

Common SIM Artifacts – Tips for Successful SIM Imaging

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imagination at work

Common Structured Illumination Artifacts

2 main artifacts & their causes

1. Haloing/doubling

- Caused by asymmetrical point spread function:
- Spherical aberration
- Photobleaching
- Cutting structures off

2. Honeycomb

- Caused by diffuse labeling or bad signal to noise
- Increase signal to noise
- Adjust Wiener filter constant



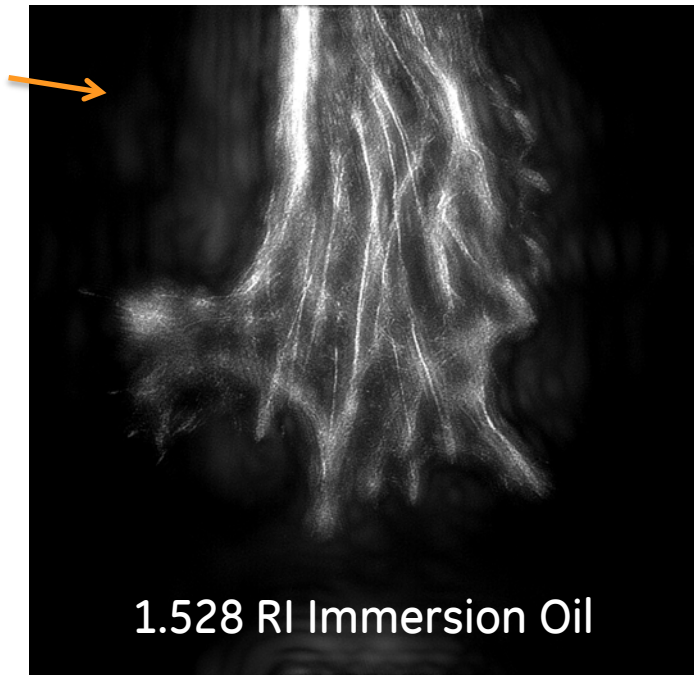
Haloing/Doubling Cause 1:
Spherical aberration due to
refractive index mismatch



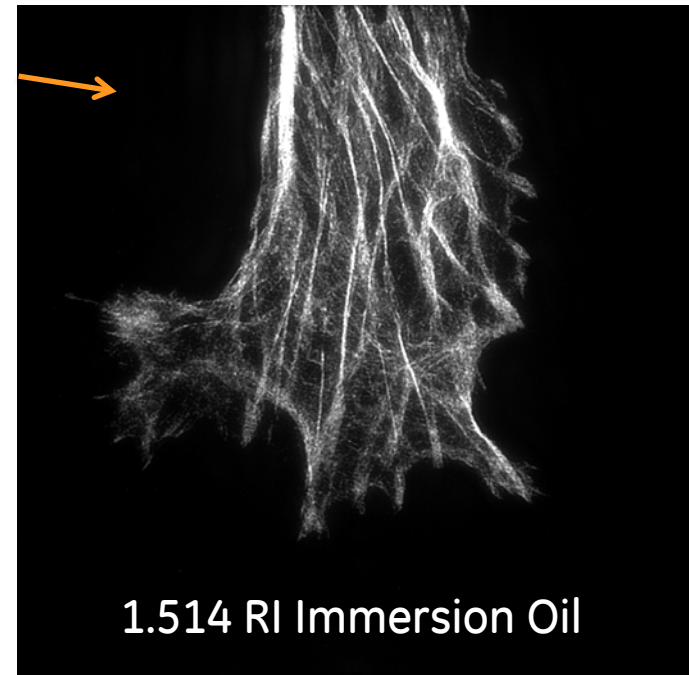
Haloing/Doubling Artifact

Caused by asymmetry in point spread function

Haloing/doubling artifact



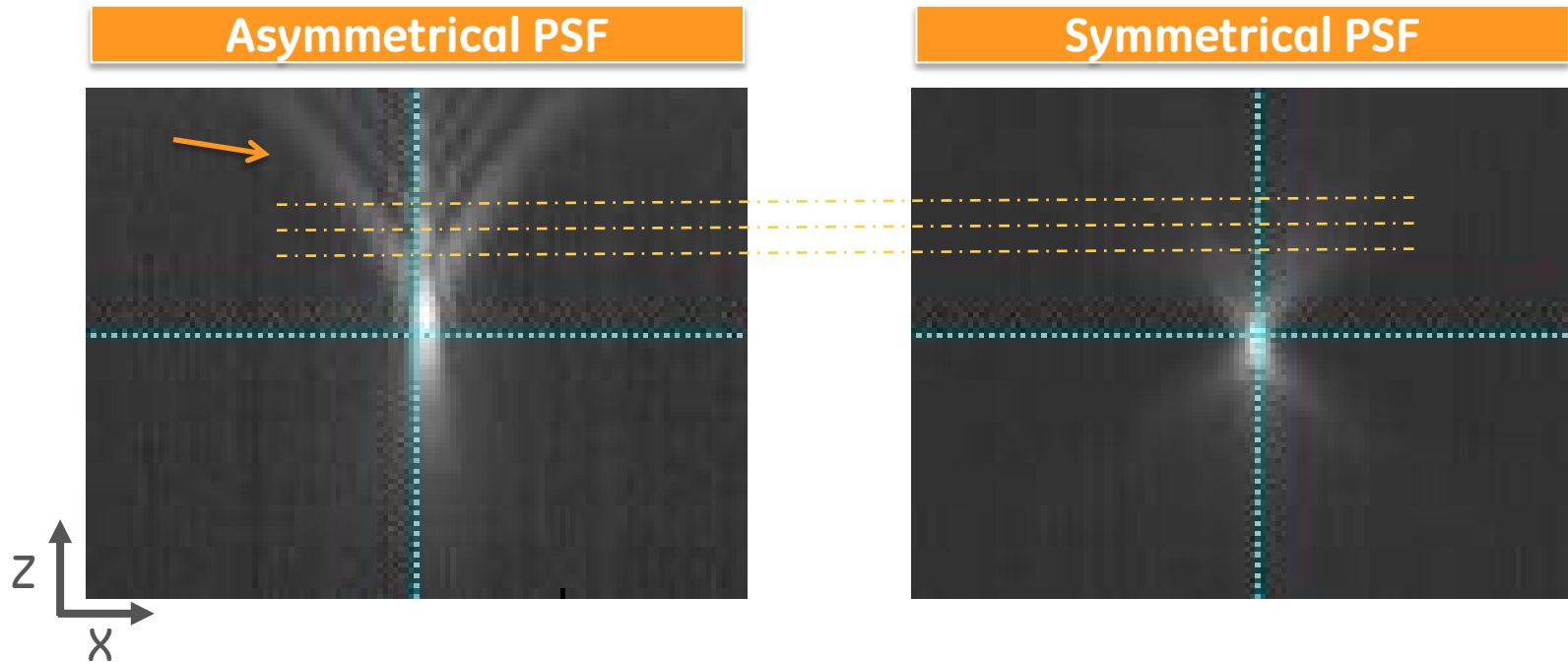
Artifact free



Spherical aberration caused by refractive index mismatch results in asymmetrical point spread function

Haloing/Doubling Artifact

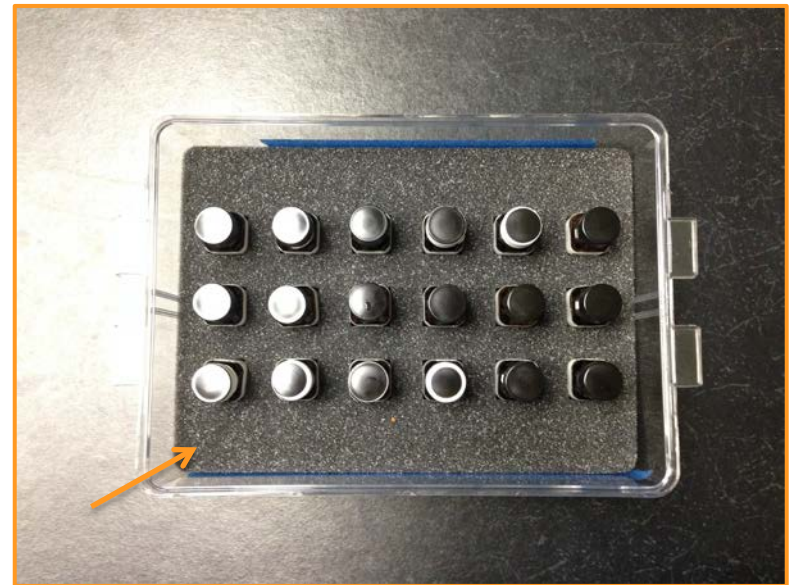
What causes this?



SIM algorithm assumes a perfectly matched PSF! When it detects out of focus light from mismatched PSF, it assumes this is real signal & reconstructs it

Haloing/Doubling caused by Spherical Aberration

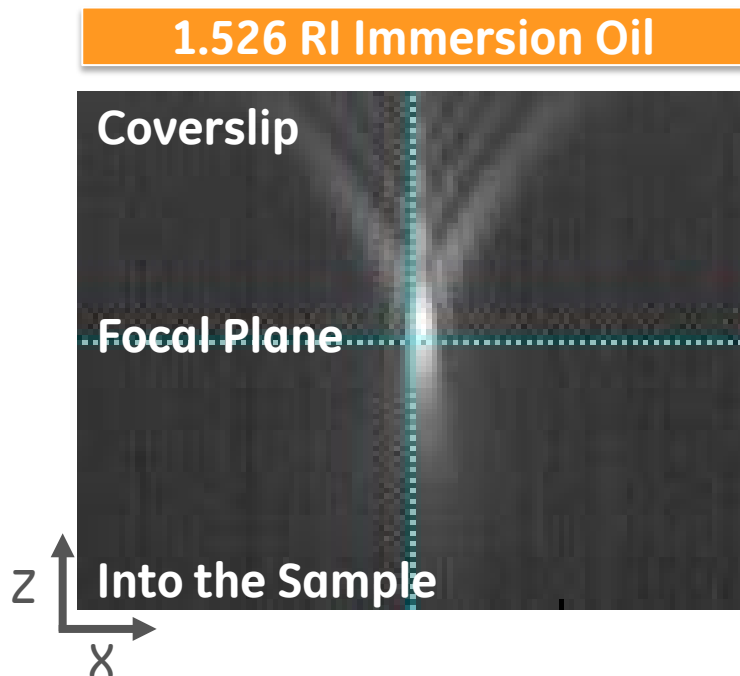
Minimize by matching the RI of immersion oil to your sample



The OMX comes with an oil kit with 18 oils with different refractive indices ranging from 1.500 to 1.534 in increments of 0.002

Haloing/Doubling Caused by RI Mismatch

How to find the best oil to minimize artifacts

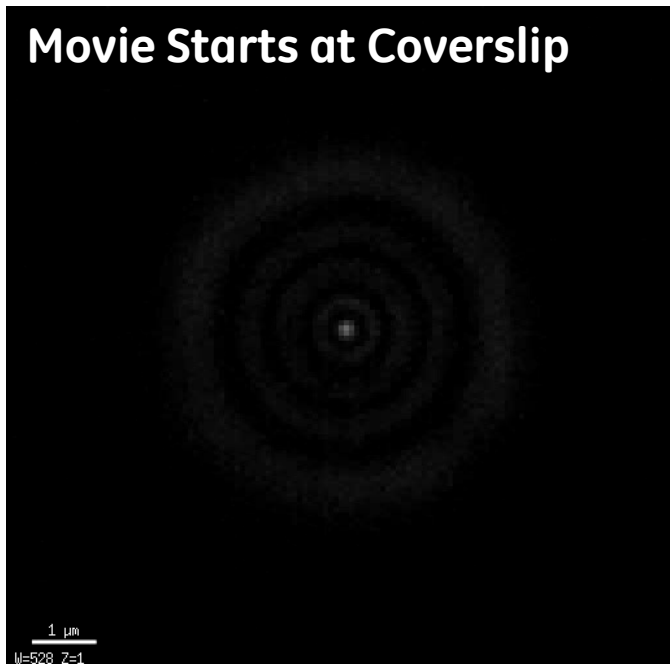


If the flaring/
asymmetry of the
PSF is towards the
coverslip or 'up', the
RI of the oil is too
high! Go down in oil!

Haloing/Doubling Caused by RI Mismatch

How to find the best oil to minimize artifacts

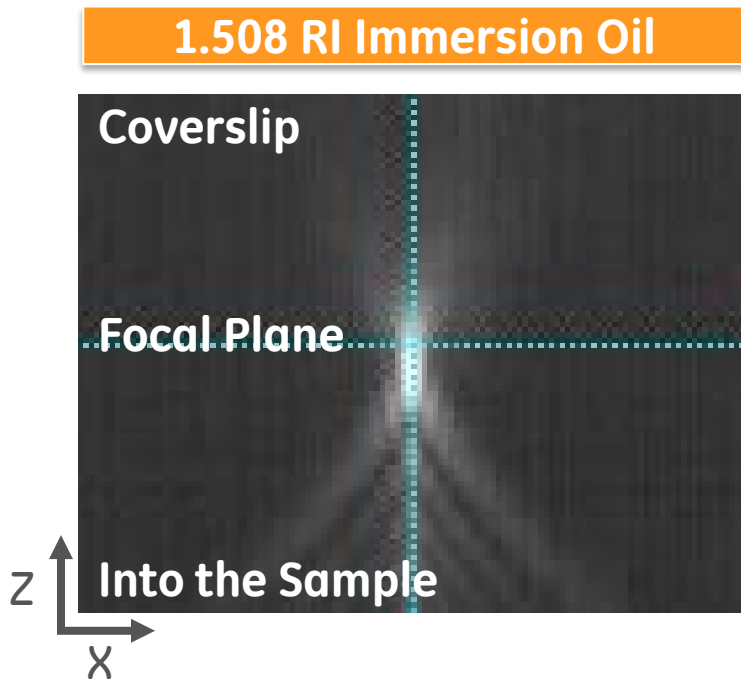
1.526 RI Immersion Oil



If the flaring/
asymmetry of the
PSF is towards the
coverslip or 'up', the
RI of the oil is too
high! Go down in oil!

Haloing/Doubling Caused by RI Mismatch

How to find the best oil to minimize artifacts

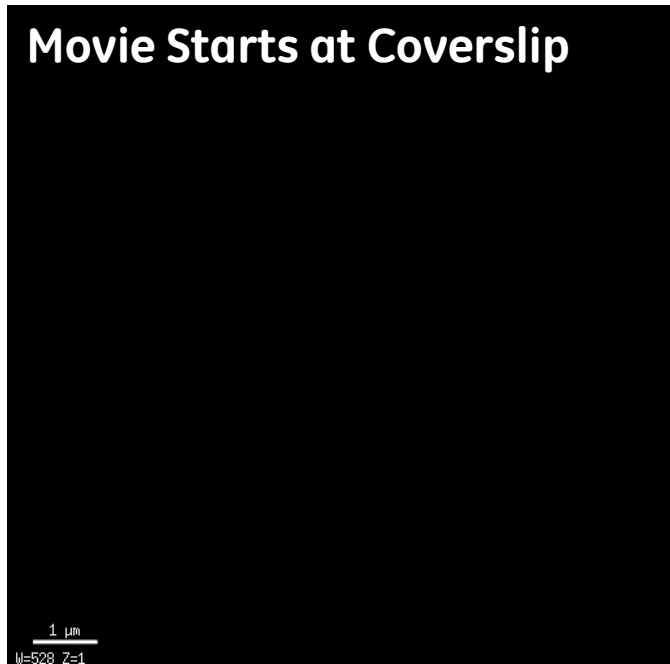


If the flaring/
asymmetry of the
PSF is away from the
coverslip or 'down',
the RI of the oil is too
low! Go up in oil!

Haloing/Doubling Caused by RI Mismatch

How to find the best oil to minimize artifacts

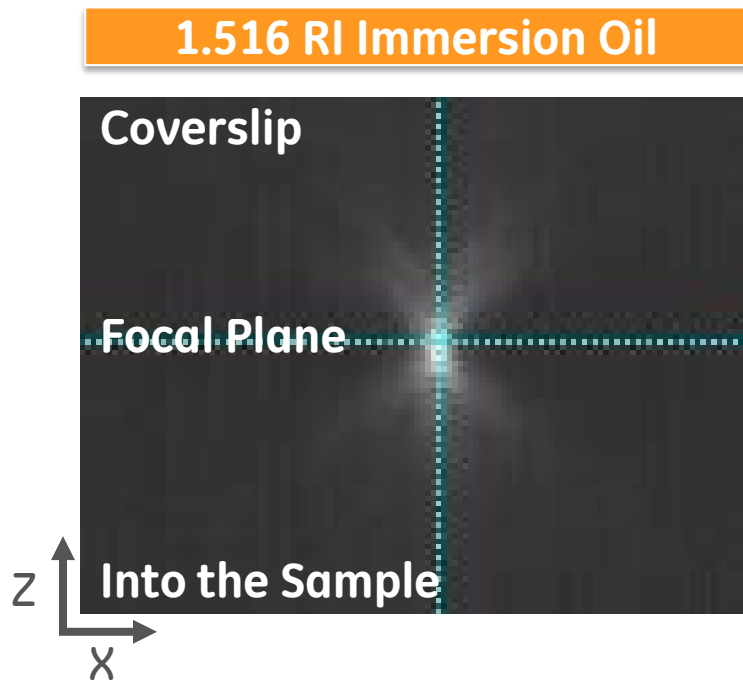
1.508 RI Immersion Oil



If the flaring/
asymmetry of the
PSF is away from the
coverslip or 'down',
the RI of the oil is too
low! Go up in oil!

Haloing/Doubling Caused by RI Mismatch

How to find the best oil to minimize artifacts

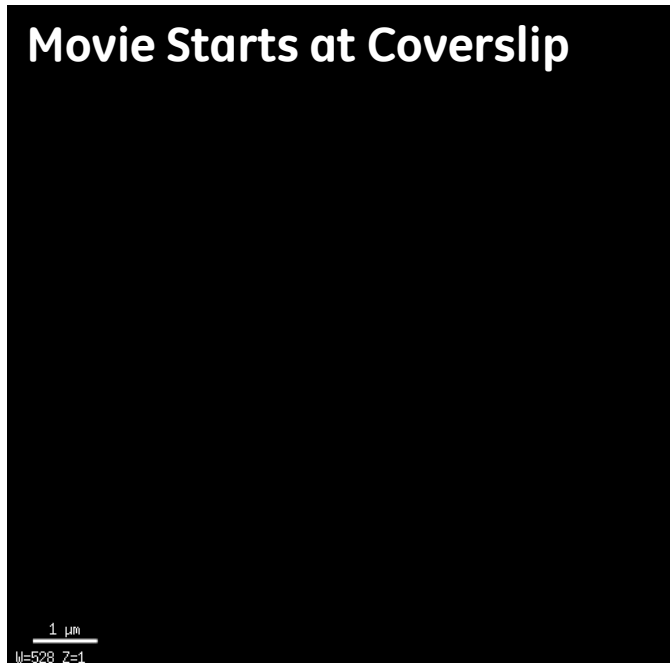


If the flaring of the PSF is symmetrical on both sides of the focal plane, the oil is matched!

Haloing/Doubling Caused by RI Mismatch

How to find the best oil to minimize artifacts

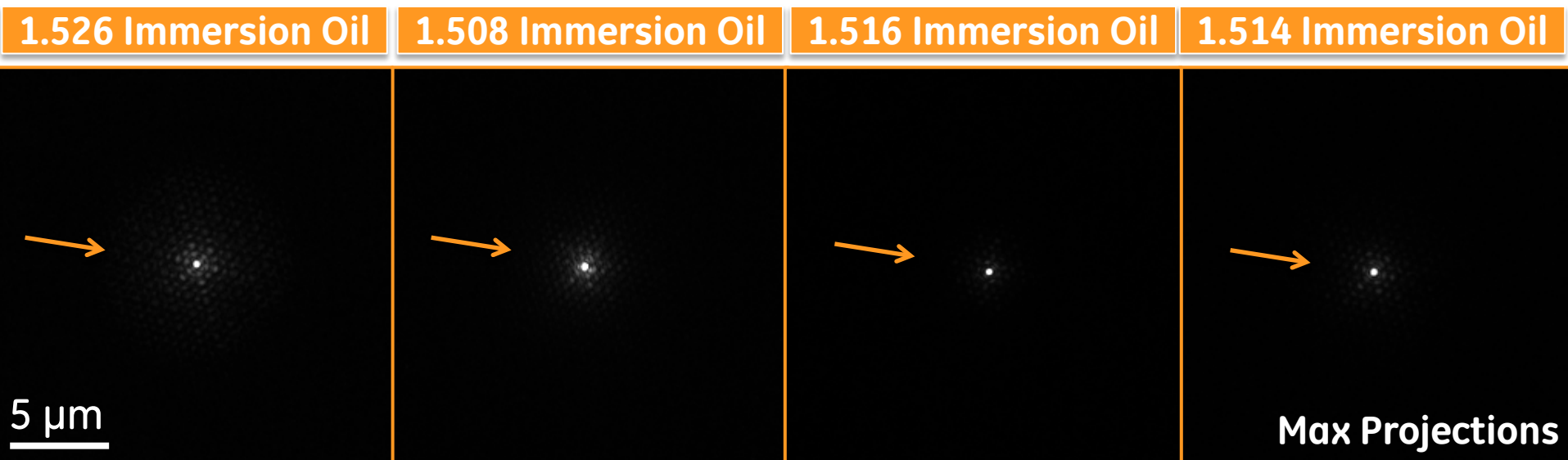
1.516 RI Immersion Oil



If the flaring of the PSF is symmetrical on both sides of the focal plane, the oil is matched!

Haloing/Doubling Caused by RI Mismatch

Affects of RI mismatch on SIM reconstructions



Haloing or doubling artifacts are minimized in SIM reconstructions when the oil used results in a symmetric PSF



Haloing/Doubling Caused by RI Mismatch

Matching oil for multi-color samples

- Can only match the oil for 1 wavelength
- Meaning the wavelengths with suboptimal oil matching will have some haloing/doubling artifacts
- What can be done about this?
 1. Match for the most red shifted wavelength because the blue shifted wavelengths are more forgiving
 2. Match for the color you care the most about



Haloing/Doubling Caused by RI Mismatch

Which oil should you start with?

Excitation Wavelength (nm)	Imaging at the coverslip	Imaging + 5 μ m from coverslip	Imaging + 10 μ m from coverslip
405	1.510	1.512	1.514
488	1.512	1.514	1.516
568	1.514	1.518	1.520
642	1.518	1.520	1.522

For imaging at 37°C increase oil by +0.008 or 4 steps in oil kit
For every + 5 μ m imaging into the sample go up 0.002 or 1 step in the oil kit



Tools to Estimate the RI Oil for your Sample

GE Oil Immersion Calculator (it's free!)

The image displays two screenshots of the GE Oil Immersion Calculator app interface. The left screenshot shows the input fields for various parameters, and the right screenshot shows the calculated Recommended Refractive Index of 1.515.

Left Screenshot (Input Fields):

- Working Distance of Objective Lens (mm): 60x/ 1.42 NA Oil PlanApoN (0.15 mm)
- Coverslip Thickness (μm): 170 μm (#1.5)
- Specimen Refractive Index: Water/Cell Culture Medium (1.333)
- Distance from Coverslip to Specimen (μm): 0
- Temperature ($^{\circ}\text{C}$): 25

Right Screenshot (Output):

- Distance from Coverslip to Specimen (μm): 0
- Temperature ($^{\circ}\text{C}$): 25
- Excitation wavelength (nm): 490
- Recommended Refractive Index: 1.515
- Find matching oil button

Search for 'GE oil immersion calculator' in the app store
Or visit www.gelifesciences.com/oilcalculator

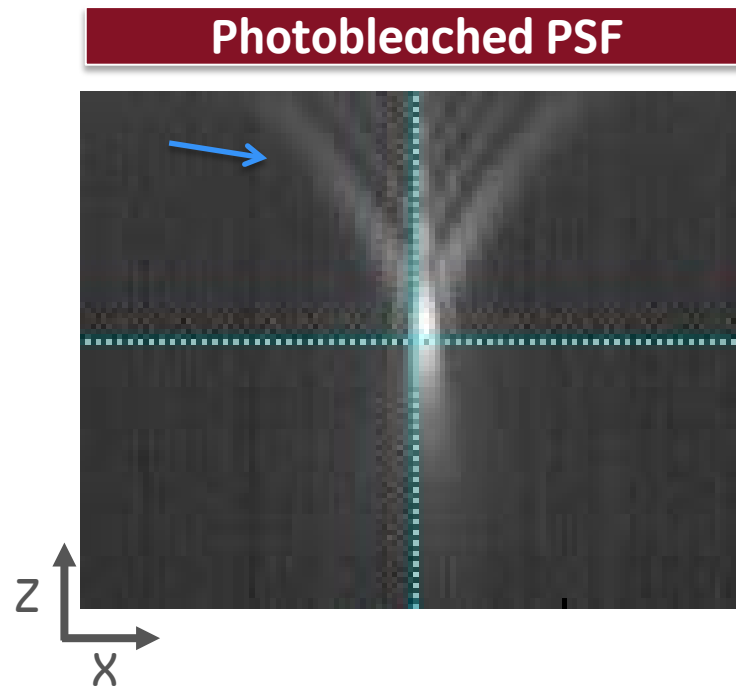


Haloing/Doubling Cause 2: Photobleaching



Common Structured Illumination Artifacts

What causes the haloing or doubling artifact?



When photobleaching occurs, lose part of PSF

Haloing/Doubling Caused by Photobleaching Gives asymmetrical PSF

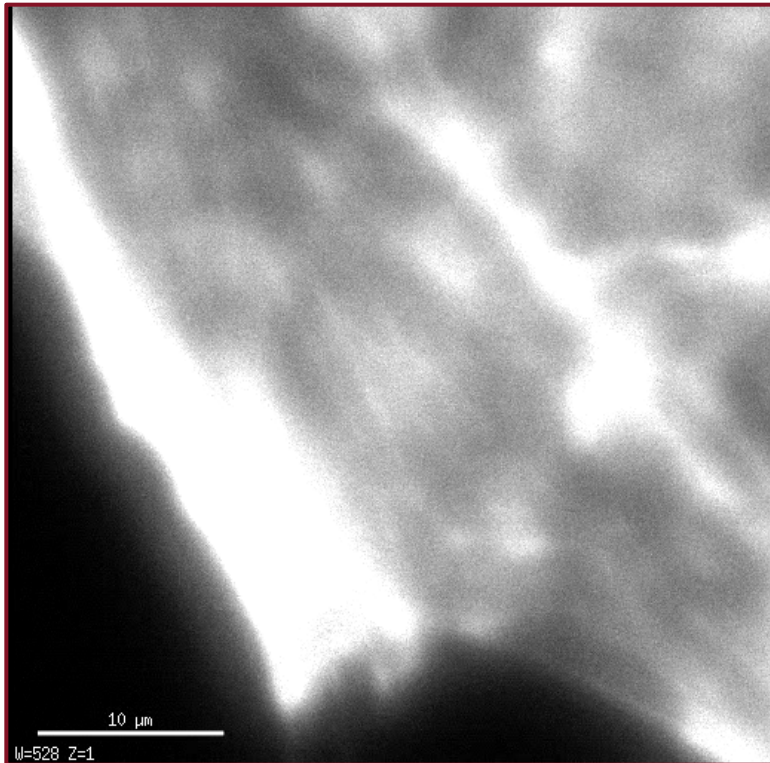
- Do not bleach more than ~30% across the stack
- Imaging parameters to change to minimize photobleaching:
 - Lower %T, increase exposure
 - Increase %T, decrease exposure
 - Smaller z stack (fewer # of optical sections)
 - Use all channel then z imaging setting
 - Use antifade reagents



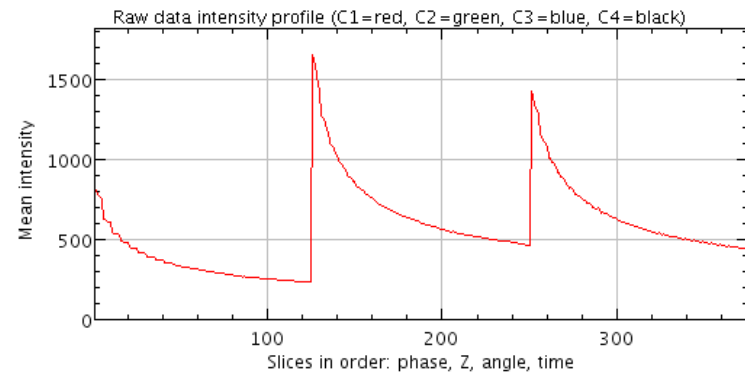
Haloing/Doubling Caused by Photobleaching

Need less than 30% from top to bottom

Raw SIM Data with Bleaching



Intensity profile

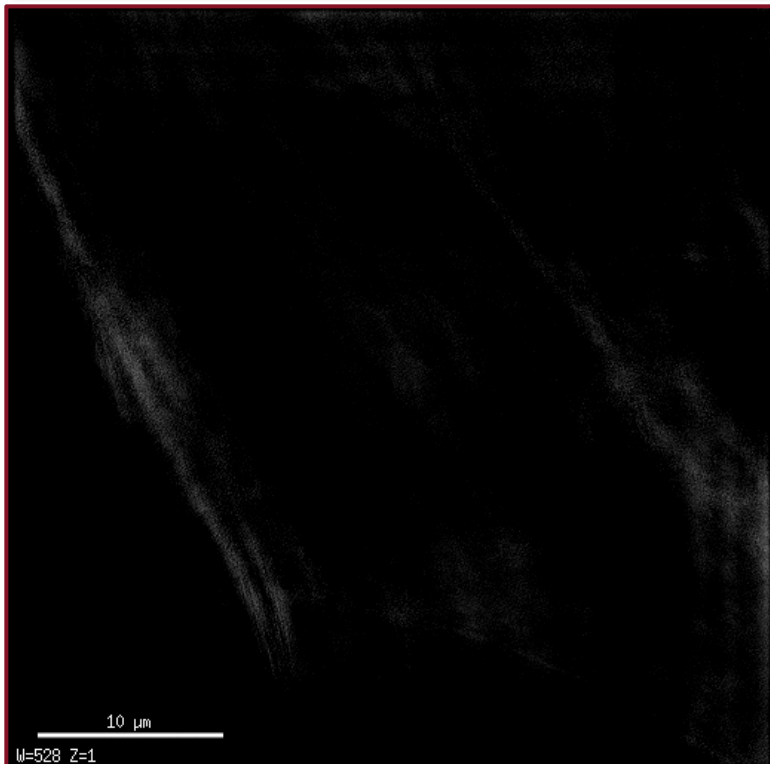


Stack starts around 1500 counts & ends at ~500 (25ms exposure, 15%T)

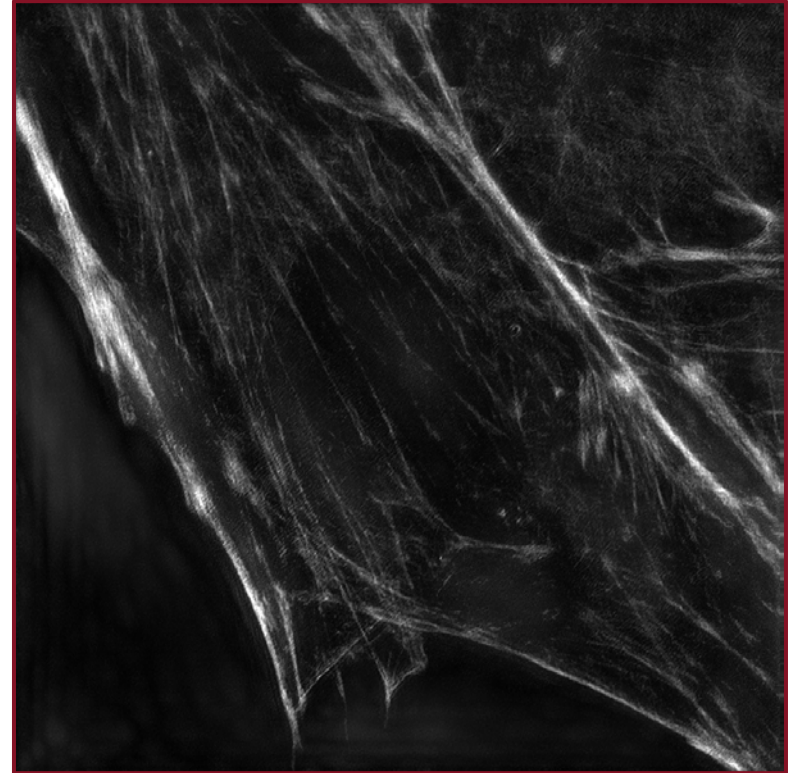
Haloing/Doubling Caused by Photobleaching

Need less than 30% from top to bottom

Z-stack Movie 3D-SIM



Max Intensity Projection

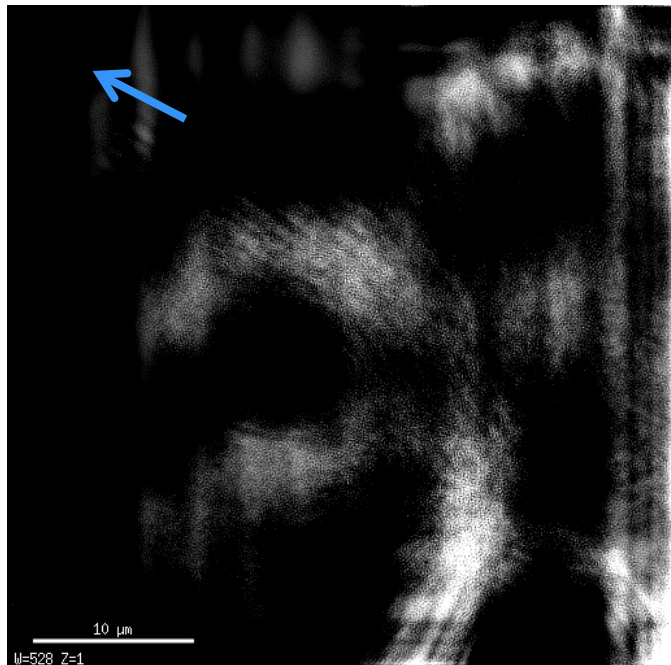


Ensure that bleaching is minimized

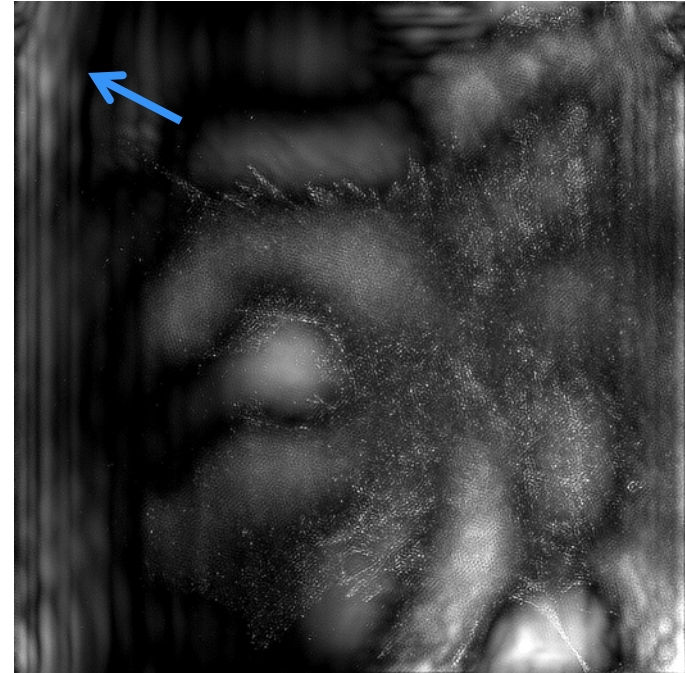
Haloing/Doubling Caused by Photobleaching

In extreme cases will get lines/square edges

Z stack movie



Max Projection

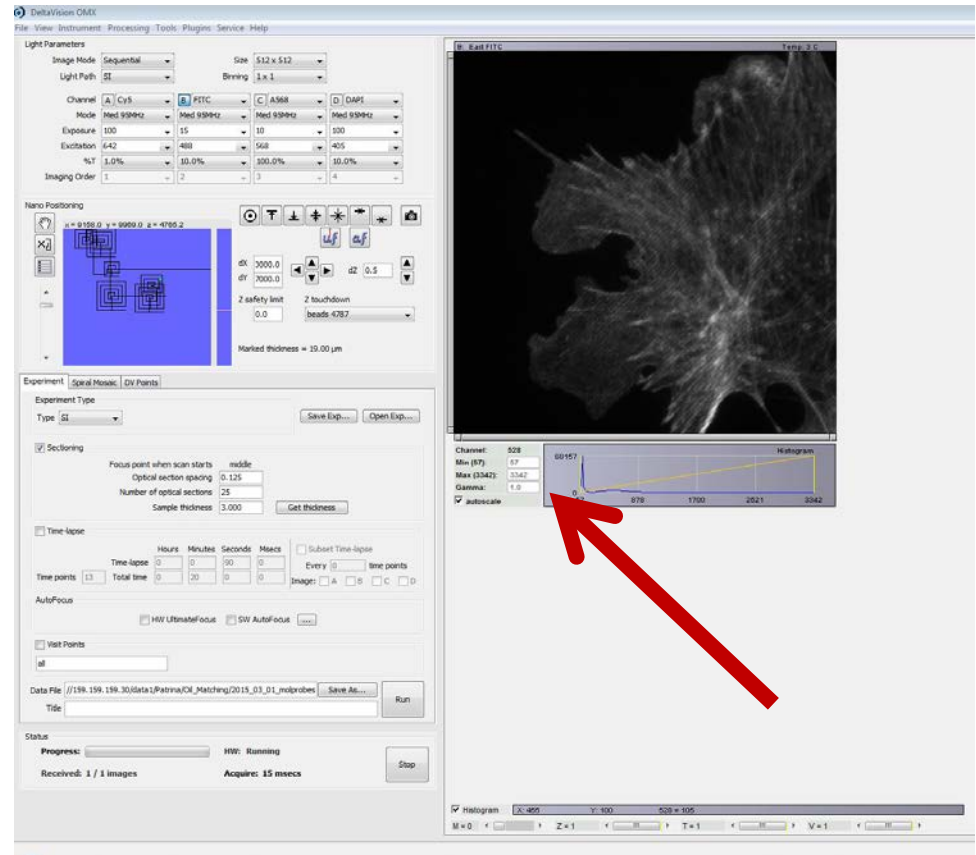


Sample bleached by ~90% while acquiring a 3 μm z stack

Haloing/Doubling Caused by Photobleaching

Pay attention to max counts during acquisition

- Take note of max intensity count before starting experiment
- Watch how values change during acquisition
- Adjust imaging parameters to keep bleaching under 30%

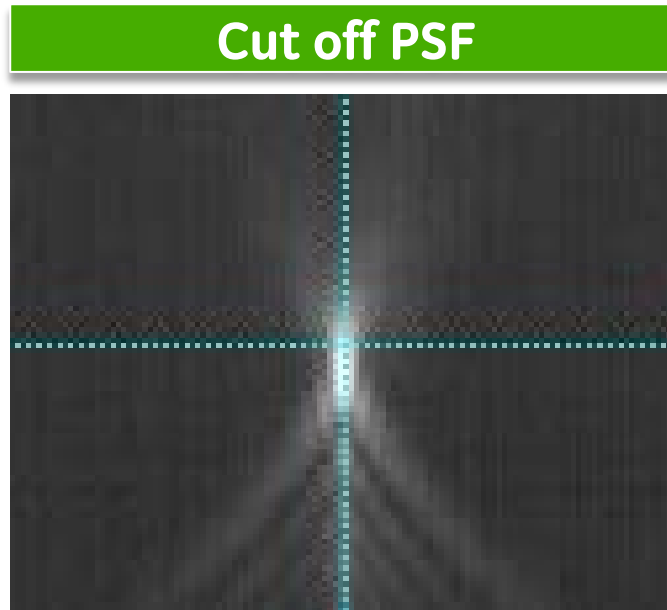


Haloing/Doubling Cause 3: Cutting Structures off



Haloing/Doubling Caused by Cutting Off

Cutting off structures give asymmetrical PSF

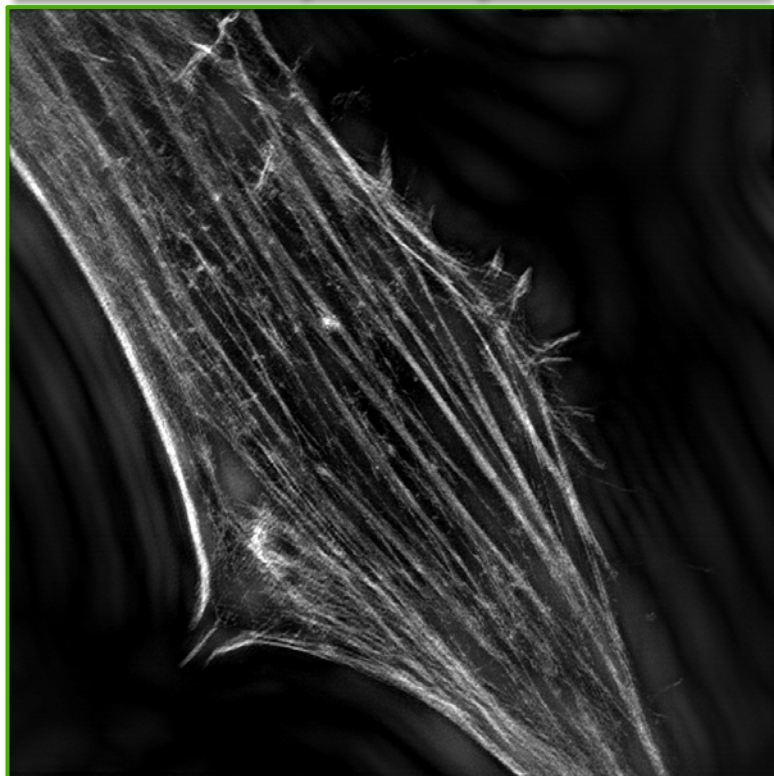


Part of the PSF is lost when cut off a bright structure

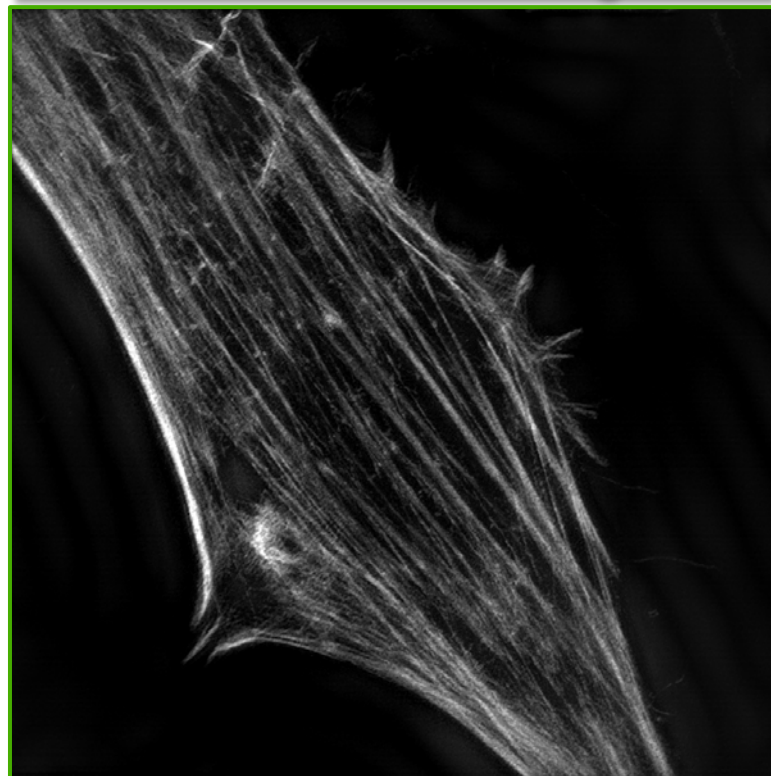
Haloing/Doubling Caused by Cutting Off

Cutting off structures give asymmetrical PSF

Z stack partially cut off



Entire structure imaged



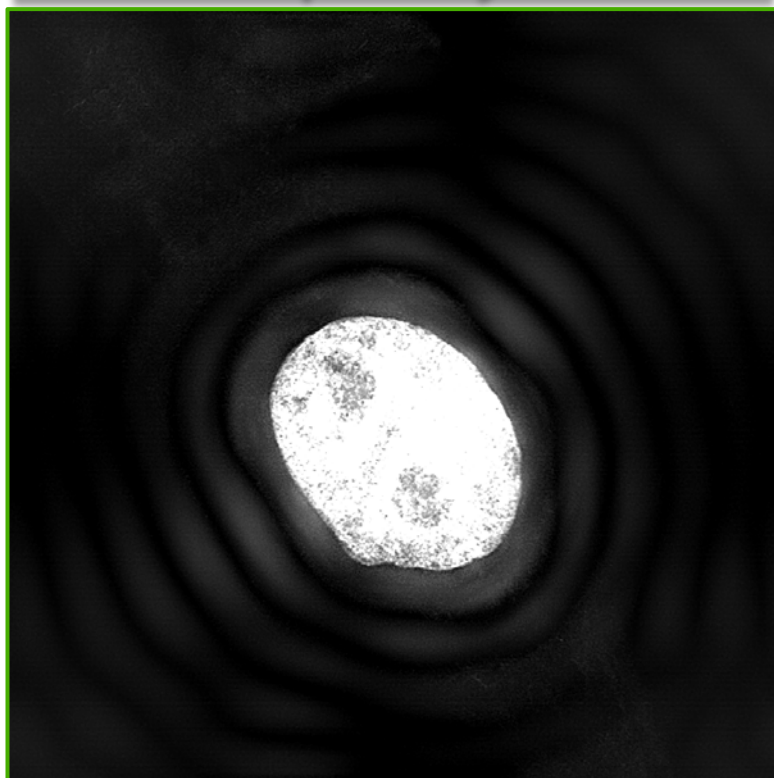
Pro tip: Set up z stack until you can no longer see the stripe pattern



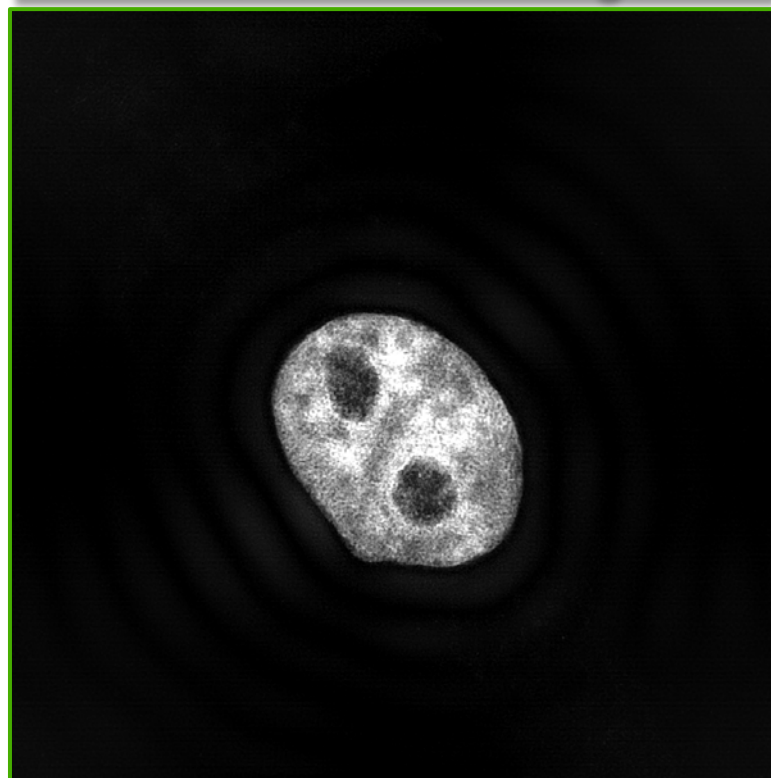
Haloing/Doubling Caused by Cutting Off

Cutting off structures give asymmetrical PSF

Z stack partially cut off



Entire structure imaged



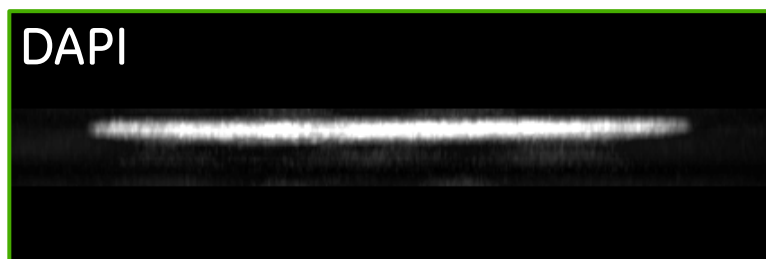
Pro tip: Set up z stack until you can no longer see the stripe pattern



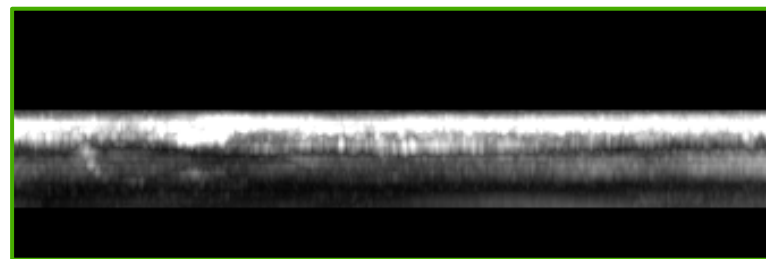
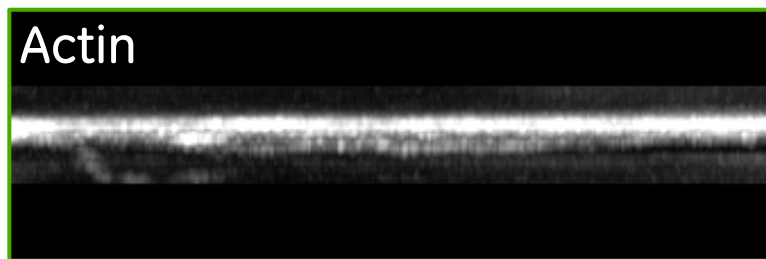
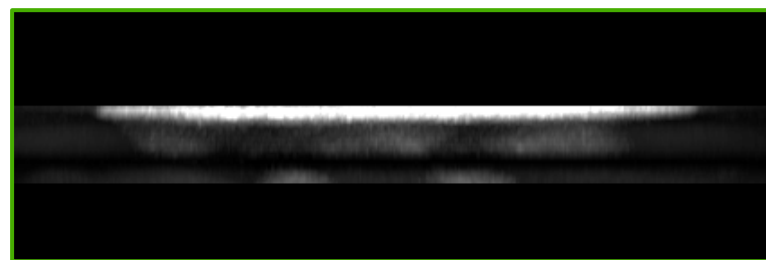
Haloing/Doubling Caused by Cutting Off

Cutting off structures give asymmetrical PSF

Z stack partially cut off



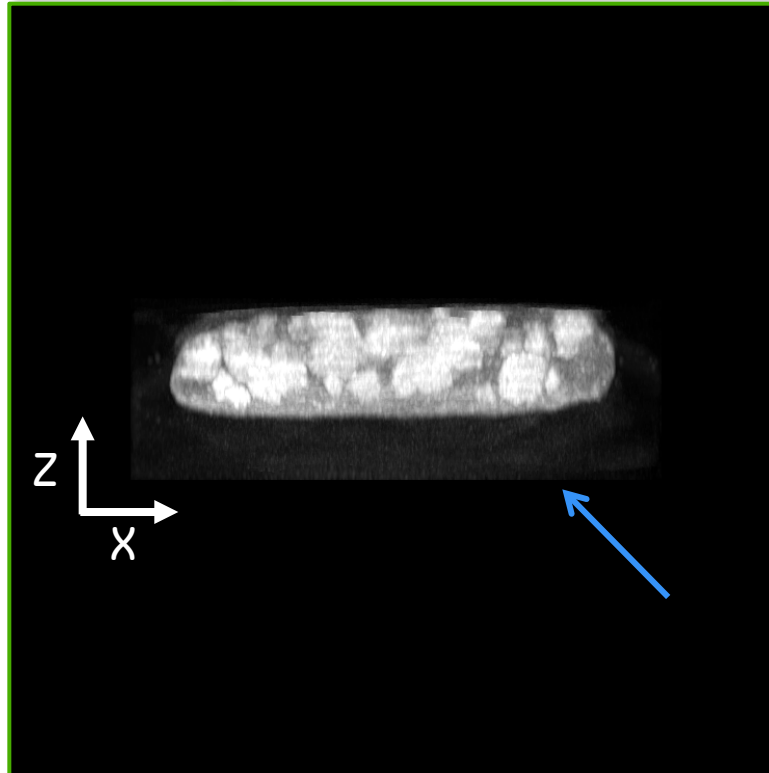
Entire structure imaged



Ensure that entire structure is captured in Z stack

Haloing/Doubling Caused by Cutting Off Image entire structure

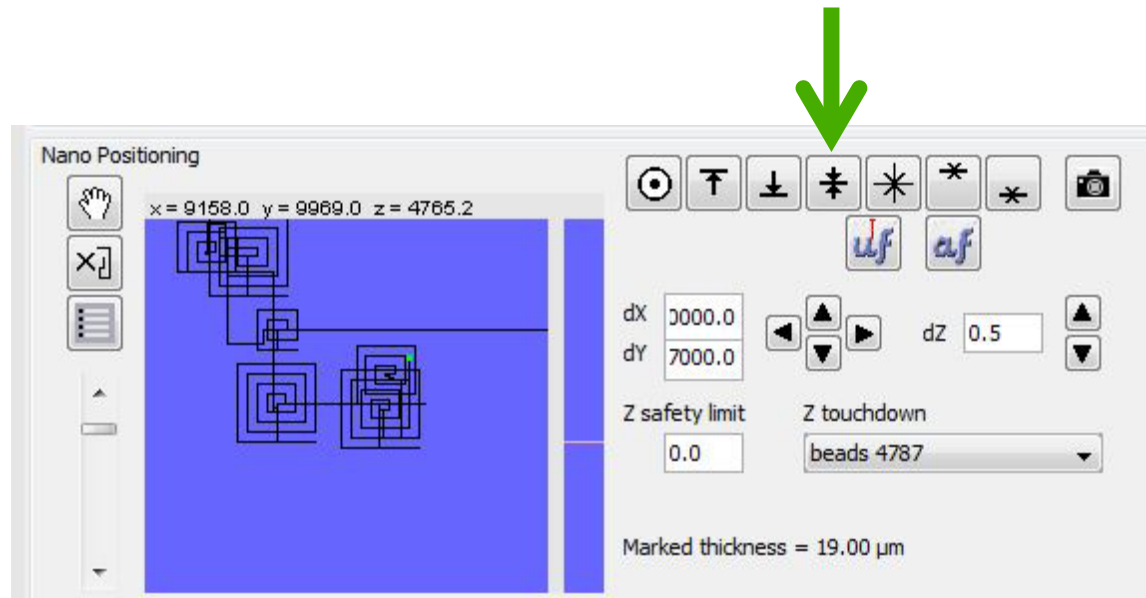
Orthogonal View - Nucleus



Part of nucleus is cut off, resulting in artifacts

Haloing/Doubling Caused by Cutting Off Image entire structure

- Common with DAPI/nuclear labels
- Set Z stack so striped pattern is no longer visible
- Make sure click 'visit middle' button



Common SIM Artifacts: Honeycomb

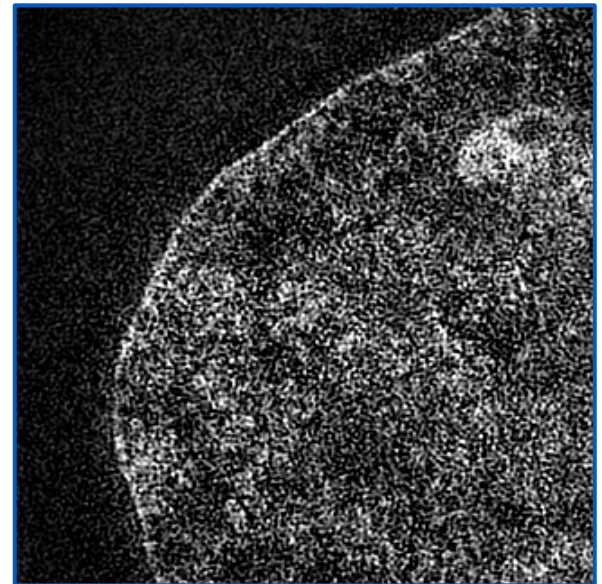


Common SIM Artifacts: Honeycomb

Diffuse labeling & bad S/N gives honeycomb

- Regular/hexagonal pattern
- Common with diffuse labeling or samples with bad S/N
- Caused by:
 - Little to no mid range frequencies to detect (imaging the SIM pattern)
 - Masking of the SIM pattern by high background
 - Going from Fourier space to real space

Typical honeycomb



Common SIM Artifacts: Honeycomb

How to minimize the honeycomb artifact

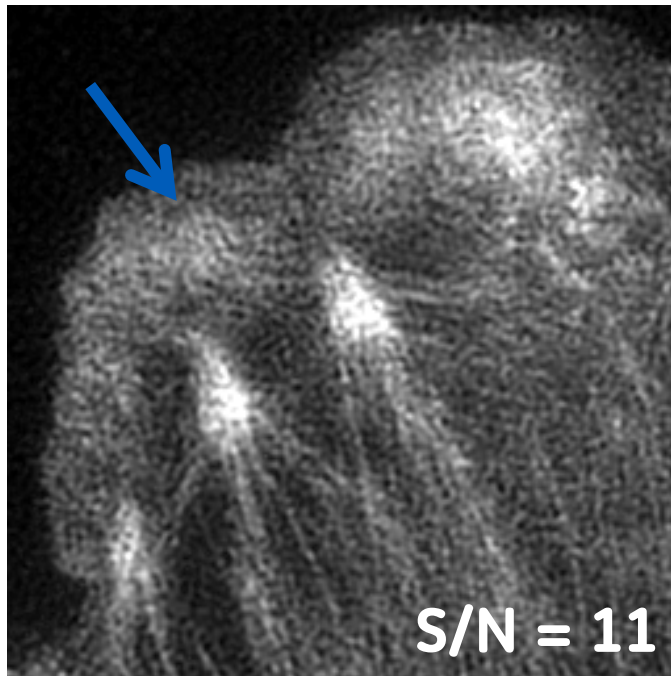
- Sometimes you can't:
 - Not all samples are appropriate for SIM
 - Diffuse labeling & high background will give honeycomb
 - SIM works best with samples that have discrete structures
- Increase S/N without photobleaching
- Increase Wiener filter constant
 - This removes high frequency data
 - Decreases resolution!
- Have good controls



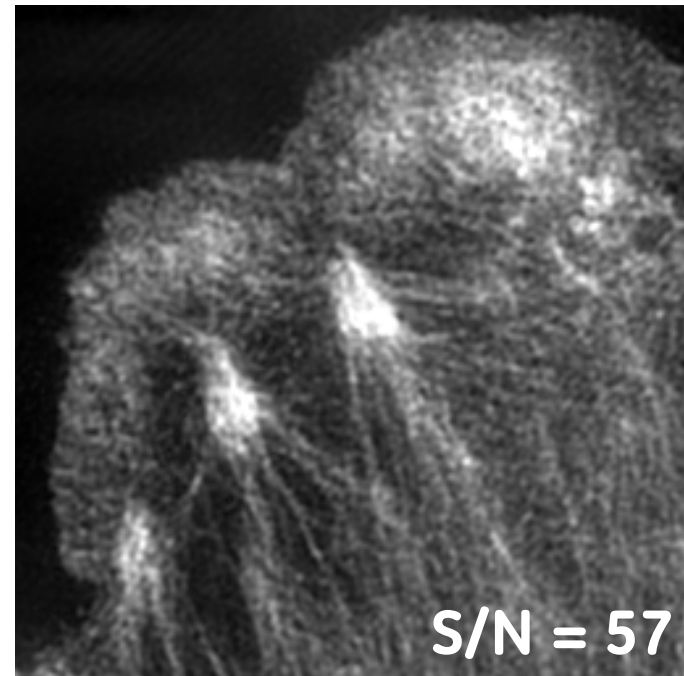
Common SIM Artifacts: Honeycomb

Get best S/N to help minimize honeycomb

Low Signal to Noise



High Signal to Noise

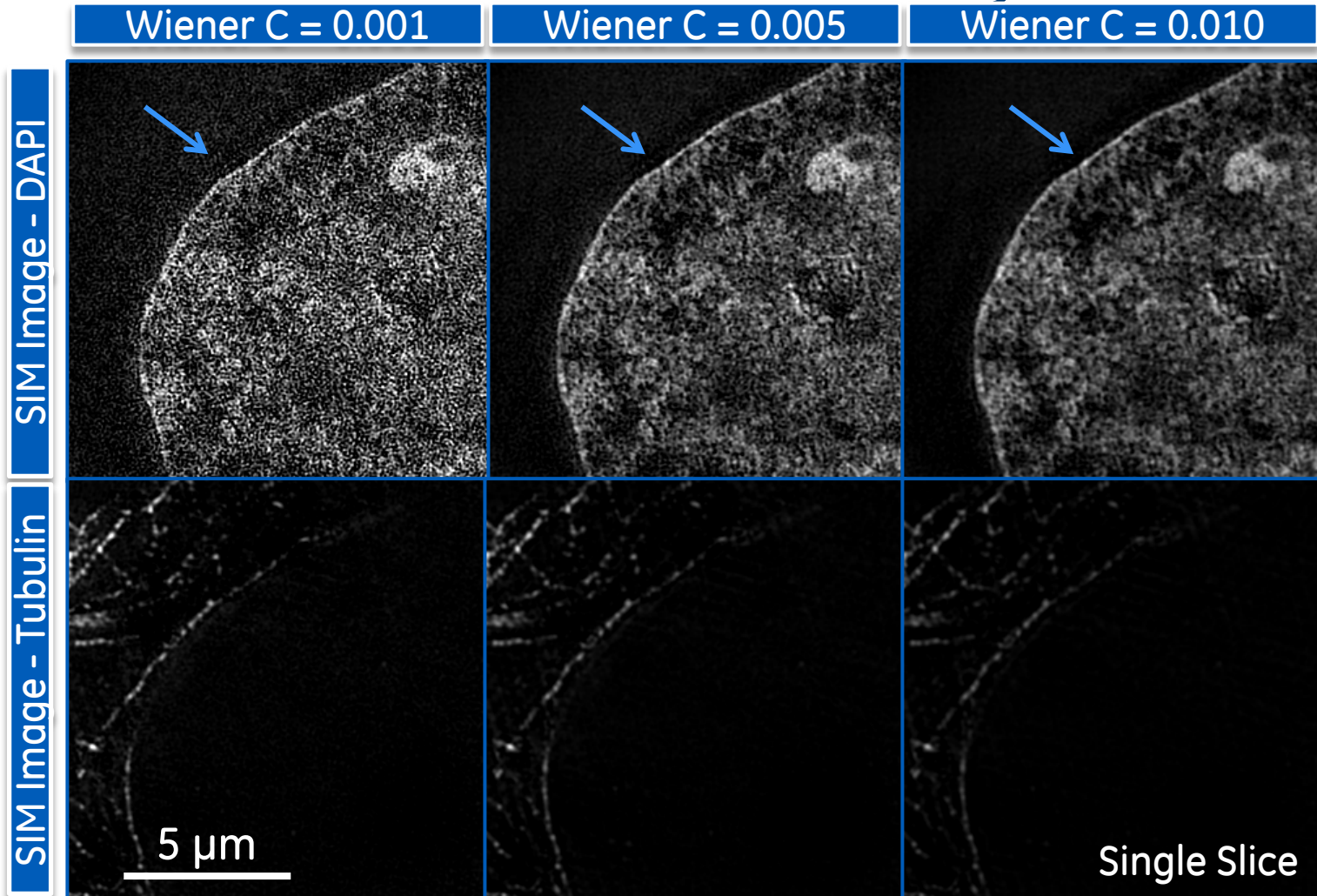


Increase %T or exposure until the background signal starts increasing



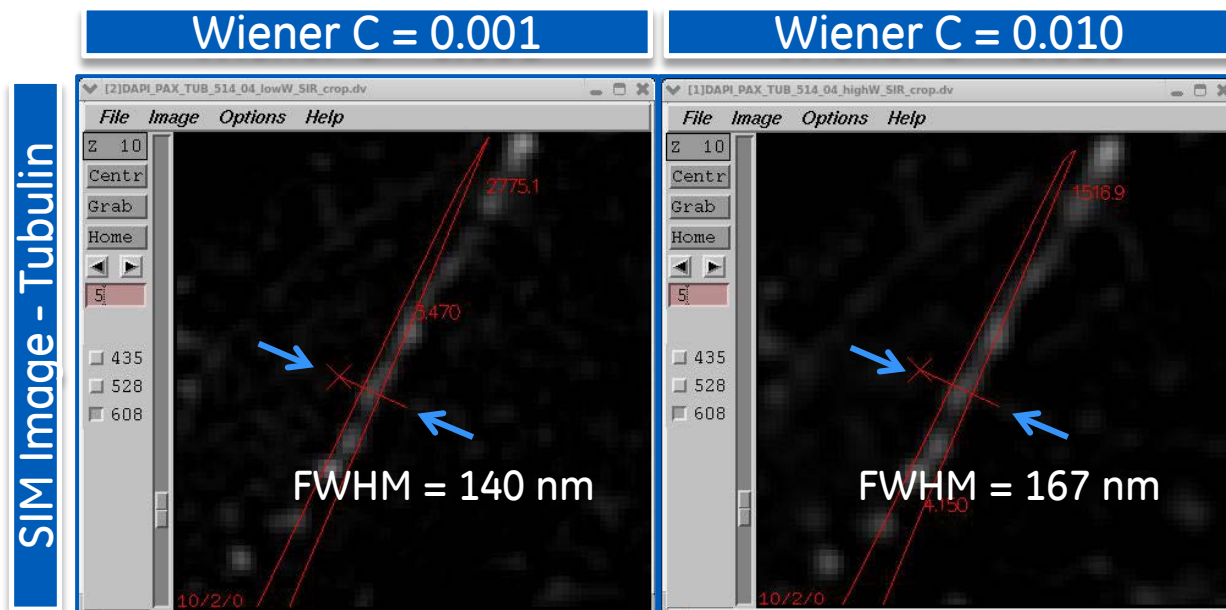
Common SIM Artifacts: Honeycomb

Increase Wiener C to reduce honeycomb



Adjusting the Wiener Filter Constant

Increasing this constant decreases resolution



Reconstructing with the higher Wiener filter constant resulted in a loss of 27nm in resolution!



Common SIM Artifacts: Honeycomb

Affect of using different Wiener Filter Values

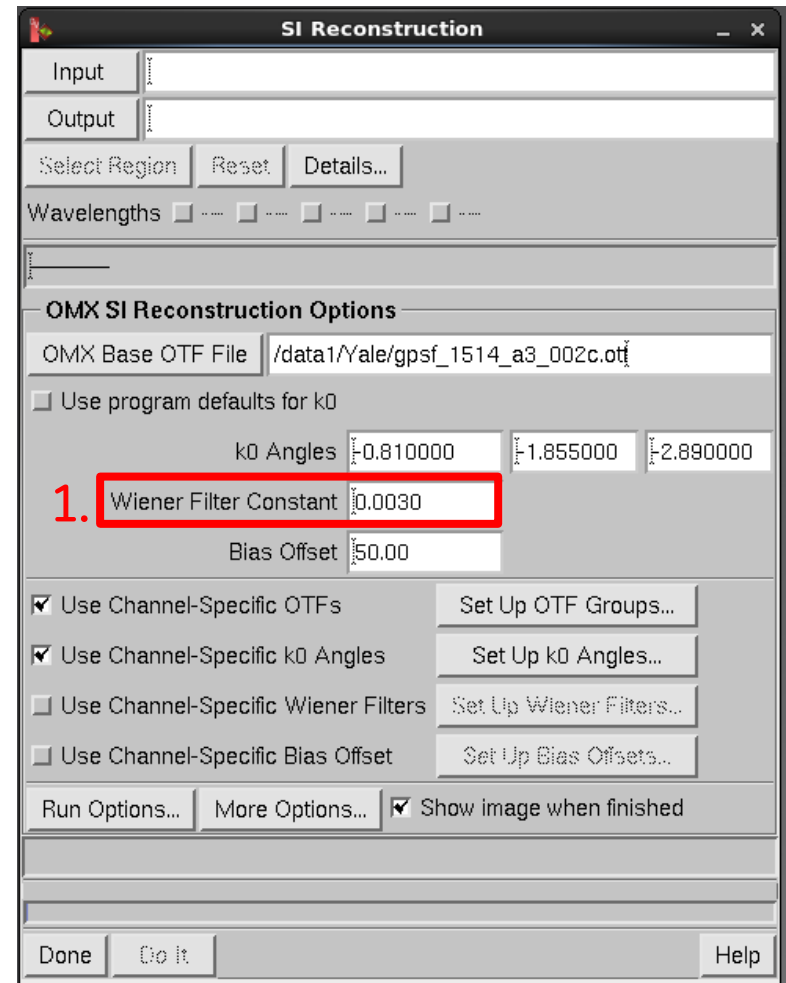
- As this constant is increased:
 - The honeycomb pattern starts to decrease
 - The DAPI signal starts to look smoother and less noisy
- **By increasing this constant you remove high frequency info! You lose resolution by doing this!**
- Typical values are 0.001-0.003
- For diffuse labeling or bad S/N use 0.005 or higher



Adjust to Wiener Filter to Minimize Honeycomb

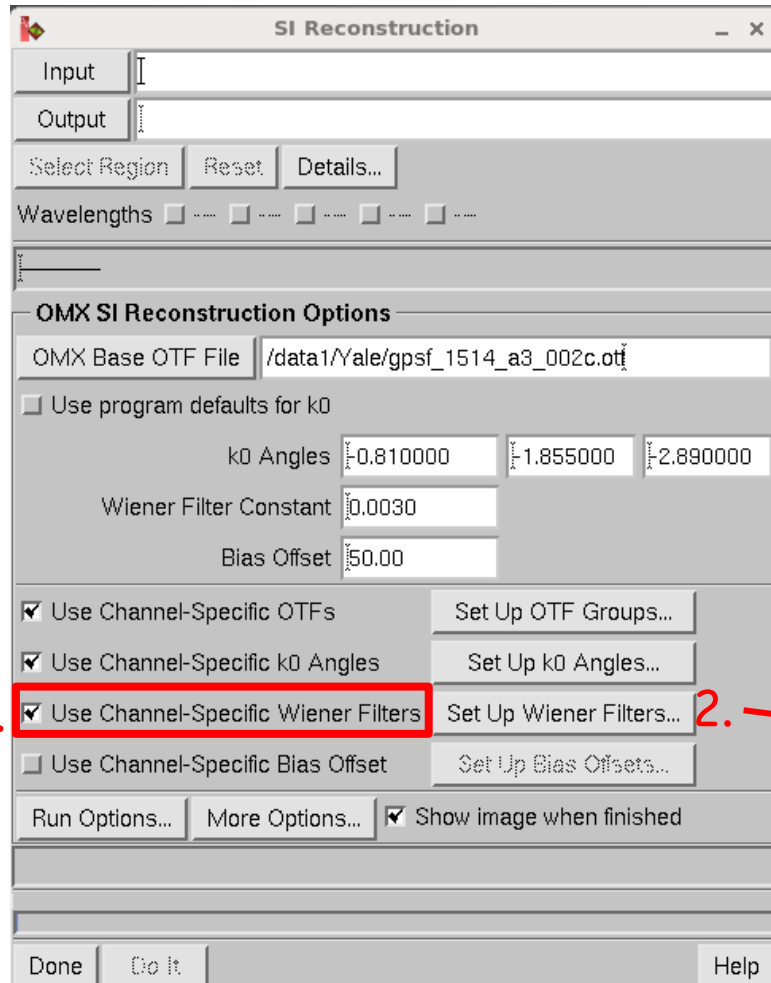
How to adjust the Wiener filter constant

1. In SI reconstruction window adjust values in the 'Wiener Filter Constant' box
2. For data with good signal to noise ratio typical values are 0.001-0.003
3. Adjusting values in this box applies the same Wiener Filter Constant to all channels
4. For structures with diffuse labeling or bad signal to noise, try increasing this value to 0.005 or higher



Adjust to Wiener Filter to Minimize Honeycomb

How to use channel specific Wiener Filters



The SI Reconstruction tool interface shows various input fields and options. A red box highlights the 'Use Channel-Specific Wiener Filters' checkbox, which is checked. A red arrow points from this checkbox to the 'OMX Channel Wiener Filter Options' dialog box.

Input:

Output:

Select Region Reset Details...

Wavelengths ☐ ☐ ☐ ☐ ☐ ☐

OMX SI Reconstruction Options

OMX Base OTF File

☐ Use program defaults for k0

k0 Angles

Wiener Filter Constant

Bias Offset

☒ Use Channel-Specific OTFs Set Up OTF Groups...

☒ Use Channel-Specific k0 Angles Set Up k0 Angles...

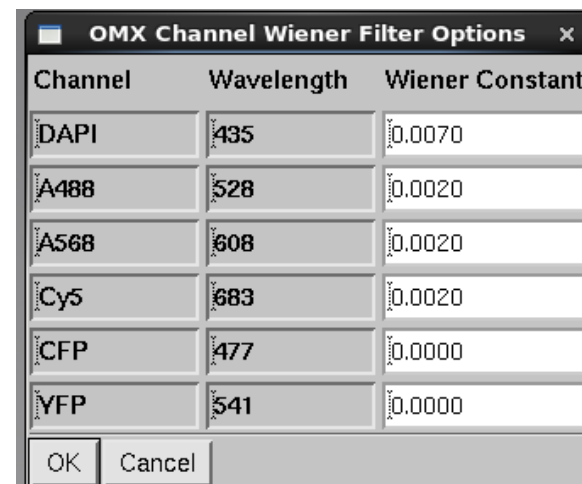
☒ Use Channel-Specific Wiener Filters Set Up Wiener Filters...

☐ Use Channel-Specific Bias Offset Set Up Bias Offsets...

Run Options... More Options... ☒ Show image when finished

Done Do It Help

1. In the SI Reconstruction tool, check the 'Use Channel-Specific Wiener Filters'
2. Click on 'Set Up Wiener Filters' to open the OMX Channel Wiener Filter Options
3. Input Wiener Filter constants for each channel, i.e. higher values for diffuse signals (DAPI) & lower values for other channels



The OMX Channel Wiener Filter Options dialog box shows a table with columns for Channel, Wavelength, and Wiener Constant. The channels listed are DAPI, A488, A568, Cy5, CFP, and YFP. The Wiener Constant values are 0.0070 for DAPI, 0.0020 for A488, A568, and Cy5, 0.0000 for CFP, and 0.0000 for YFP. The dialog box has OK and Cancel buttons.

Channel	Wavelength	Wiener Constant
DAPI	435	0.0070
A488	528	0.0020
A568	608	0.0020
Cy5	683	0.0020
CFP	477	0.0000
YFP	541	0.0000

OK Cancel



Common SIM Artifacts: Honeycomb

How to minimize the honeycomb artifact

- Not possible with all samples!
- Increase signal to noise without photobleaching
- For diffuse labeling increase Wiener filter constant
 - Use 0.001-0.003 for normal samples
 - Use 0.005+ for diffuse labeling (results in a loss of resolution!)
 - **Report this value in the methods section of your paper!**



SIM Summary



Tips for Successful SIM Imaging

Summary

- 3 things to master:
 - 1) Determining the best immersion oil for your sample
 - 2) Adjusting imaging parameters to minimize bleaching
 - 3) Imaging the entire structure
- Get highest signal to noise ratio possible without photobleaching



Have questions? Need help? Need support?
Call or email me!

Patrina.pellett@ge.com

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