Zeiss LSM 900 Confocal Training

Neuroscience Imaging Facility The Ohio State University Rightmire Hall 614-292-1367

Neuroscience Imaging Core

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Access to the Confocal Microscope

	Supervised	Unsupervised		
	Mon-Thurs 9a-5p	Mon-Fri 9a-5p	Evenings and Weekends	Required Experience
Trainee	Х			Seminar & minimum of 2 supervised sessions. Advancement decided on case by case basis
Standard user		х		Seminar & minimum of 10 unsupervised sessions. Advancement decided on case by case basis
Experienced user		Х	Х	Advancement decided on case by case basis

Access to the room

-Key card access. Contact Paula for access.

Scheduling time

-Use Google Calendar. Provide a gmail address to Paula for access.

Contact numbers

Question about how to use the microscope, i.e. software questions -Contact Paula 614-292-1367 9a-5p, M-Th -Zeiss Hotline 800-233-2343 10a-6p, M-F

Physical problem with microscope, i.e., it's broken -Contact Paula 614-292-1367 9a-5p, M-Th

Problem after hours –Contact Paula 614-270-3239

Computer

Data storage

-Archive to an external hard drive USB ports, including USB 3.0

Users

- -All users will have a user name and password
 - User name PI name + lab (eg, "McKinney lab")
 - · Password last five digits of the lab phone number

Rules

- All files must be saved to the user files on the <u>D drive</u>, files stored elsewhere will be deleted
- Files saved to the D drive will be deleted after one week

What is fluorescence?



Excite with high energy photon (shorter wavelength) Emit lower energy photon (higher wavelength)

How does a fluorescence microscope work?



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The principal behind confocal microscopy:

Rejection of out of focus light via the pinhole allows us to capture optical sections.



Limits of Pinhole Rejection

The size of the pinhole is a compromise between thin optical sections and reasonable signal levels.





Laser Scanning Microscopy

The laser scans across the sample in a raster pattern, building the image pixel by pixel.



The Airyscan Principle

Instead of rejecting light at the pinhole, a 32-channel detector collects all light of an Airy pattern. During post-processing, the light in the outer rings of the Airy pattern can be reassigned to the center point.





Benefits of Airyscan

Airyscan can provide up to a 1.7 fold increase in resolution, down to 120nm, as compared to ~250nm for conventional confocal.



Airyscan can improve the signal to noise (SNR) or contrast in an image. Improvements in SNR make it easier to see dim objects and make the background appear darker.



Inside the LSM 900

Our LSM 900 has 3 detectors – two GaAsP and one Airyscan. The Variable Secondary Dichroics (VSDs) allow us to direct the light to any detector, giving us a lot of flexibility in imaging.



Choices

	ę	So m	any c	hoices		
Image Resolution	Switch Mode	Speed	Resolution & Sensitivity	GaAsP 1	Airyscan	GaAsP 2
Confocal	Sequential	++	++	Blue, Green, Red		
Confocal	Semi- Sequential	++++	++	Blue & Red		Green
Confocal	Simultaneous	++++++	++	Blue	Green	Red
SR	Sequential	+	++++		Blue, Green, Red	
SR + Confocal	Sequential	+	++++/++	Blue	Green, Red	

The Microscope



Why use an inverted microscope?

Benefits

- -More flexibility, can image both open and closed chambers
- -Better suited to imaging living cells than upright microscope
- -Can image live cells and organisms in open dishes using high NA objectives
- Can accommodate a variety of different chambers

Cautions

- -Location of objective makes it more possible for liquids (oil, water, media, etc) to find their way inside the objective, possibly rendering it unusable
- -More care must be taken using the immersion objectives
- -Specimens on slides are inverted to take advantage of the best optics and must be sealed to prevent leakage
- -More difficult to apply oil when specimen is in place

Objectives

Objective specifications						
Mag	2.5x	10x	20x	40x Water	63x Water	63x Oil
Numerical aperture	0.085	0.3	0.8	1.1	1.2	1.4
Correction	EC Plan Neofluar	EC Plan Neofluar	Plan Apochromat	LD C- Apochromat	C- Apochromat	Plan Apochromat
Working distance	8.8 mm	5.2 mm	0.55 mm	0.62 mm	0.28 mm	0.19 mm
Resolution (520 nm)	1 µm	1.1 µm	0.40 µm	0.29 µm	0.26 µm	0.23 µm



The water immersion objectives have a correction collar for coverslip thickness. A #1.5 coverslip is nominally 0.17mm, but the range of acceptable thickness is 0.16-0.19mm. It is possible to compensate for this variation in thickness empirically.



PMT

Understanding Objectives

Quality

-Objectives are highest quality = expensive -Image is only as good as the objective

Numerical Aperture (NA)

Measure of the width of the cone of light that the objective can gather

-High NA objectives have:

- •High resolution
- Brighter image
- Shorter working distance
- -NA > 1.0 generally are immersion objectives

Care of Objectives

DO NOT REMOVE OBJECTIVES!

Immersion objectives

- -Use the CORRECT IMMERSION FLUID
- -Use oil on the 63x Oil objective
- -Use water on the 63x Water and the 40x Water objectives

Using Immersion fluid

- -Use what is supplied in the room - do not mix oils
- -Use minimum amount necessary
- -Clean slides after use
- -Use only lens tissue to clean objectives -
- blot, don't rub
- -Always clean objective after use with oil
- -Don't drag dry objectives through the oil on the slide



Specimen Mounting

Fixed Specimen

- -Coverslip #1.5 ~0.17mm
- -Permanent mounting media -Mounting media that does not dry should be sealed with nail polish







Brightfield & DIC

Brightfield

-Available with all objectives -Very low contrast

DIC

- -Available with 20x dry, 40x water, 63x water, and 63x oil objectives
- -Reveals gradients in refractive index, so the resulting image tends to show edges
- -Generates low contrast
- -Better for overlaying with fluorescent images than brightfield





Molecular Biology of the Cell 5/e

Epifluorescence Filter Cubes

Filter Cube	Example Fluorochromes
Blue	Alexa 405, DAPI
Green	Alexa 488, Cy2, Fluoroscein
Red	Alexa 568, Alexa 546, Cy3, Rhodamine



The Confocal Microscope and Workstation Layout



Stage Power supply (top) Microscope power supply (bottom)

-4 lasers

Lasers

Our system has 4 laser lines, allowing us to excite a wide range of fluorochromes.

Laser line	Example fluorochromes	
405	Alexa 405, DAPI	
488	Alexa 488, Cy2, Fluoroscein	
561	Alexa 568,Alexa 546, Cy3, Rhodamine	
640	Alexa 647, Cy5, TOTO-3, TO-PRO-3	

Getting Started

Start up in numerical order. All of the switches are labeled.



Start the Zeiss Confocal Software



Double click on the Zen 3.0 icon.

Software Startup



Stage and Focus Calibration

The system will ask to calibrate the stage and the focus upon software startup. This should be done BEFORE you put a sample on the stage. The objectives will move up and down. The stage will move left /right and front/back. If this step is skipped, the software may not be able to remember stage or focus positions.

Stage/Focus not Calibrate	d
The stage and focus are r It is recommended to per	not yet calibrated. rform an automatic calibration before working with the system
Caution!	
clear of the instrur	ngers and damaging the instrument. Ensure that people stand ment and that the full travel range is not obstructed by any le carrier, stage insert, TL condensor or other special device
clear of the instrur objects (e.g. sampl	ment and that the full travel range is not obstructed by any le carrier, stage insert, TL condensor or other special device

When the stage and focus are calibrated, the "Calibrate" buttons will be greyed out.

Microscope	Marks
Stage Show All	- 🛇 Focus - Show All 📝
A Caution! Risk of Crushing	A Caution! Risk of Crushing
Stop	Current 50.00 µm 🐑 <u>50.00 µm</u> 🕄 Stop
X-Position 41124 μm 🗧 41124 μm	✓ Handwheel on
Y-Position 37148 μm 🗘 37148 μm	Step Size 0.10 µm 🗘
Speed 100 %	Home Work Load
Acceleration 100 %	Measure
X/Y Position Set Zero Calibrate	Distance 3.85 µm Reset
Marks	Z-Position Set Zero Calibrate
Show All	

The Locate Tab

The Locate tab contains presets for viewing your sample thru the eyepieces. You can shutter the light and change filter cubes here.

	1 3.0 (ZEI	N system)						
File	Edit	View	Acquisition	Graphics	Tools	Window	Help	
6								
Loc	_	Acqui	sition Proce		nalysis			
Transn	nitted	Light	Off On	Reflec	cted Ligh	nt Off	On	
Switch	То	⊁ Fluo	rescence	⊁ Transmis	sion			
Favori	tes 🕻	Configu	re					
Brig	htfield	C	API	GFP	DsRed	All C	Off	
	IC II	D	IC III					

Acquisition Tab



Image Capture Settings - Channels



Choose which channels to capture.

Adjust the laser power, master gain, digital offset and pinhole size.

Image Capture Settings – Acquisition Mode

👻 🕿 Acquisit	ion Mode	🗸 Show All 🛛 📝
LSM		
	rame	Line
Crop Area	۹.1	3.0 x 1
Scan Area		
Jean Area		
Image Size:	33.8 µm × 33.8 µm	Pixel Size: 0.05 µm
Frame Size	715 px 🛟 × 7	15 px 🛟 Presets 🔻
Sampling		Confocal
Frame Time:	5.82 s	Pixel Time: 0.61 µs
Scan Speed	0. .	10 🗘 Max
Direction		>
Correction	Auto	
	Correction X	0.00 * :
	Correction Y	0.00 * :
Line Step	1	
Averaging	None 2x	4x 8x 16x
Mode	Repeat per Line	Repeat per Fra
Method	Mean Intensity	Sum Intensity
Bits per Pixel	8	16

Here is where you can set the imaging parameters – zoom, number of pixels, scan speed, averaging, and bits per pixel.

Flexible Layout

Zen has a flexible layout. Dialogs can be "popped" out and moved around. Whole sections of the software can be hidden. The entire workspace can be zoomed in or out.



Microscope Controls - Objectives

The objectives can be changed from the software. Upon selecting an objective button, the objectives will lower, change position, and raise up to the working position.



Microscope Controls - Stage



The stage can be moved in this menu. This is also where stage positions can be memorized and revisited.

Microscope Controls - Focus



The focus can be changed in the Focus dialog. The objectives can be completely lowered with the "Load" button

Preview Window

Your images show up in this window. Tabs on the side of window show different views, including single channels, orthogonal views, and image information.



Display Settings

The histogram of pixel intensities is located here. You can change how the image looks on the screen – brightness, zoom, z-slices.



Capture a Confocal Image



Load settings from template

Choose the gear icon and "New from Template". Load the "Confocal405_488_561_640_Frame" setting.

ZEN 3.0 (ZEN system)	
File Edit View Acquisition Graphics Tools Win	Vindow Help
Co	*
Confocal_4_color *	🔹 🐲 🔹 Acquisition Parameter
	New Show All
* Smart Setup	New from Template AirySR405_488_561_647_Frame
AF C Continuous	Rename Confocal405_488_561_640_FRAME Sna Save Save Acquisition Reload Acquisition State State State State </td
Experiment Regions Experiment Designer Auto Save Automated Image E Bleaching Automation	Delete
E Imaging Setup ✓Show Far Red Red Green +LSM Conf Confocal Confocal @	how All 🗷

You will need to save the setting with a new name. This will only show up under your login.

If you have previous images with settings that you want to use again, you can load the image and choose the "Reuse" button.

	1		
TEN 3.0 (ZEN system)			
File Edit View Acquisition Graphics Tools	Window Help		
🖻 🖩 📖 🗡			
	Ì		
Locate Acquisition Processing Analysis	<u> </u>		
	8 ×	a Acquisition Parameter	
The file name must be at least one character long!		🔿 🛥 Acquisition Mode	Show All
✗ Smart Setup		Channels	🗸 Show All 🛛 🗹
		Focus Strategy	🗸 Show All 🛛 📝
AF I I I I I I I I I I I I I I I I I I I	ous Snap	Software Autofocus	🗸 Show All 🛛 📝
7-Stack	us snap	 Multidimensional Acquisition 	

Select channels to image

🕩 🛥 Acquisition M	۸ode			✓ Show /	All 🛃
👻 丛 Channels				✓ Show /	
Far Red Conf	ocal	AF647		Ref.	
Red Conf	ocal	AF568			
Green Conf	ocal	AF488			
DAPI Conf		AF405			
~ ^ +	🛱 Foc	us Ref.			* -
🗏 High Intensity	Laser R	lange			
Red					
Lasers	4 0	5 🔲 488	☑ 561	640	
561 nm	0			0.03 %	
Pinhole	-0			28 µm	
1.00 Airy Units ≜	1.2 μm	section		1 AU	Max
Alexa Fluor 568		-	-	_	
Master Gain	_		-0	745 V	
Digital Offset	-			∩ − [o	•
Digital Gain	-0			1.0	
	-				
Display Setting	Defa	ult			
O Focus Strated	av			Show /	
Software Aut				✓ Show /	

Open the "Channels" dialog and check the boxes next to the fluorochromes you want to image. I've chosen red and green here.

Optimize the channel settings

You will need to adjust the gain and laser power for each channel. To start:

1 – Choose a channel. Green is selected here. It appears as a lighter grey than the other channels.

2 – Choose the "1 AU" button. This will set the pinhole size to 1 Airy Unit for optimal confocality. If the button is blue, that setting will be maintained until you change it.

3 – Choose the "Live" button. This will scan with the selected channel (green in this case) with a 512x512 pixel resolution and at the highest scan speed.





The live image. It will be in the same orientation as thru the eyepieces.

Range Indicator



Click the "Range Indicator" checkbox to change the display so that pixels of maximum value are red and pixels of minimum value are blue.

A good target is to use the full range – have a few pixels of blue and and few of red.

If you will be measuring pixel intensities, there should be no red pixels in areas that will be analyzed.



!!! Be careful !!!

Make sure to choose the "Reset" button to display the full range. Adjusting the brightness will give a false sense of saturation!

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Adjust the Master Gain and Laser Power

🕩 🛥 Acquisition	Mode			🗸 Show	All 🛃
A Channels				🖌 Show	All 🛃
Far Red Con	focal	AF647		Ref	
Red Con	focal				
Green Con	focal				
DAPI Con	focal	AF405			
× ^ +	Foci	us Ref.			* •
Lasers 488 nm Pinhole 1.00 Airy Units ≙	-0- -0	section		640 - 0.1 % - 25 μm	
Alexa Fluor 488 Master Gain Digital Offset Digital Gain	-		0	- 750 V - 0 - 1.0	•
Display Setting	Defa	ult			•

Adjust the Master Gain up to 800, then adjust the laser power so that histogram fills up the full 16 bit range and there are a few red pixels in the "Range Indicator" view.

If you will be measuring pixel intensities, there should no red pixels.

If you need more laser power, click the "High Intensity Laser Range". This allows you to use higher laser powers. It changes ALL the channels to the higher laser range.



The adjusted image. The histogram is filling the range without a peak at the maximum values (right hand side).

Repeat these steps for all the channels.

Detectors are Sensitive!



The detectors can be damaged by high intensity light. They will shut off if the brightness of you sample exceeds a certain range. The image will be black and there will be a message that the detectors are switched off.

# Acquisition Parameter				
🕨 🛥 Acquisition Mode 🛛 🗸 Show All 🛃				
- 🔺 Channels	Show All			
Far Red Confocal AF647	Ref. 📕 🔻			
Red Confocal AF568	<u> </u>			
Green Confocal AF488				
DAPI Confocal AF405	• ▼			
High Intensity Laser Range				
Far Red				
Lasers 405 488 561	640			
640 nm	0.01 %			
Pinhole -0	31 µm 🛟			
1.00 Airy Units ≜ 1.4 µm section	1 AU Max			
Alexa Fluor 647				
Master Gain	654 V 🛟			
Digital Offset	0			
Digital Gain	1.0			
Display Setting Default				
Focus Strategy	Show All			
Software Autofocus	Show All			

To adjust the setting so that the image is not too bright:

- 1. Stop the scanning.
- 2. Reduce the Master Gain and Laser Power to minimum levels
- 3. Choose Live to start scanning

While watching the histogram:

4. Slowly bring up the Master Gain (up to 800) and adjust the Laser Power

so the histogram is full and there are a few red pixels and a few blue pixels in the Range Indicator image.

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The adjusted image. Notice how the histogram fills the full range.

Acquisition Mode



These are the scanning parameters for your image.

Scan Area

Choosing "Scan Area" allows you to rotate the scanning field. "Crop Area" is zoom. Choose the "1" button to go back to a zoom of 1.



Averaging and Bits per Pixel

You can choose to average several frames or several lines. This will result in a smoother looking image.

The detectors are monochrome detectors. The confocal settings default to 16 bits per pixel. This is the number of levels of grey. 8-bit images measure black to white as 0-255 and 16-bit images measure the same black to white as 0-65,535. A 16-bit will give you more control over brightness & contrast. If you are measuring intensities, a 16-bit image will allow you measure smaller differences. 16-bit images use a little more hard drive space, but are worth it.



Image Size, Scan Speed & Direction

You can choose the number of pixels in your image. There are presets (up to 6144x6144), but you are not limited to those. Selecting the "Confocal" button will fill in a frame size with optimal pixel sampling for confocal resolution.

You can change the Scan Speed. It will indicate how long a frame will take. Reducing the scan speed will increase the signa, I as well as increase photobleaching, but can have the same smoothing effect as averaging.

Direction is how the scanner captures signal. It can either capture signal unidirectionally (only from left to right on the scan) or bidirectionally (from both left to right and right to left). Bidirectionally is recommended for speed.

Image Size:	319.5 μm × 319.5 μm Pixel Size: 0.15 μm
Frame Size	2146 px 🗘 × 2146 px ♀ Presets ▼
Sampling	1.0 x Confocal
Frame Time:	5.31 s Pixel Time: 0.49 μs
Scan Speed	
║───	
Direction	

Correction & Line Step

Please don't use Correction or Line Step. We've chosen settings that automatically do these functions. Adjusting them in this dialog will fight against the automatic processes.



Continuous Button

Choosing the "Continuous" button will scan all of the channels with the number of pixels in the final image. It will continue scanning until it is stopped.



Snap Button

Choosing the "Snap" button will capture one image with all of the channels with all of the pixels in the final image. This is your final image.





Saving Images

Images will show up in the "Images and Documents" dialog.Right-clicking on the image name will give many options. Choosing the diskette icon will allow you to save the file.

Images are saved as .czi files. These are Zeiss proprietary files. They can be opened in Zen and ImageJ (with the Bio-Formats Importer). Zeiss offers a light version of Zen for free. Images can be converted to .tiff files (or other formats) in Zen, Zen Lite and ImageJ/FIJI.

Images and Documents		
Container 1		
	C:\Useive.czi $\star \star \star \star \star$	
	512 kB / 1.74 MB	
	C:\Us61.czi*	
	C 🗖	
	17.57 MB / 18.63 MB 🏼 🌛	
	C:\Us34.czi* ★★★★★	
	С 🗆	
	17.57 MB / 18.63 MB 🏼 🍠	
🔚 🖙 ×		
Save Acquistion Settings

The settings used to acquire your image can be saved. Click on the gear icon to see the menu. These settings will only be seen under your login, but it very simple to share them with another user.

If anything has changed about the setting since last saved, there will be an asterisk next to the name of the setting.

Locate	Acquisition	Tocessing	Analysis				
Confocal_4_c	olor *				* •	" Acquisition Par	amet
* Smart Setup						from Template	•
AF		[]	<u></u>	6	Rena	ime	
Find Focus	Set Exposure	Live	Continuous	Snaj	Save		
					Save	As)-
Z-Stack					Relo	ad	
Time Series	5				Set A	As Startup Default	
					Impo	ort	
			Start Ex	kperimen	Ехро	ort	
					Dele	te	
Experiment	t Regions	🗌 Exp	xperiment Designer				
Auto Save		Aut	omated Image	Export			

Capture an Airyscan Image

Capturing an Airyscan image starts by loading Airyscan settings. Load the "AirySR405_488_561_640_Frame" setting and save it.

488_561_640				* Acquisition Para			
			-	New	•		Show All
∦ Smart Setup			🎄 R	New from Template	A	irySR405_488_561_647_	Frame
AF 🙆	()	1	Ó	Rename	C	onfocal405_488_561_64	0_FRAME
Find Focus Set Exposure	Live	Continuous	Sna	Save		AF568	Ref. 📕 🔻
Z-Stack				Save As Set As Startup Default	l Foi	AF488 cus Ref.	<mark>□</mark> ▼ * ▼
Time Series		-	>	Import Export	er f	Range	
		Start Exp	perimer	Delete			

Choose Airyscan Channels

The steps for optimizing the settings are the similar, but the dialogs look a little different. You will still choose which channels to capture in the "Channels" dialog.

Notice that there is no option for the size of the pinhole. Digital Offset is also gone.

🕨 🛥 Acquisition Mo	✓s	ihow All			
Channels				√ S	ihow All 🛛 🗹
✓ 405 SR		DAPI			Ref. 🗖 🔻
✓ 488 SR		AF48	8		
✓ 561 SR		AF56	8		— —
✓ 640 SR		AF64	7		
× × + 🖮	Foc	us Ref.			* -
 High Intensity La 640 Lasers 640 nm 	aser R		488 🔲 561	✓ 640 —0 <u>5.0</u>	
Alexa Fluor 647 Master Gain Digital Gain Display Setting	 Defa	ult	0	80	

Airyscan Acquisition Mode

The minimum zoom for Airyscan images is 1.3 x.

Choose the "SR" button to choose a number of pixels that is optimal for Airyscan. There is still the option to use few pixels, however, you will sacrifice the increase in resolution.

Airyscan setting default to 8 bit. This is due to how the images are processed. Using 8 bit reduces the chances of having a saturated image.

LSM Crop Area Scan Area Image Size: 78.0 µm × 78.0 µm Pixel Size: 0.04 µm Frame Size 2210 px * 2210 px Presets * Sampling 2.0 x SR Confocal Frame Time: 21.88 s Pixel Time: 0.96 µs Scan Speed	👻 🛥 Acquisit	ion Mode	🗸 Show All 🛛 📝
 Scan Area Image Size: 78.0 μm × 78.0 μm Pixel Size: 0.04 μm Frame Size 2210 px * 2210 px Presets Sampling 2.0 x SR Confocal Frame Time: 21.88 s Pixel Time: 0.96 μs Scan Speed S	LSM		
Frame Size 2210 px → × 2210 px → Presets ▼ Sampling 2.0 x SR Confocal Frame Time: 21.88 s Pixel Time: 0.96 µs Scan Speed 6 Max Direction Auto Correction X 0.00 ↔ Correction Y 0.00 ↔		۹ ()	- <u>1.3 x</u> 1
Sampling 2.0 x SR Confocal Frame Time: 21.88 s Pixel Time: 0.96 µs Scan Speed 6 Max Direction Auto Correction X 0.00° ↓ Correction Y 0.00° ↓ Averaging None 2x 4x 8x 16x	Image Size:	78.0 μm × 78.0 μm l	Pixel Size: 0.04 μm
Frame Time: 21.88 s Pixel Time: $0.96 \ \mu s$ Scan Speed 6 Max Direction Auto Correction X 0.00° Correction Y 0.00° Averaging None 2x 4x 8x 16x	Frame Size	2210 px 🛟 × 2210 px	Presets 🔻
Scan Speed 6 Max Direction Auto Correction X 0.00°; Correction Y 0.00°; Averaging None 2x 4x 8x 16x	Sampling	2.0 x	SR Confocal
Direction Correction Auto Correction X 0.00° Correction Y 0.00° Averaging None 2x 4x 8x 16x	Frame Time:	21.88 s P	ixel Time: 0.96 µs
Correction Auto Correction X 0.00° Correction Y 0.00° Averaging None 2x 4x 8x 16x	Scan Speed	· · · · · · 6	Aax Max
Correction X 0.00° Correction Y 0.00° Averaging None 2x 4x 8x 16x	Direction		
Correction Y 0.00 ° ¢	Correction	Auto	
Averaging None 2x 4x 8x 16x		Correction X	0.00 ° 🗘
		Correction Y	— 0.00 ° 🗘
Bits per Pixel 8 16	Averaging	None 2x 4x	8x 16x
	Bits per Pixel	8	16

The software will let you know if Airyscan is configured optimally. If it is not, it will tell you what is missing. Airyscan is optimized for the 63x, but it is possible to capture Airyscan images on lower magnifications, however, the gains in resolution will be less.

	614.83 MB Start Experiment
Airyscan Acquisition is c	optimally configured.
Experiment Regions	Experiment Designer
🗌 Auto Save	Automated Image Export
Bleaching	Automation

Optimize the Channel Settings

Just like for a confocal image, you'll choose the "Live" button to see an image of your sample.



trat	Channels		Chow All	Show All
e A				
	405			Ref. 🗖 🔻
nen	488	SR	AF488	•
en	561	SR		-
	640	SR	AF647	•
	~ ^	+ 🛈 Foo	us Ref.	* ▼
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	Display Set	ting Defa		

Adjust the Master Gain (up to 900) and the laser power until the pixel intensities fill approximately half of the 8 bit range. Sometimes an image will show up as black on the screen. Remember to check the histogram. Here there is signal in the histogram, so the image only looks black because the brightness is set incorrectly.



Use the "Best Fit" or "Min/Max" buttons to adjust the scaling in order to see the image.





This image has been adjusted so that the histogram takes up about half of the range

Airyscan Detector Adjustment

When using an Airyscan setting there will be a honeycomb icon at the bottom of the software.



Open the dialog and focus on your sample. If the brightest spot is not in the middle hexagon, use the adjustments to make sure it is in the middle. The sample must be in focus for this to be effective.



Process the Airyscan Image

Airyscan images require post-processing to reassign the pixel values.

After capturing an Airyscan image, the software will automatically do this processing and display the image as a super resolution ("SR") image. If you would like to see the raw data, choose the "Sum Channel" button.

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Dimensions Graph	ics Custom Graphics Airyscan	- Displa
Display Mode	Sum Channel SR	Histo
Super Resolution	7.00 🗘 🖌 Auto Filter	□ A
Process	Current Image (2D) Create Processed Image(s)	
	43 Neuroscience l	maging Co

10/3/2019

The processed image is only displayed on the screen. To save the processed image, you can choose the "Create Processed Image" button. This will create a new file in the file manager.



Airyscan Processing Tab

The processing that happens in Display Controls uses auto settings. If you would like to have more control over the settings that are used for the processing, go to the Processing Tab and choose "Airyscan Processing" for the Method.

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Method			_	
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In the Parameters dialog, unclick "Auto Filter" and you will have lots of options. You can adjust the strength of the processing based on the channel. Stronger processing might give a more artifactual look to the image, while weaker processing will give a "softer" image.

" Method Parameters	
✓ Parameters ✓ Show All	
Settings	
✓ 2D SR Processing	
Auto Filter	
🗹 Adjust per Channel	
AF488-T2	
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	4

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When you have chosen all the settings, choose the "Apply" button to create new files.

Capture a transmitted light image



To capture a transmitted light image, first make sure all of the settings on the microscope are correct. Then choose a channel in the channels dialog (here I've chosen green). At the bottom of the Imaging Setup dialog, you will see a "T-PMT" option. Click the checkbox next to it to make it active.

Now there is an extra line in the green channel, labelled "T-PMT". This is the transmitted light PMT.

🕨 🛥 Acqui	🕨 🛥 Acquisition Mode 🛛 🗸 Show All 📝					
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The T-PMT also has independent Master Gain, Digital Offset and Digital Gain. Adjust the Master Gain of the transmitted light channel until the image looks good.

Make sure that the image is not saturated by checking the histogram.

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e	
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: Picti	User: APEER Offline

Adding a Scale Bar

Once you have captured an image, you can add a scale bar, by choosing the "Graphics" tab in the Display Controls area.

Choose the ruler icon and a scale bar will be displayed on your image. It is possible to change the length, font, color, etc.

Zen saves this scale bar as a different layer, rather than making it a permanent part of the image. If you would like to show it on exported images, there is an option to "burn in graphics" in the Processing Tab

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Image: Second	Keep Tool H Auto Color Snap to Pixel
Layers Annotations/Measurements	Dimension
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💿 🔹 = 1 🗖 🔽 🗖 Scale Bar	X 1743.7: 🗘
	Y 2038.7(🗘
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H 🕞	<u>∡ 0.0</u> €

The size of pixel can be found in the Info tab of the image. You can use this information to draw your own scale bar.

	C Channels	2	
L A Unmix	Scaling (per Pixel)	0.149 μm x 0.149 μm	
	Image Size (Pixels)	2146 x 2146	
Measure	Image Size (Scaled)	319.45 μm x 319.45 μm	
-	Bit Depth	16 Bit	
Analysis	Image Center Position	X: 0.00 μm, Y: 0.00 μm	
	ROI Center Offset	X: 0.00 μm, Y: 0.00 μm	
	Acquisition Information		
	Acquisition Start	9/18/2019 2:41:54 PM	
	Microscope	Axio Observer.Z1 / 7	

Capture a Z-stack

To capture a Z-stack, click the "Z-stack" checkbox. This will open a Z-stack dialog

AF	0	Ē	9 2	6
Find Focus	Set Exposure	Live	Continuous Snap	
		_		
🗹 Z-Stack	5 Slices			
Time Series	;			
All Tracks per Slice				
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			·	

🔻 🗏 Z-Stack	✓:	Show All 🛛 🛃
First / Last	Center	
	Set Last 1302.94	µm 🛟
F	Range 11.57 µn	
	Slices 12	+
	Interval 1.00 µm	\$
	Optimal 0.5	4 µm
	Keep 💿 Interva	al
1300.5	Slice	
	Set First 1314.51	µm 🗘
Position 1302.9 µm		
Slice # 1		
Optimize Sectioning and Ste	0	
Auto Z Brightness Correction		

Focus to the top of your sample and choose "Set First", focus to the bottom of your sample and choose "Set Last". The software will suggest an optimal Interval (step size). The optimal value oversamples (takes more slices than is really necessary), so it is safe to choose a bigger value for the Interval.

First / Last	Center
1317.0	Set Last 1302.94 µm
	Range 11.57 µm
- ÷	Slices 12
	Interval 1.00 µm
	Optimal 0.54 µm
	Keep 💿 Interval
1300.5	Slice
	Set First 1314.51 µm
Position 1302.9 µm	

Once you have set the first & last, as well as choosing how many slices, you can start the Z-stack acquisition by clicking "Start Experiment". The software will give you an idea of how the capture time will be.

✓ Z-Stack	5 Slices	
Time Series		
All Tracks per Slice		87.84 MB
		► Start Experiment

You can see all the slices in your Z-stack by choosing the "Gallery" tab.



The Ortho tab will display an orthogonal view of the stack.



Neuroscience Imaging Core 10/3/2019 The Ortho tab will also show a Maximum Intensity Projection.

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	- Ma	easure 3D Di					
ıp							
	New I	mage from	Current View			Create	

The maximum intensity projection (or orthogonal views) can be saved as a separate file in this dialog as well. Choose what kind of view (Current View, X-Y Plane, etc) and then choose the "Create" button to make a new image file.

Show All Display Ortho D	isplay
Cut Lines * 1 Z	Line Width
Cut Line Opacit Maximum In Measure 3D	Current View
New Image from	Current View Create

More Features: Tiles

Our system has a motorized stage, which allows us to do all sorts of neat things. We can do montages:



The software knows the dimensions of common sample carriers. We can capture a set of regular or randomized regions in each well. We can ask the software to go to 5 points in each well and capture a Z-stack. There are a multitude of possibilities.



More Features: Multiplex

Multiplex allows us to capture images with improved resolution (down to 140nm resolution) with drastically increased speed. Because the Airy pattern is known, the reassignment algorithm can be altered so that it can determine values for two (or more) rows of pixels for each single scan of the laser.



More Features: Sample Navigator

Sample Navigator allows us to capture a very low power (2.5x) image and use that as a "map" for higher magnification images.



Software Shutdown

The Zen software takes a little while to shutdown.



If you try to restart the software too quickly after shutting it down, you might get a message that it is already running. Just wait a little bit and try again.



Signing out of the PC

- 1. Click on the Windows icon on the lower left of the monitor.
- 2. Click on the 3 lines icon.
- 3. Click on your login icon (round Zeiss icon in this example.)
- 4. Choose "Sign out".



Shutdown

Shutdown in reverse numerical order. Start at #3 if you want to continue using the pc (to transfer files, export images, etc).All of the switches are labeled.

