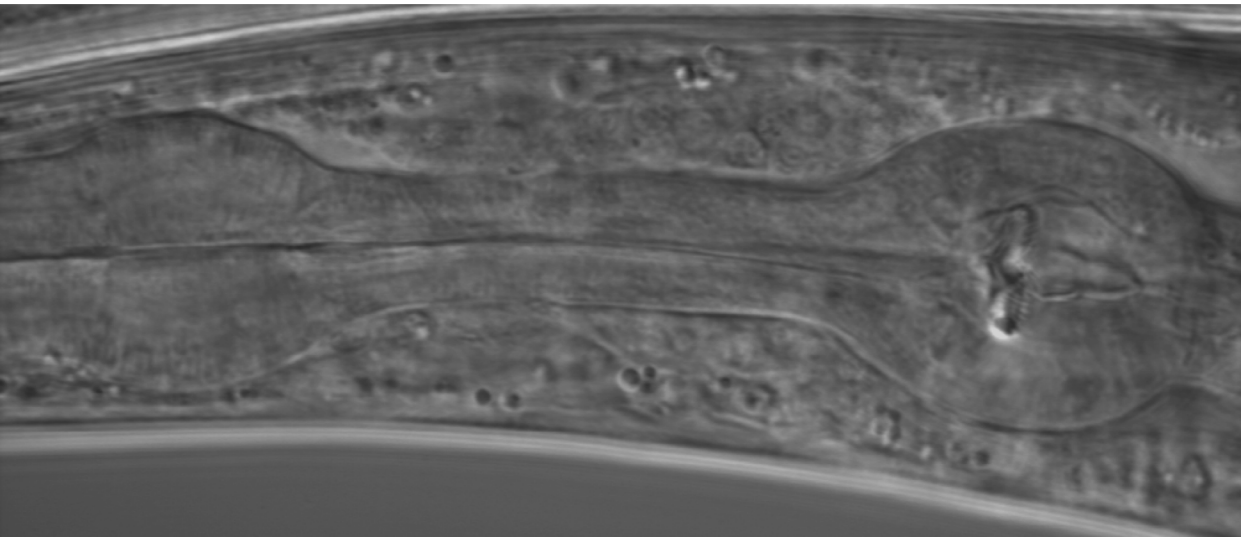


Adjusting Your Microscope Settings:

White Pseudocolored Panneuronal TagRFP-T (Part 1)

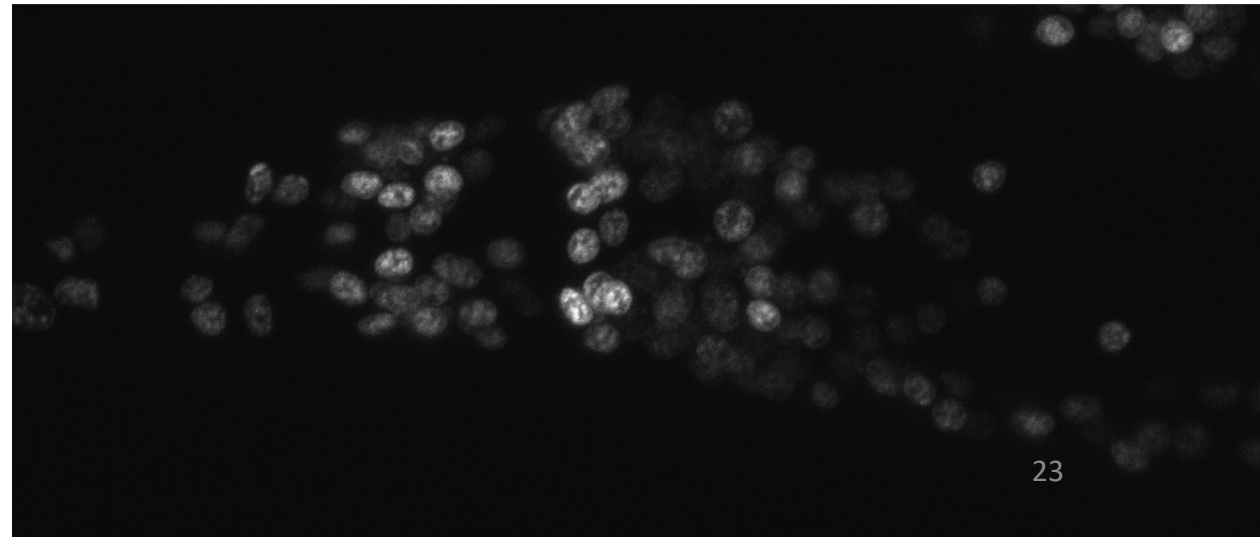
- We will start by adjusting the panneuronal marker TagRFP-T.
- The section titled, “Configuring Your Microscope” (page 18), can help you locate your TagRFP-T channel.
- **Please ensure your TagRFP-T channel is pseudocolored WHITE.**
- Find the head of an L4 or young adult.
- Increase the TagRFP-T illumination/gain/exposure to the point where you see bright neuronal nuclei, but not too far beyond the saturation limits of your photosensor/camera.

The head of a young adult worm (DIC).



A **Maximum Intensity Projection** of, correctly configured, panneuronal TagRFP-T on a Zeiss Airyscan LSM800.

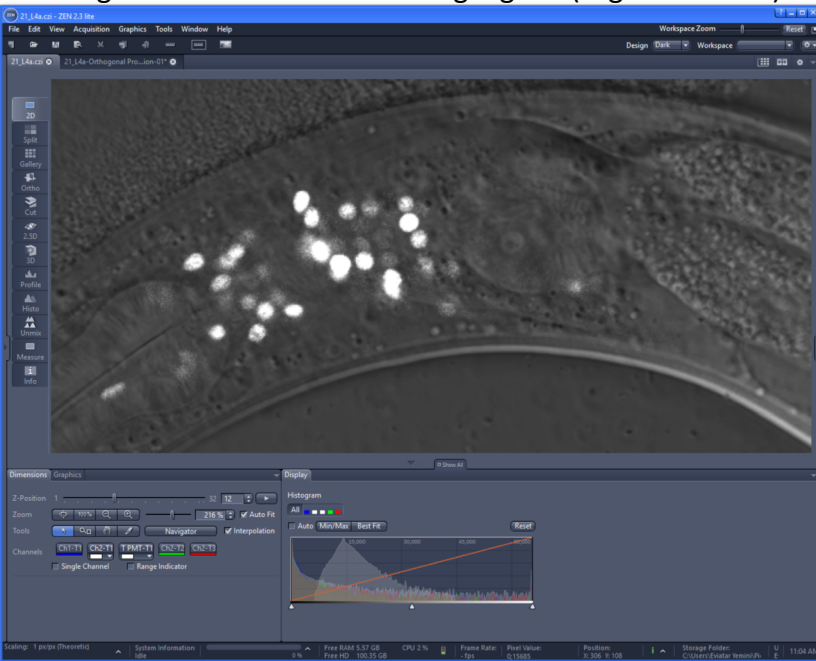
Note: your setup likely needs more saturation (see next page).



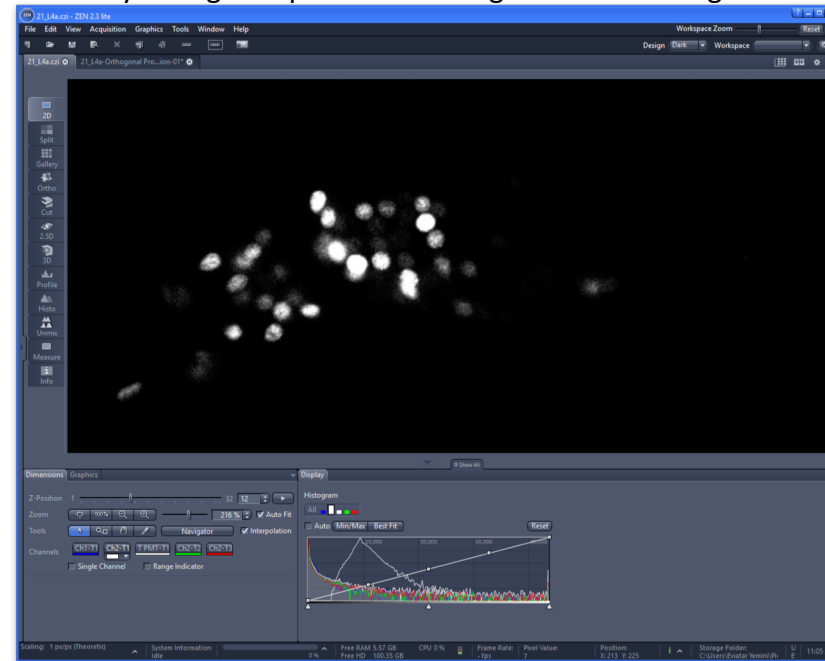
Adjusting Your Microscope Settings: White Pseudocolored Panneuronal TagRFP-T (Part 2)

- The world is not a perfect place 😊
- Ideally, if you restrict TagRFP-T emission to $< 615\text{nm}$ (although, up to $< 640\text{nm}$ can work well in practice), you will get the beautiful picture shown on the previous page: nearly identical brightness in every neuron.
- In reality, most of the configurations in the section titled, “Configuring Your Microscope” (page 18), use a long-pass red filter that passes all emitted light $> 600\text{nm}$. We collect more red light & simplify the design. But, we pay the price by permitting the mNeptune2.5 landmark to bleed into the panneuronal TagRFP-T.
- Therefore, neurons expressing the mNeptune2.5 landmark will appear brighter in the panneuronal TagRFP-T channel.
- Luckily, we can find a balance, wherein all neurons are visible but some saturate the photosensor/camera.

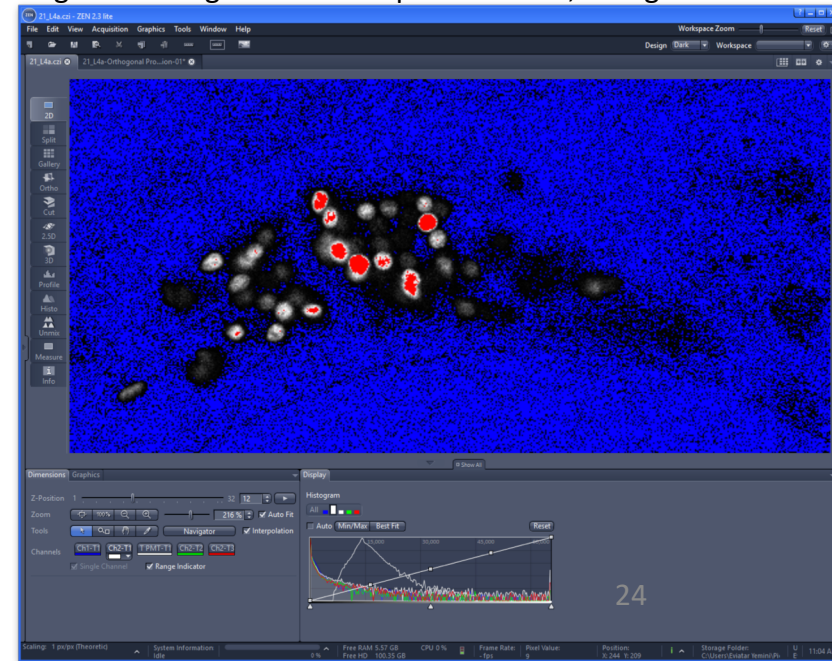
A single Z slice of an L4's Lateral ganglion (TagRFP-T + DIC).



Correctly configured panneuronal TagRFP-T in this single Z slice.



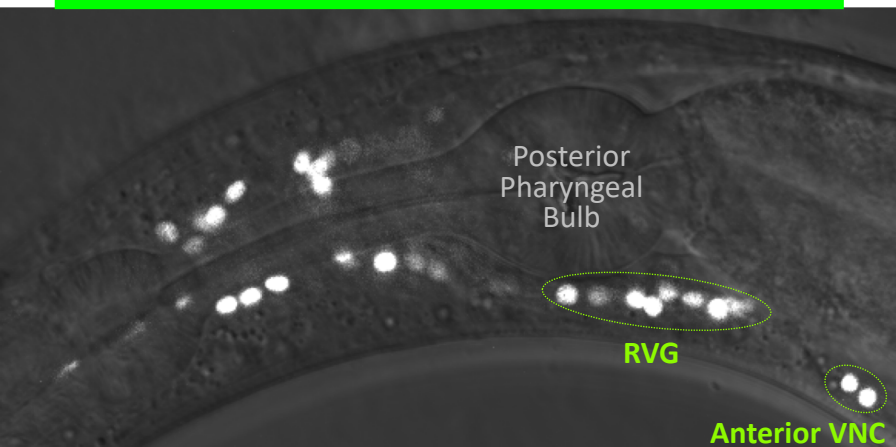
TagRFP-T “range”: saturated pixels are red, background is blue.



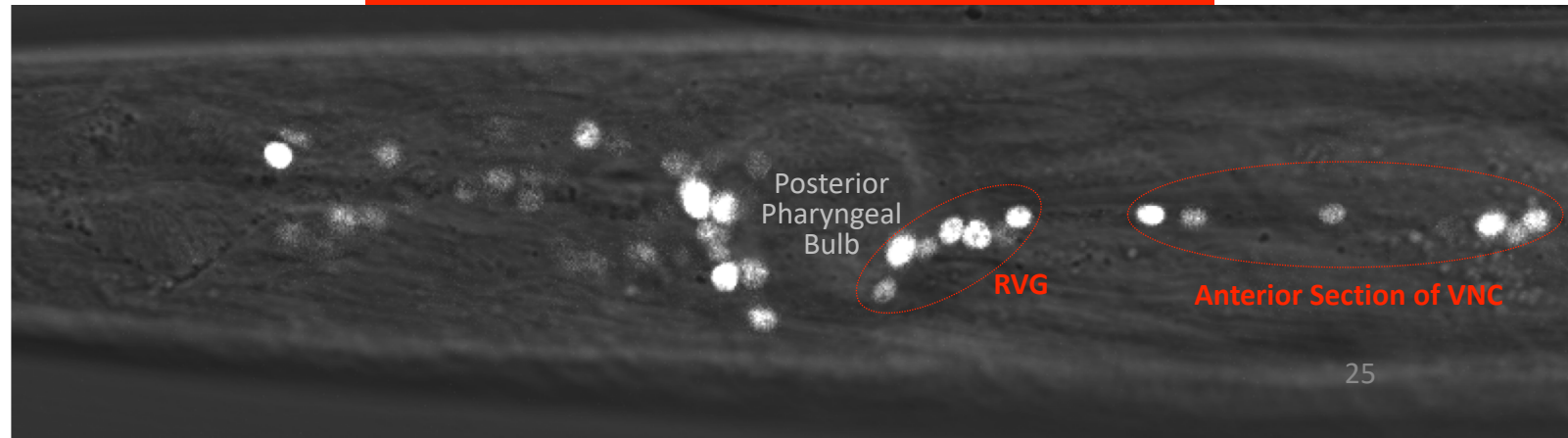
Adjusting Your Microscope Settings: Orienting Your Worm

- In the next few pages, we will be configuring the brightness for the pseudocolored landmarks using 3 neurons in the dorsal ganglion: ALA, CEPD, & URX.
- Locate the head of a young adult or L4.
- Use the panneuronal TagRFP-T to orient your worm laterally, ventral-side down in the image:
 1. To do this, find the worm's retro-vesicular ganglion (RVG) & ventral nerve cord (the "VNC"): a long string of neurons extending from the posterior pharyngeal bulb all the way towards the tail.
 2. Make sure the RVG & VNC neurons are positioned at the bottom of your worm (the worm should be in a lateral orientation & its ventral side should be oriented at the bottom of your image).
 3. If the RVG & VNC appears at the midline of your worm (roughly, halfway between the worm's top & bottom), you have a poorly oriented worm. Ignore it & find another worm that has the correct orientation.

CORRECTLY oriented worm: lateral orientation
with RVG & VNC at bottom of the worm.

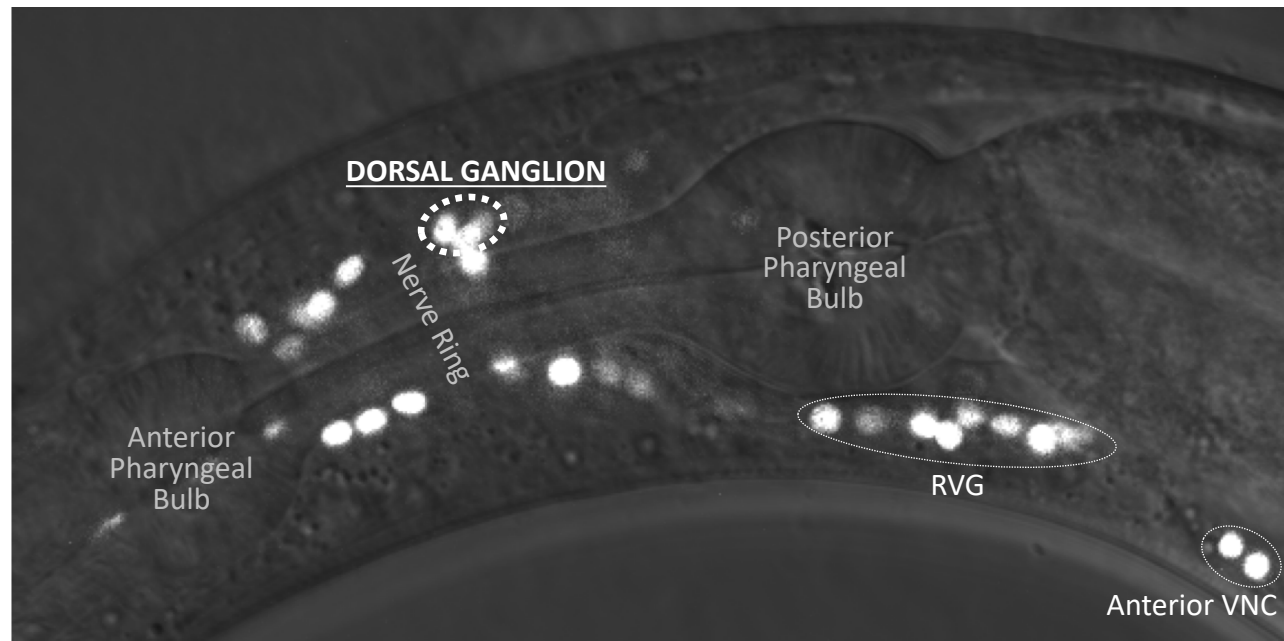


BADLY oriented worm: dorsal-ventral orientation
with RVG & VNC at the midline of the worm.



Adjusting Your Microscope Settings: Finding the Dorsal Ganglion

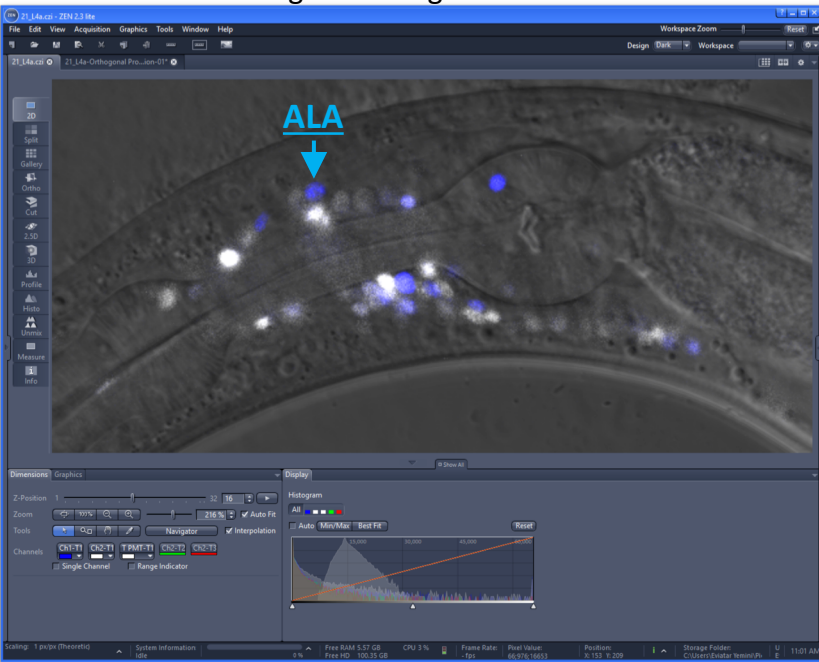
- Now that your worm is oriented correctly (lateral view, ventral side down) we will be locating the dorsal ganglion.
- The dorsal ganglion (DG) is a small package of 4 cell classes (ALA, CEPD, RID, URX) at the top of the head.
- With your worm oriented correctly (lateral orientation, with RVG & VNC at the the bottom of the image):
 1. Find the lateral (left-right) midline of the worm, the location where the RVG & VNC are in clearly in view.
 2. Locate the nerve ring, a space devoid of neurons between the anterior & posterior pharyngeal bulbs.
 3. Immediately posterior to the nerve ring, at the dorsal (upper) most portion of the worm, you will see a dense group of neurons, these represent the Dorsal Ganglion (DG).
 4. You will be using **ALA to adjust blue (mTagBFP2)**, **URX to adjust green (CyOFP)**, & **CEPD to adjust red (mNeptune2.5)**.



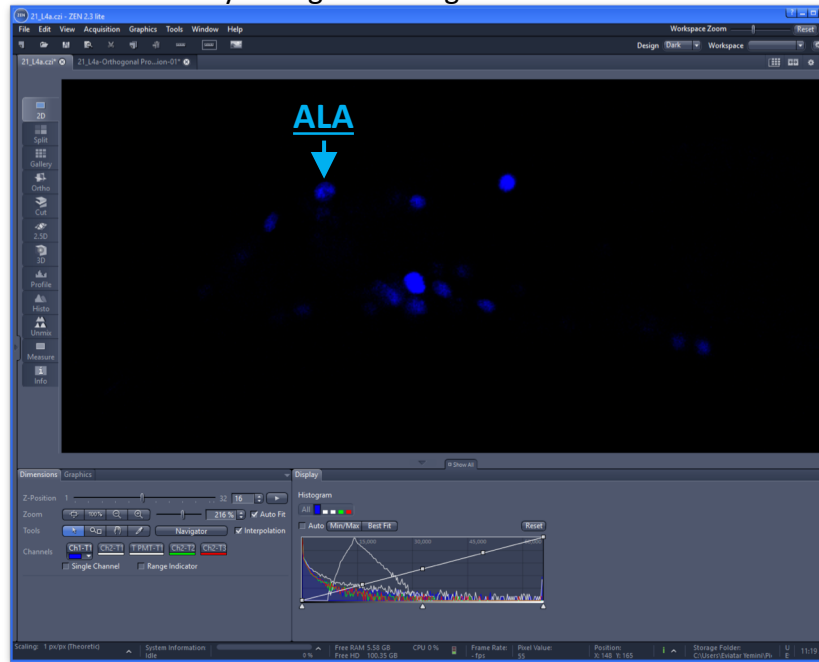
Adjusting Your Microscope Settings: Blue Pseudocolored Landmark mTagBFP2

- Next we will adjust the blue landmark mTagBFP2.
- The section titled, “Configuring Your Microscope” (page 18), can help you locate your mTagBFP2 channel.
- **Please ensure your mTagBFP2 channel is pseudocolored BLUE.**
- Increase the mTagBFP2 illumination/gain/exposure to the point where you see bright blue neuronal nuclei.
- Locate ALA, the brightest blue neuron in the dorsal ganglion.
- Configure the mTagBFP2 illumination/gain/exposure to the point where ALA just begins to saturate the photosensor/camera.
- Note: other blue neurons will now appear saturated, this is the correct configuration.

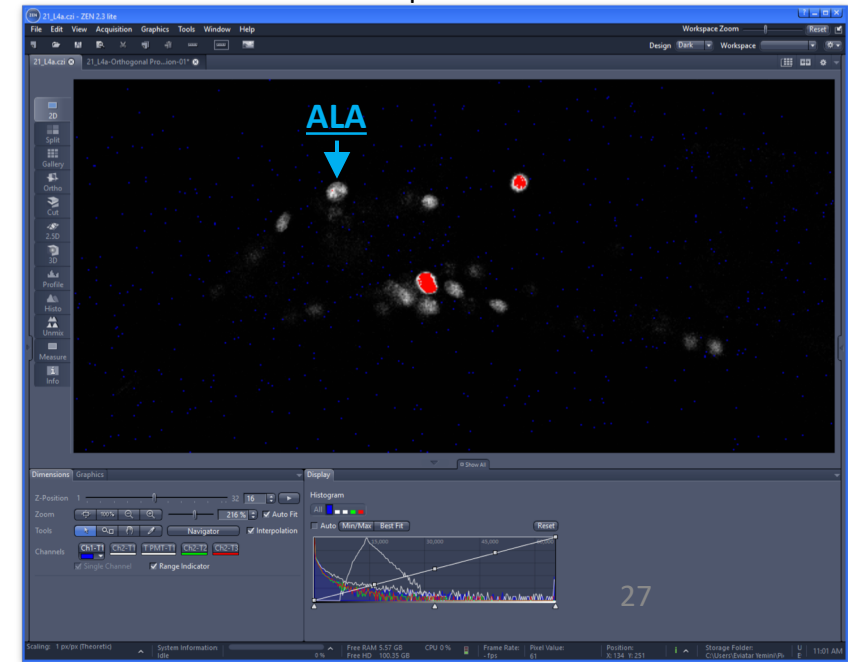
mTagBFP2 + TagRFP-T + DIC



Correctly configured mTagBFP2 blue landmark.



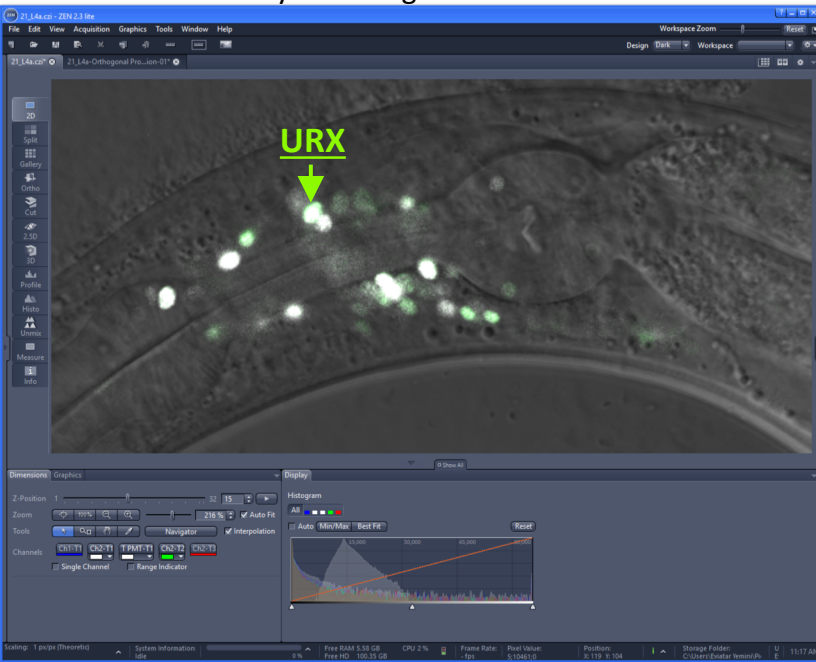
Correctly configured mTagBFP2 “range”:
Saturated pixels are red.



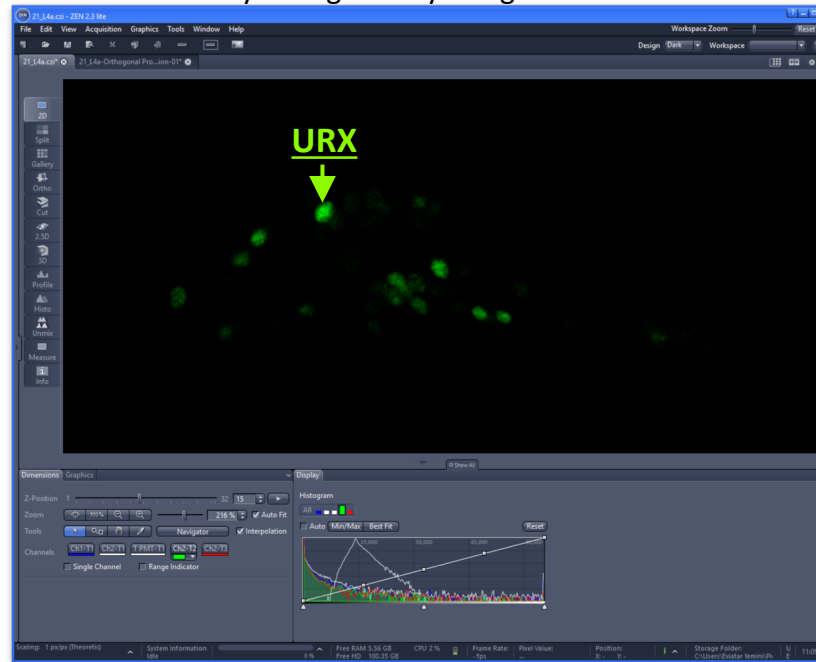
Adjusting Your Microscope Settings: Green Pseudocolored Landmark CyOFP

- Next we will adjust the green landmark CyOFP.
- The section titled, “Configuring Your Microscope” (page 18), can help you locate your CyOFP channel.
- **Please ensure your CyOFP channel is pseudocolored GREEN.**
- Increase the CyOFP illumination/gain/exposure to the point where you see bright green neuronal nuclei.
- Locate URX, the brightest green neuron in the dorsal ganglion.
- Configure the CyOFP illumination/gain/exposure to the point where URX just begins to saturate the photosensor/camera.
- Note: other green neurons will now appear saturated, this is the correct configuration.

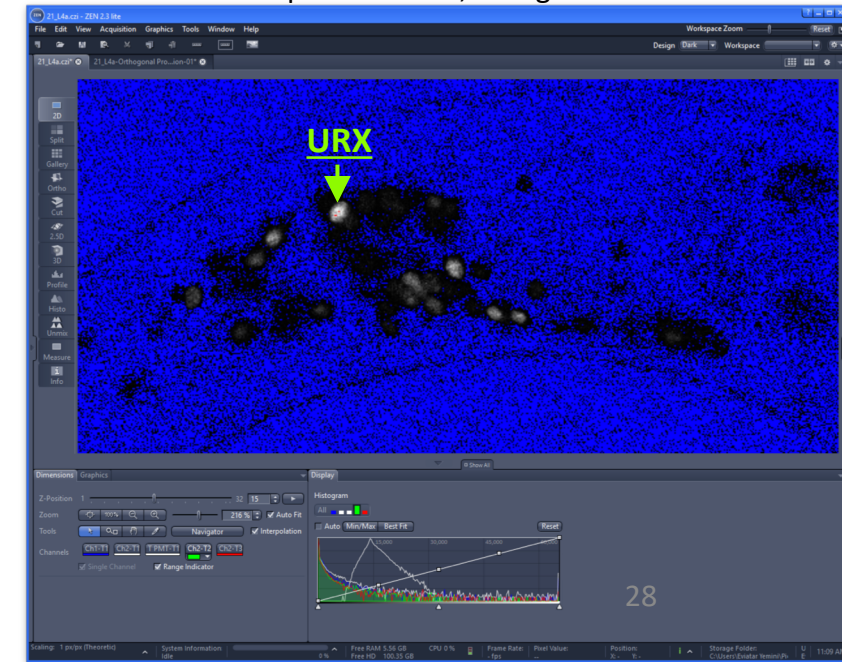
CyOFP + TagRFP-T + DIC



Correctly configured CyOFP green landmark.



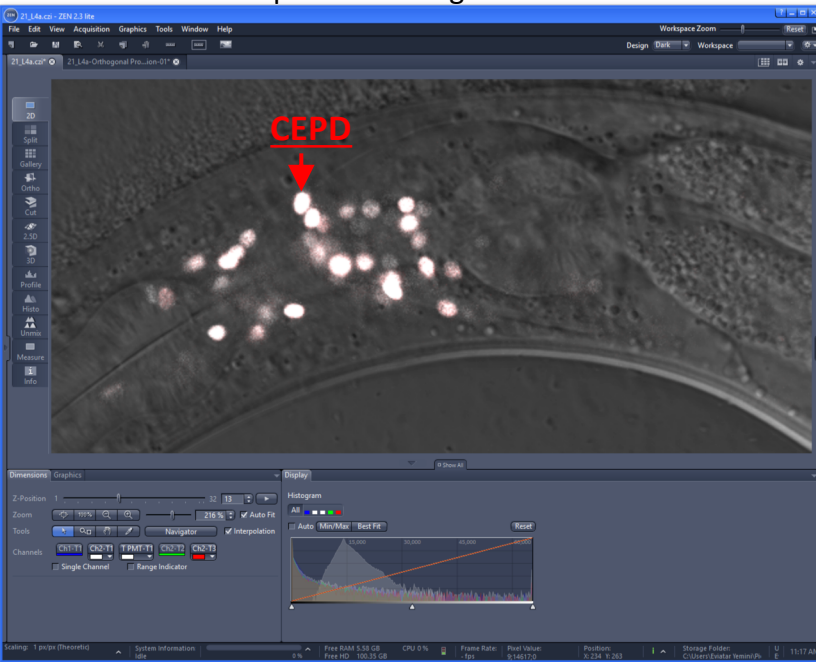
Correctly configured CyOFP “range”:
Saturated pixels are red, background is blue.



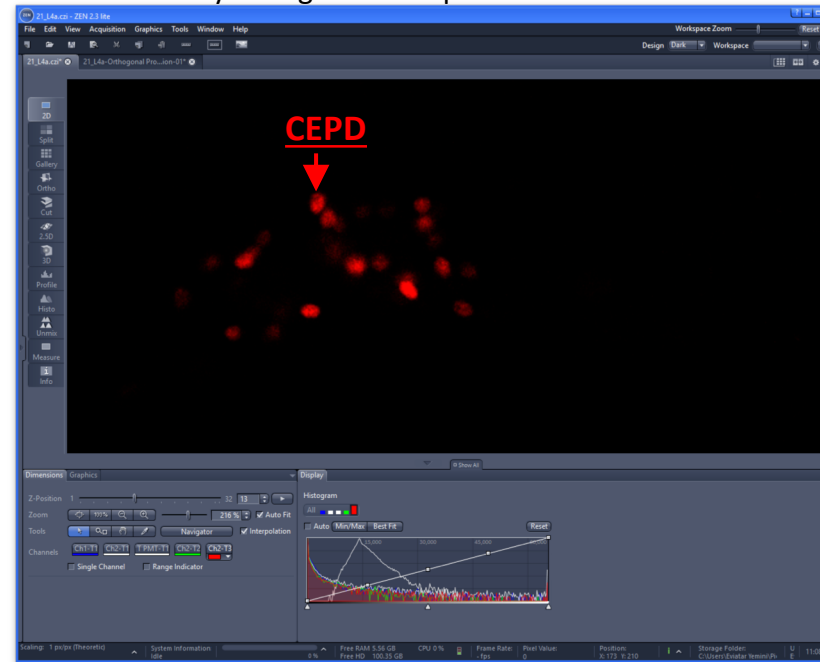
Adjusting Your Microscope Settings: Red Pseudocolored Landmark mNeptune2.5

- Next we will adjust the red landmark mNeptune2.5.
- The section titled, “Configuring Your Microscope” (page 18), can help you locate your mNeptune2.5 channel.
- **Please ensure your mNeptune2.5 channel is pseudocolored RED.**
- Increase the mNeptune2.5 illumination/gain/exposure to the point where you see bright red neuronal nuclei.
- Locate CEPD, the brightest red neuron in the dorsal ganglion.
Note: URX also expresses red, if you confuse URX & CEPD, your red mNeptune2.5 channel will still be configured correctly.
- Configure mNeptune2.5 illumination/gain/exposure to the point where CEPD just begins to saturate the photosensor/camera.
- Note: other red neurons will now appear saturated, this is the correct configuration.

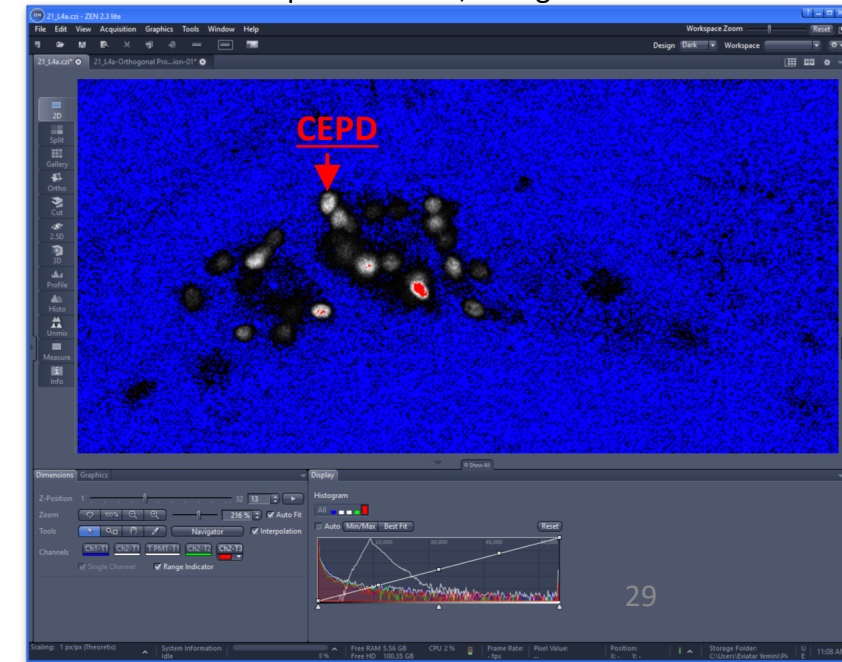
mNeptune2.5 + TagRFP-T + DIC



Correctly configured mNeptune2.5 red landmark.



Correctly configured mNeptune2.5 “range”:
Saturated pixels are red, background is blue.



Adjusting Your Microscope Settings: White Pseudocolored Reporter GFP/YFP/CFP

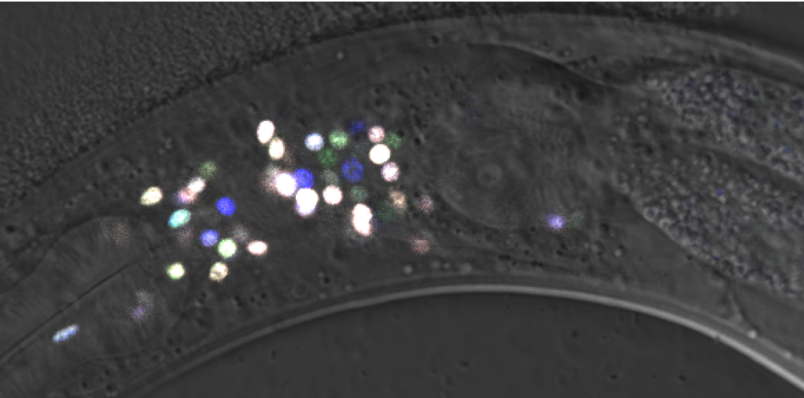
- When you've crossed a GFP/YFP/CFP reporter into NeuroPAL we can adjust the GFP/YFP/CFP channel.
- The section titled, "Configuring Your Microscope" (page 18), can help you locate your GFP/YFP/CFP channel.
- **Please ensure your GFP/YFP/CFP channel is pseudocolored WHITE.**
- Configure GFP/YFP/CFP illumination/gain/exposure to the point where your reporter expression is clearly visible.
- Important notes, **PLEASE READ THE FOLLOWING:**
 - 1. Some microscopes may link your landmark tracks to your GFP/YFP/CFP channel. Be careful that you're not reconfiguring the NeuroPAL landmark channels when adjusting the GFP/YFP/CFP channel.**
 - 2. Every reporter line has a different brightness, you will need to re-adjust this channel per reporter strain.**
 - 3. Bright GFP/YFP/CFP can bleed into the green CyOFP landmark. In practice, this is unlikely to confuse you since the saturated expression usually show obvious bleed through & there are plenty of nearby neurons in NeuroPAL to help orient you.**
 - 4. Due to the limits of our RGB palette, the panneuronal TagRFP-T marker & your GFP/YFP/CFP reporter will share WHITE coloring. I suggest taking your pictures normally then, later, viewing these channels one at a time, but never at the same time, to avoid confusion. You can also reconfigure either channel to be the color of your choice but this is likely to be even more confusing. In practice, the choice to color both channels identically, then view them separately, has never presented any problems.**

Visualizing Multicolor NeuroPAL Volumes

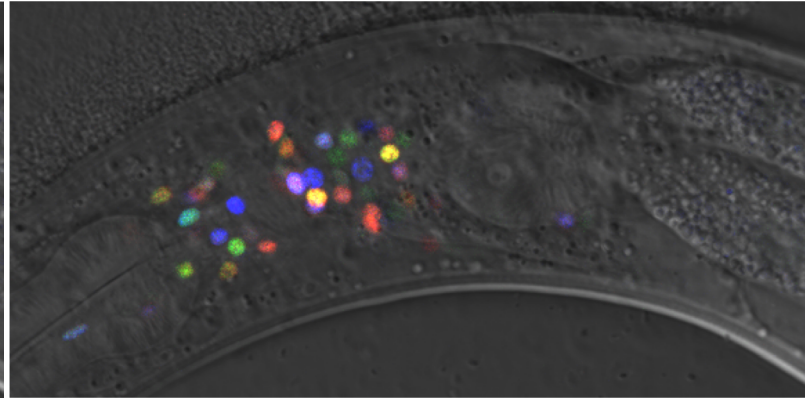
- While configuring your landmark channels, you may have bleached the fluorophores. *** Please image several new worms to ensure that your channels are correctly configured. Readjust them as necessary.

*** Note: lasers & arc lamps grow dimmer as they age. If the NeuroPAL colors look like they're dimming, please check your equipment, you may need to replace it or readjust for the aging of your illumination components.

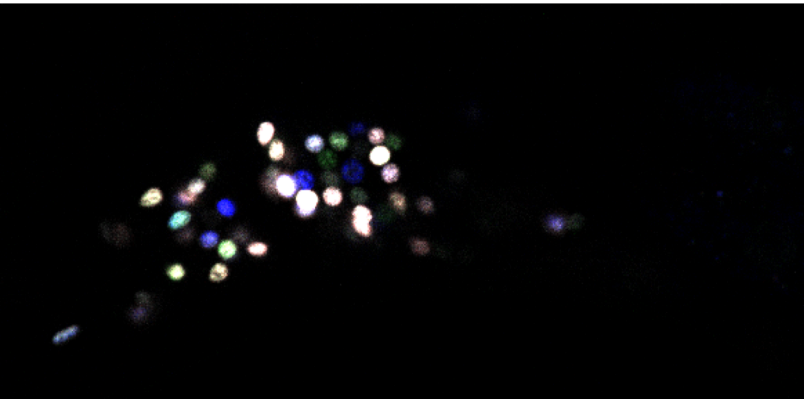
Panneuronal + Landmarks + DIC (Single Z-Slice)



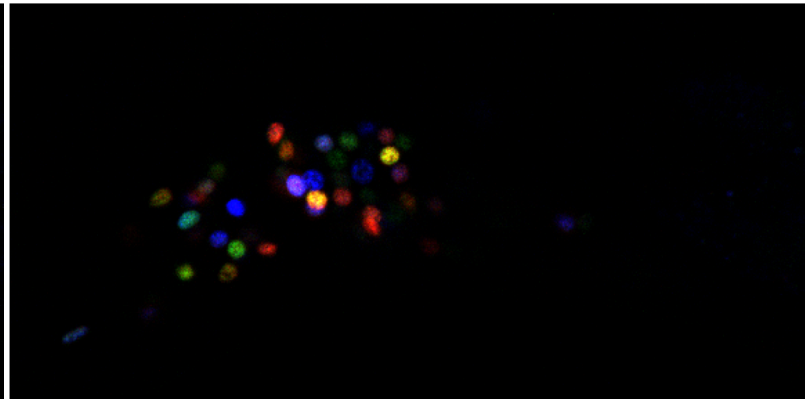
Landmarks + DIC (Single Z-Slice)



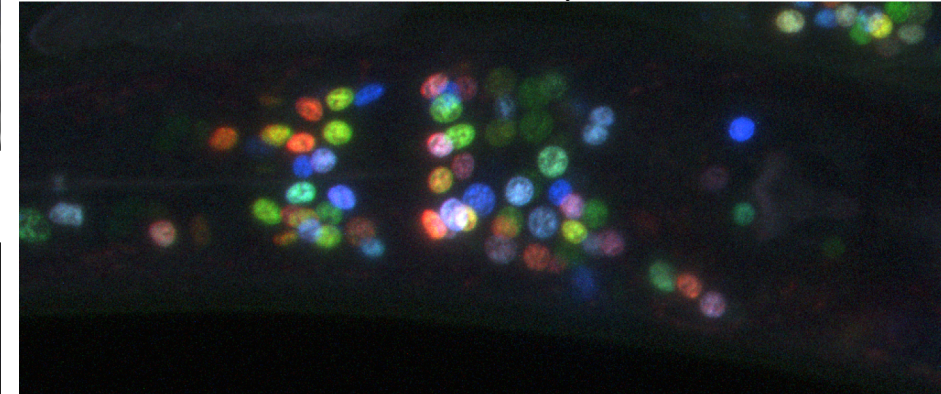
Panneuronal + Landmarks (Single Z-Slice)



Landmarks (Single Z-Slice)

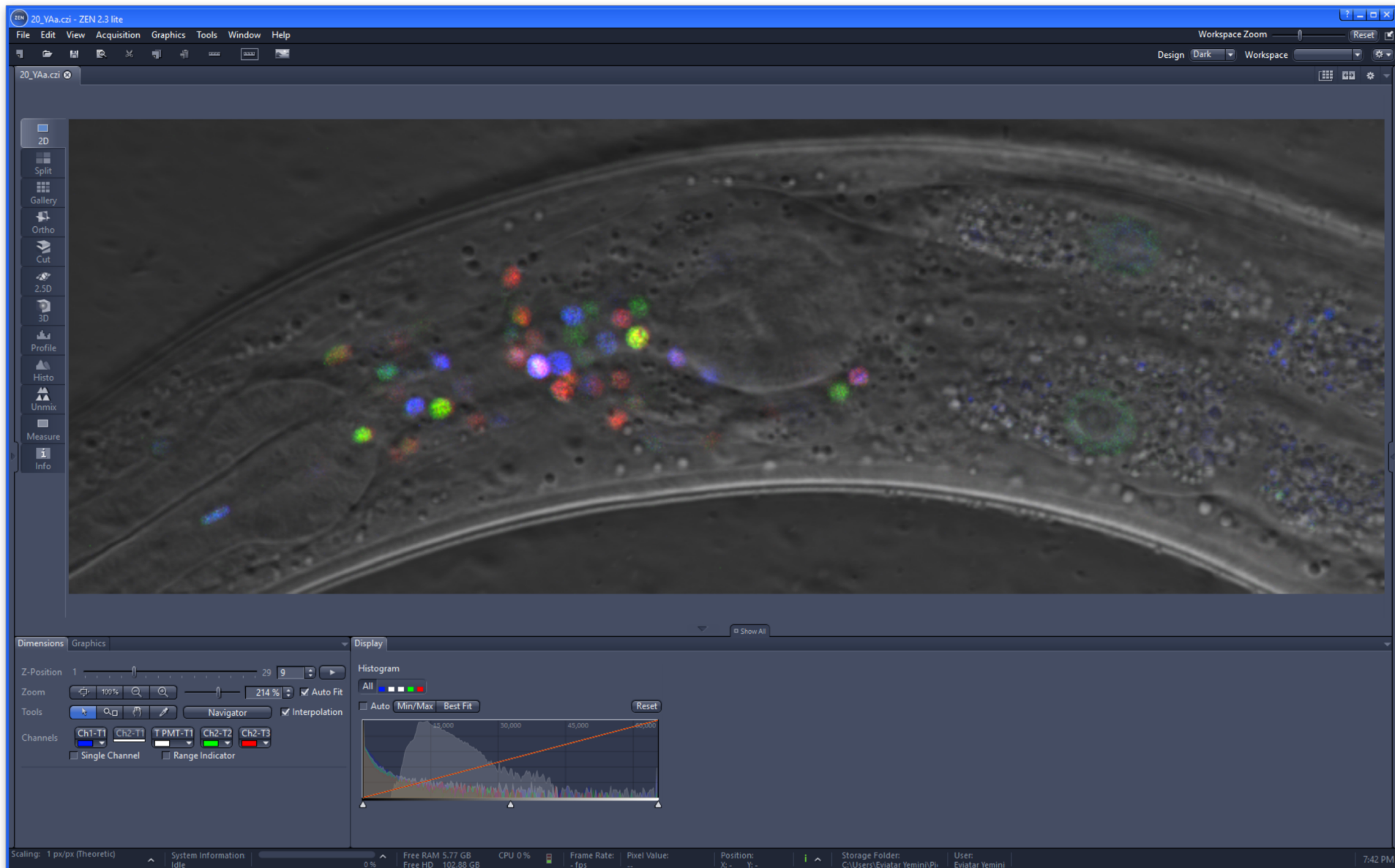


Maximum Intensity Projection of Landmarks on a Zeiss LSM800 Airyscan



Tricks of the Trade:
Making the NeuroPAL Colors
More Visible

Adding the DIC Channel Brightens the NeuroPAL Colors



Adjusting the Gamma is Better than Adjusting the Histogram's Min/Max

If you turn off the DIC, you can improve the colors by increasing the gamma. To do this in Zen, slide the **gamma** adjustment (**red arrow**) to increase the coloring. Avoid going too far as this will increase the background noise & confuse you.

