



Jacqueline Ross, jacqui.ross@auckland.ac.nz

MICROSCOPY NEW ZEALAND INC. CONFERENCE WORKSHOP 2017

MEDICAL AND

SCHOOL OF MEDICAL SCIENCES

TH SCIENCES

Working with stacks using Fiji

31 January 2017

1. Image – Stacks menu

Example 1 – Widefield fluorescence microscopy

1. Open the file called Test_z_28Feb2011.seq (inside the Light Microscopy-Neuron folder);



- 2. Use the scroll bar at the bottom of the image to look through the images. Click the arrow head to make the series play automatically. Or use the left/right arrows on the keyboard.
- 3. Go to *Image Stacks Make montage* to create a gallery image of the stack. This can be saved as an image file. This can be used for any stack of images and is also useful for creating thumbnails from a set of images.

4. Select the options you want and click **OK** to create the montage.



- 5. Close the montage and select the stack again.
- Go to Image Stacks Z Project to create a z series projection file. Try out the different options. Note that you can choose to delete any slice from the stack by Image Stacks Delete Slice if you think that will improve the projection.

Which one works best for this data set?

Test_z_28Feb2011.seq (50%) 2/7; 4.27x3.20 inches (1280x960); RGB; 33MB			
The A	ZProjection	×	
Nord Start	Start slice:	1	
	Stop slice:	7	14
	Projection type	Average Intensity	
and and and		Min Intensity Sum Slices	
		Standard Deviation Median	
Non I wanted			

7. Now try out the **Extended Depth of Field** macro. You will find it under the **Plugins** menu. How does it compare with the Z Projections?

Example 2 – Confocal microscopy

- Go to *File Import Image Sequence*, select the first TIFF file of the data set inside the Membrane Labelling folder/dataset (=dc_c4_red01.tif) and click Open;
- 2. Make sure the number of images in the stack (20) is correct and click OK.

📴 Sequence Options 🛛 🔀		
Number of images: 20 Starting image: 1 Increment: 1 Scale images: 100 % File name contains:		
(enclose regex in parens)		
 Convert to 8-bit Grayscale Convert to RGB Sort names numerically Use virtual stack 		
512 x 512 x 20 (5.0MB)		
OK Cancel Help		

3. Open the file called **dc_c4_red.info.** This information file is used to calibrate the stack. Note that the format is 512 x 512 pixels and the pixel sizes are x = 0.26; y = 0.26; z = 0.49. These values are in microns.

The set and infe	
File Edit Font	
format 512 512	^
set_wrk_win 0 0 511 511	
range 1 20	
set_v_norm 1	
set_disp_pos 110 38	
set_disp_win 0 0 511 511	
set_v_acqu_win 0 0 774 511	
set_page 1	
lut glow	
set_v_interlaced 1	
compress 0 1 13	
voxelview 0 /usr/people/bin/volume 0 1	
tiff 1 0 1 1938	
huffman 0	
set_plane xy	
set_field_factor 10000.0	
set_obj 40.0 "/1.0 OIL "	
set_hv 4 250	
set_hv 3 250	
set_hv 2 250	
set_hv 1 682	
set_hv 0 350	
set_offset 41	
set_offset 31	
set_offset 21	
set_offset 1 -1	
set_offset 0 0	
set_pinhole_size 0 85	
set_scan_pos_z -6.88	
set_scan_pos_y -38.68	
set_zoom 1.886	
set_step_size 0.49	
$pixelsize_x = 0.26$	
$pixelsize_y = 0.26$	
pixelsize_z = 0.49	
laser_power = 2	
date 23.05.1900	

4. To calibrate the stack, go to *Image – Properties* and enter the **Pixel width**, **Pixel height** and **Voxel depth** 0.49um. Then click **OK**.

💵 Membrane Labelling 🛛 🖾
Channels (c): 1
Slices (z): 20
Frames (t): 1
Note: c*z*t must equal 20
Unit of length:
Pixel width: 0.2600000
Pixel height: 0.2600000
Voxel depth: 0.4900000
Frame interval: 0 sec
Origin (pixels): 0,0
🗖 Global
OK Cancel

5. Go to *Image – Stack – Orthogonal Views* to get a cross-section view of the stack (XZ and YZ views). You can move the position of the cross and also move through the XY stack to get different views through the stack. This option is good for looking for structure/distribution/colocalisation.



- 6. Close the XZ and YZ windows.
- 7. Try out the z projection options for this stack. Which works best?
- 8. Go to Image Stacks 3D Project. Use the settings below. Click OK to create the animation.

3D Projection	X	Projections of Membrane 5/36: 133.12x133.12 um (512x512): 8-bit: 9MB	
Projection method:	Brightest Point 💌	JAYAN .	STAT)
Axis of rotation:	Y-Axis 💌		NW A
Slice spacing (pixels):	1.00	(LLIM	
Initial angle (0-359 degrees):	0		CI No
Total rotation (0-359 degrees):	360		m
Rotation angle increment:	10	IN IN I	ALL
Lower transparency bound:	1	and have	CHA.
Upper transparency bound:	255		YII
Opacity (0-100%):	0	TINK	
Surface depth-cueing (0-100%):	100	MI DIN	
Interior depth-cueing (0-100%):	50		-AM
☐ Interpolate	Cancel Help	A A A	XX
			▶

- Click the Arrow head to play through the animation. If you want to change the speed, go to Image Stacks – Tools – Animation Options. If you want to save the animation as a movie, go to File – Save As AVI.
- 10. Or go to *File Save As Movie*. This provides additional output options, AVI, MOV or MP4. It also has compression options and allows you to specify the frame rate (speed) and quality for the movie.

👿 saveMovie	🐺 saveMovie
Frame rate 5.0	Frame rate 5.0
container format .avi 💌	container format 🛛 .avi 💌
using codec .mov	using codec MPEG4 💌
video quality .mp4	video quality RAW
custom bitrate(kb/s) 400	custom bitrate(kb/s)
Use less memory (slower)	H.264
OK Cancel	OK Cancel

1. Plugin – Volume Viewer

1. Go to Plugins - Volume Viewer;

Mode: Slice (0)	▼ Interpolation: Trilinear (1) ▼ z-Aspt	Reset
xy slice z=10 yz slice x=255 xz slice y=255	Transfer Fun Original (0) Draw LUT RG 0.0 Distance	ction (TF): Color & Alpha
xy yz yz	1.0 Scale	
Rotation: x:	115 $\frac{1}{2}$ y: 41 $\frac{1}{2}$ z: 17 $\frac{1}{2}$ xy yz xz Show: Axes \checkmark Clipping Slice pos	itions 🗌

- 2. Choose the view by clicking on the buttons at the bottom XY, YZ, XZ.
- 3. Make sure that the **Z-aspect** is correctly displayed (1.88). If not, change the **Z-Aspect** to 1.9 (X, Y pixel size = 0.26um, Z = 0.49um) so that the correct depth is being displayed.



- 4. Try out the different **Modes** and **Interpolation** methods. You can also use different LUTs and change the **Background** colour. Lighting effects are available in the bottom right-hand corner (yellow arrow).
- 5. Click **Hide TF** to hide the options on the right-hand side. Click **Snapshot** to create an image.
- 6. The bottom left-hand side controls (red arrows) are for navigation through the stack, shown by the grayscale images at the top.



7. Close the Volume Viewer window.

2. 3D Surface Plot

- 1. Go back to the original dataset. Move through the slices until you are at slice 9.
- 2. Go to *Analyze 3D Surface Plot*. There are lots of options here to optimize the display of your dataset, such as different LUTs, smoothing, perspective and lighting.
- 3. If you want to save an image, click **Save Plot.** Try out the different options with this dataset.



4. Close the **3D Surface Plot** window.

3. Surface Plot

1. Using the same data set, go to *Analyze – Surface Plot*. This creates a series of surface distribution images of your labelling.



2. The **Surface Plot** can be saved as an image sequence or a movie. You can also save individual images.



3. Close the Surface Plot window.

4. 3D Viewer

- Published article and instructions on USB stick.
- Used for visualization, volume measurements, etc.

• Located under **Plugins** menu.

Open the data set called **C_unknown_x_ed.lei.** Select the highest resolution option. Then launch the **3D Viewer** (**Plugins – 3D Viewer**);



Select the options you want and click OK.



5. Temporal Colour Coding

 Go to *Image – Hyperstacks – Temporal Color Code* and choose from the Look Up Tables listed, e.g. Fire. This will treat the z stack as a time series so you get different colours for different depths = depth-coded projection.



6. Extended Depth of Field plugins

Two versions – **Complex wavelet-based** method (Easy and Expert) and **Model-based** method Plugins available here: <u>http://bigwww.epfl.ch/demo/edf/index.html</u>

7. Extended Depth of Field Macro

Author - Richard Wheeler

Available here

Creates a focused image from a widefield fluorescence stack.

Installation - place into Plugins folder

8. Stack Contrast Adjustment plugin

Authors – Jan Michalek, Martin Capek, Jiri Janacek

Adjusts for drop off in signal through a z stack

Available here

Documentation and demo stacks also available on the website

Installation - place into Plugins folder

9. Plane Brightness Adjustment Plugin

Authors – Jan Michalek, Martin Capek, Jiri Janacek

Available <u>here</u>

Adjusts for uneven intensity in XY planes, e.g. darker corners.

Documentation and demo stacks also available on the website

Installation - place into Plugins folder

10. Analyzing Stacks as individual slices

- Generally, the same methods are used for measuring stacks as for individual images.
- 1. When thresholding the images, you select **Stack histogram** as below;

Threshold
30.66 %
↓ 308
624
Default 💌 Red 💌
🔽 Dark background 🔽 Stack histogram
Auto Apply Reset Set

2. The parameters you want to measure are selected under *Analyze – Set Measurements*. Make sure that you select **Stack position**;

🗊 Set Measurements	8
🔽 Area	🗌 Mean gray value
Standard deviation	Modal gray value
🔲 Min & max gray value	Centroid
Center of mass	Perimeter
Bounding rectangle	☐ Fit ellipse
Shape descriptors	🔽 Feret's diameter
Integrated density	🗖 Median
Skewness	☐ Kurtosis
Area fraction	✓ Stack position
Limit to threshold	Display label
Invert Y coordinates	Scientific notation
Add to overlay	NaN empty cells
Redirect to:	None 🔽
Decimal places (0-9):	1
	OK Cancel Help

3. When you make measurements of a stack, you use the **Measure Stack** macro instead of **Analyze** – **Measure**. This macro can be installed as a plugin in your **Plugins** menu.

- 4. If you are using the **ROI Manager**, then you go to *More Multi Measure* to measure all of the images. You could also use this option by creating a selection of the entire image and adding it into the **ROI Manager**.
- 5. Select **Measure all slices** and click **OK.**

🕎 Multi Measure 🔀	
 Measure all 118 slices One row per slice Append results 	
Enabling both options will result in a table with 14 columns.	
OK Cancel	

1. The **Label** field will include the name of the stack, the ROI name and the slice name.

11.3D Objects Counter

- Located under Analyze menu.
- Documentation <u>here</u>
- 1. Go to Analyze 3D OC Options to select the options you want;

3D-OC Set Measurements	23
Parameters to calculate:	
Volume	Surface
Nb of Obj. voxels	Nb of Surf. voxels
Integrated Density	Mean Gray Value
🔲 Std Dev Gray Value	🗖 Median Gray Value
🥅 Minimum Gray Value	Maximum Gray Value
Centroid	Mean distance to surface
Std Dev distance to surface	Median distance to surface
Centre of mass	Bounding box
Image parameters:	
Close original images w	/hile processing (saves memory)
Show masked image (re	edirection requiered)
Mana' parametera:	
maps parameters.	
Dots size 5	
Font size 10	
🔽 Show numbers	
White numbers	
ResultsTable parameters:	
Store results within a tak	ble named after the image (macro friendly)
Redirect to:	-
Indicate. Indice	<u> </u>
	OK Cancel

- 2. Then go to Analyze 3D Objects Counter,
- 3. Threshold the image and select the options you need;



4. Click **OK** to run the analysis.

🗊 C_unknown_x_ed.lei - unknown_x_ed_Series007 💶 💷 🗾	D Surface map of C_unknow	n_x_ed.lei - unknown_x		x
1/22 (z:1/22 - unknown_x_ed_Series007_5x); 3000x3000 microns (512)	14/22; 3000x3000 microns (512x512); 8-bit; 5.5MB		
		1		
		. .		
				_ <u>32</u>
i i alla d				
	· · · · ·			æ
>		26		
	28			24
				÷
			¢	
				•
🔟 Objects map of C_unknown_x_ed.lei - unknown_x 🗖 🔲 🔀	Statistics for C_unknown_x	_ed.lei - unknown_x	- 0 1	x
Objects map of C_unknown_x_ed.lei - unknown_x Image: Control of the second sec	Statistics for C_unknown_x File Edit Font	_ed.lei - unknown_x		X
Objects map of C_unknown_x_ed.lei - unknown_x Image: Control in the second sec	File Edit Font Volume (micron^3)	surface (micron^2)	Nb of obj	X
Objects map of C_unknown_x_ed.lei - unknown_x	Statistics for C_unknown File Edit Font Volume (micron*3) 329.303	_ed.lei - unknown_x Surface (micron^2) 6821773.500	Nb of obj	×3
Objects map of C_unknown_x_ed.lei - unknown_x 22/22; 3000x3000 microns (512x512); 8-bit; 5.5MB	Statistics for C_unknown_x File Edit Font Volume (micron*3) 1 329.303 2 5.814	surface (micron*2) 6821773.500 111583.484	Nb of obj	×3
Dbjects map of C_unknown_x_ed.lei - unknown_x	Statistics for C_unknown x File Edit Font Volume (micron^3) 1 329.303 2 5.814 3 1.577	Surface (micron^2) 6821773.500 111583.484 33027.691	Nb of obj 0 0 0	×)
Dbjects map of C_unknown_x_ed.lei - unknown_x	Statistics for C_unknown File Edit Font Volume (micron*3) 329.303 329.303 2 5.814 31.577 4 0.304 304	Surface (micron^2) 6821773.500 111583.484 33027.691 9544.430	Nb of obj 0 0 0 0	×
Dbjects map of C_unknown_x_ed.lei - unknown_x Image: Comparison of C_unknown_x_ed.lei - unknown_x 22/22; 3000x3000 microns (512x512); 8-bit; 5.5MB Image: Comparison of C_unknown_x_ed.lei - unknown_x	Statistics for C_unknown x File Edit Font Volume (micron*3) 1 329.303 2 5.814 3 1.577 4 0.304 5 0.172	Surface (micron^2) 6821773.500 111583.484 33027.691 9544.430 6454.499		×
Dbjects map of C_unknown_x_ed.lei - unknown_x Image: Comparison of C_unknown_x_ed.lei - unknown_x 22/22; 3000x3000 microns (512x512); 8-bit; 5.5MB	Statistics for C_unknown x File Edit Font Volume (micron^3) 1 329.303 2 5.814 3 1.577 4 0.304 5 0.172 6 0.318	Surface (micron^2) 6821773.500 111583.484 33027.691 9544.430 6454.499 12428.358	Image: Nb of obj 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	×
Dbjects map of C_unknown_x_ed.lei - unknown_x Image: Comparison of C_unknown_x_ed.lei - unknown_x 22/22; 3000x3000 microns (512x512); 8-bit; 5.5MB	Statistics for C_unknown > File Edit Font Volume (micron*3) 329.303 3 1 329.303 3 2 5.814 3 3 1.577 4 4 0.304 5 5 0.172 6 6 0.318 7 7 0.522 5	Surface (micron^2) 6821773.500 111583.484 33027.691 9544.430 6454.499 12428.358 17509.578	Nb of obj 0 0 0 0 0 0 0 0	×
Dbjects map of C_unknown_x_ed.lei - unknown_x Image: Comparison of C_unknown_x_ed.lei - unknown_x 22/22; 3000x3000 microns (512x512); 8-bit; 5.5MB	Statistics for C_unknown File Edit Font Volume (micron*3) 329.303 3 1 329.303 3 2 5.814 3 3 1.577 4 4 0.304 5 5 0.172 6 6 0.318 7 7 0.522 8 8 0.244	Surface (micron*2) 6821773.500 111583.484 33027.691 9544.430 6454.499 12428.358 17509.578 9544.441	Nb of obj 0 0 0 0 0 0 0 0 0	
Dbjects map of C_unknown_x_ed.lei - unknown_x Image: Comparison of C_unknown_x_ed.lei - unknown_x 22/22; 3000x3000 microns (512x512); 8-bit; 5.5MB	Statistics for C_unknown y File Edit Font Volume (micron^3) 329.303 329.303 2 5.814 31.577 3 1.577 0.304 5 0.172 0.318 7 0.522 8 0.244 9 0.314 314	Surface (micron*2) 6821773.500 111583.484 33027.691 9544.430 6454.499 12428.358 17509.578 9544.441 9887.765	Image: Nb of obj 0	
Dbjects map of C_unknown_x_ed.lei - unknown_x Image: Comparison of C_unknown_x_ed.lei - unknown_x 22/22; 3000x3000 microns (512x512); 8-bit; 5.5MB	Statistics for C_unknown > File Edit Font Volume (micron*3) 329.303 3 1 329.303 3 2 5.814 3 3 1.577 4 4 0.304 5 5 0.172 6 6 0.318 7 7 0.522 8 8 0.244 9 9 0.314 10 10 0.978 10	Surface (micron^2) 6821773.500 111583.484 33027.691 9544.430 6454.499 12428.358 17509.578 9544.441 9887.765 26710.631	Nb of obj 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	
Dbjects map of C_unknown_x_ed.lei - unknown_x Image: Comparison of C_unknown_x_ed.lei - unknown_x 22/22; 3000x3000 microns (512x512); 8-bit; 5.5MB	Statistics for C_unknown > File Edit Font Volume (micron^3) 1 329.303 2 5.814 3 1.577 4 0.304 5 0.172 6 0.318 7 0.522 8 0.244 9 0.314 10 0.978	Surface (micron*2) 6821773.500 111583.484 33027.691 9544.430 6454.499 12428.358 17509.578 9544.441 9887.765 26710.631	Image: bold state Nb of obj 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	
Dbjects map of C_unknown_x_ed.lei - unknown_x Image: Comparison of C_unknown_x_ed.lei - unknown_x 22/22; 3000x3000 microns (512x512); 8-bit; 5.5MB	Statistics for C_unknown y File Edit Font Volume (micron^3) 329.303 329.303 2 5.814 31.577 3 1.577 40.304 5 0.172 60.318 7 0.522 80.244 9 0.314 100.978 1 0.978 100.978	Surface (micron*2) 6821773.500 111583.484 33027.691 9544.430 6454.499 12428.358 17509.578 9544.441 9887.765 26710.631	Image: Nb of obj State 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	
Dbjects map of C_unknown_x_ed.lei - unknown_x Image: Comparison of C_unknown_x_ed.lei - unknown_x 22/22; 3000x3000 microns (512x512); 8-bit; 5.5MB	Statistics for C_unknown > File Edit Font Volume (micron^3) 1 329.303 1 329.303 2 2 5.814 3 3 1.577 4 4 0.304 5 5 0.172 6 6 0.318 7 7 0.522 8 8 0.2444 9 9 0.314 10 10 0.978 10 File Edit Font	Surface (micron*2) 6821773.500 111583.484 33027.691 9544.430 6454.499 12428.358 17509.578 9544.441 9887.765 26710.631	Nb of obj 0	
Objects map of C_unknown_x_ed.lei - unknown_x Image: Comparison of C_unknown_x_ed.lei - unknown_x 22/22; 3000x3000 microns (512x512); 8-bit; 5.5MB Image: Comparison of C_unknown_x_ed.lei - unknown_x	Statistics for C_unknown y File Edit Font Volume (micron^3) 1 329.303 2 5.814 3 3 1.577 4 0.304 5 0.172 6 0.318 7 0.522 8 0.244 9 0.314 10 0.978 Image: Constraint of the state of the sta	Surface (micron^2) 6821773.500 111583.484 33027.691 9544.430 6454.499 12428.358 17509.578 9544.441 9887.765 26710.631		
Dbjects map of C_unknown_x_ed.lei - unknown_x Image: Comparison of C_unknown_x_ed.lei - unknown_x 22/22; 3000x3000 microns (512x512); 8-bit; 5.5MB Image: Comparison of C_unknown_x_ed.lei - unknown_x	Statistics for C_unknown > File Edit Font Volume (micron*3) 1 329.303 1 329.303 2 2 5.814 3 3 1.577 4 4 0.304 5 5 0.172 6 6 0.318 7 7 0.522 8 0.244 9 0.314 10 10 0.978 - ✓ Log - File Edit Font C_unknown_x_ed.lei - unk -	Surface (micron*2) 6821773.500 111583.484 33027.691 9544.430 6454.499 12428.358 17509.578 9544.441 9887.765 26710.631	Nb of obj 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0 0	
Dbjects map of C_unknown_x_ed.lei - unknown_x Image: Comparison of C_unknown_x_ed.lei - unknown_x 22/22; 3000x3000 microns (512x512); 8-bit; 5.5MB Image: Comparison of C_unknown_x_ed.lei - unknown_x	Image: Statistics for C_unknown_x File Edit Font Volume (micron^3) 1 329.303 1 329.303 2 2 5.814 3 3 1.577 4 4 0.304 5 5 0.172 6 6 0.318 7 7 0.522 8 8 0.244 9 9 0.314 10 10 0.978 10 File Edit Font C_unknown_x_ed.lei - unk	Surface (micron*2) 6821773.500 111583.484 33027.691 9544.430 6454.499 12428.358 17509.578 9544.441 9887.765 26710.631	Nb of obj 0 </th <th></th>	