BOMP seminar series:

Introduction to 3D bioimage analysis

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This seminar will…

1. Guideline for proper 3D image acquisition
   - suitable for user who has been using microscopy but new to 3D imaging

2. Introduce the features of 3D image analysis software
   - NOT cover 'step by step' methods but will show what is possible on those software

Further workshops on image analysis

- Extensive tutorials and manuals are available on web
  - Volocity Playlists in YouTube 'Volocity 3D Image Analysis Software Training'
  - Imaris http://www.bitplane.com/learning

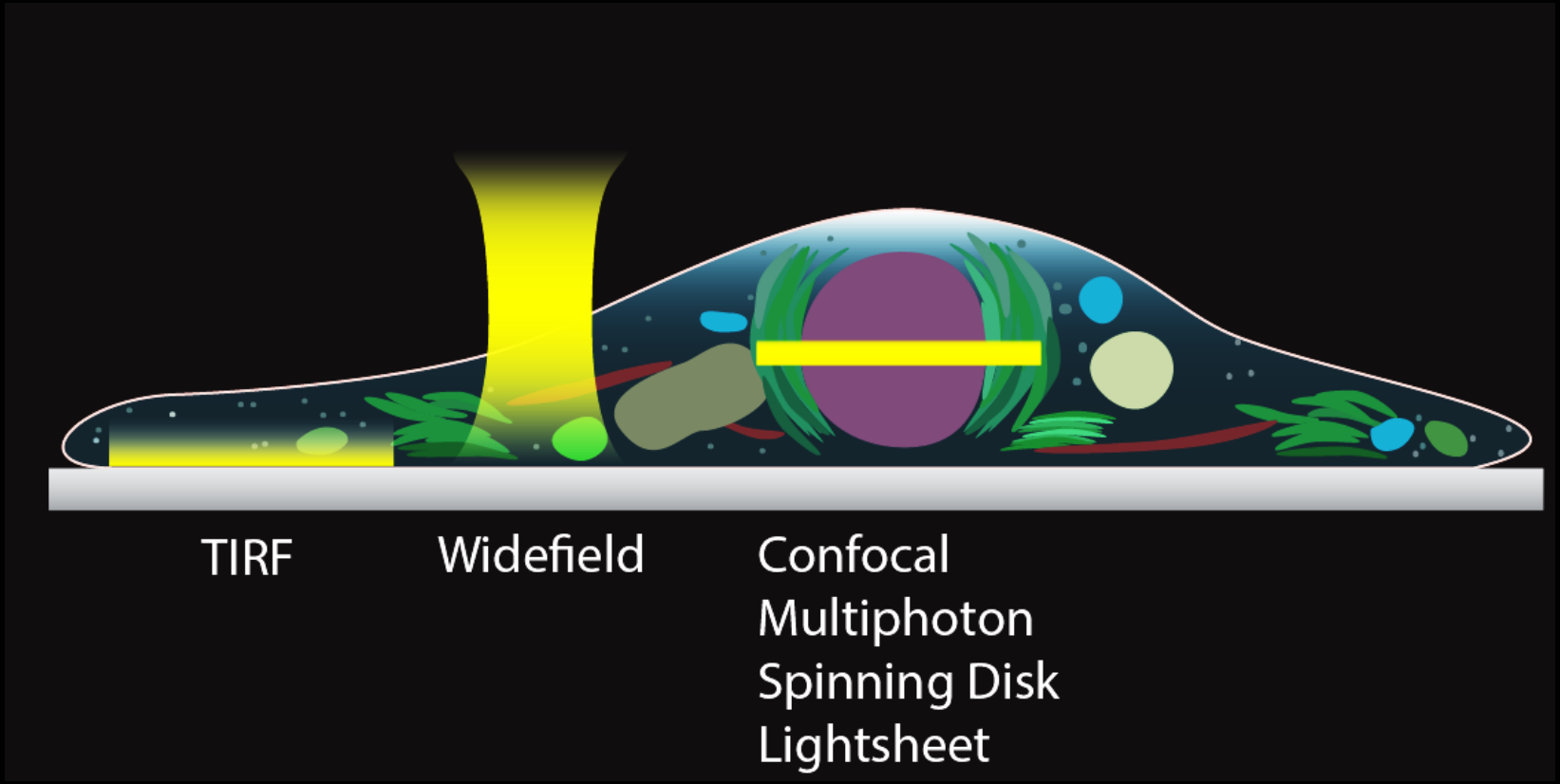
- BOMP Hands on workshop for
  - IMARIS
  - FIJI/Image J for Beginners
  - FIJI/Image J for Quantification
  - FIJI/Image J for Batch

- Contact BOMP (bomp-enquiries@unimelb.edu.au) to be included in the info mailing list

3D image acquisition

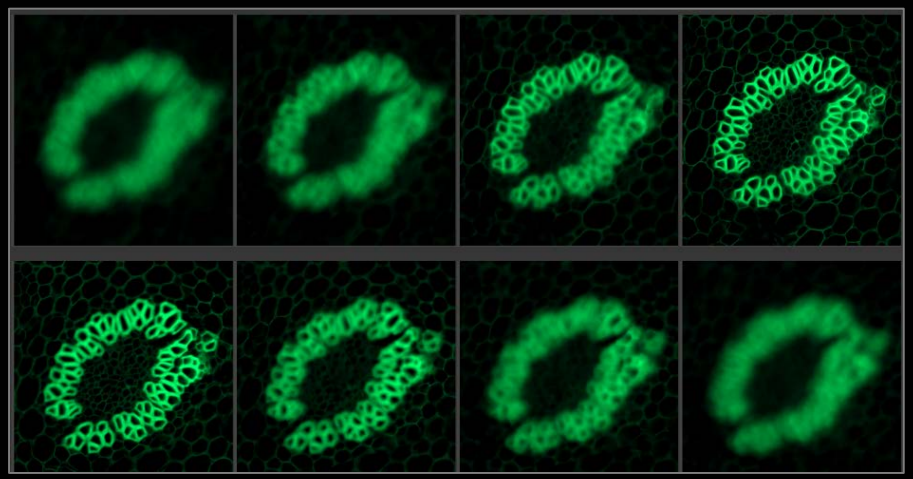
3D Microscopy options

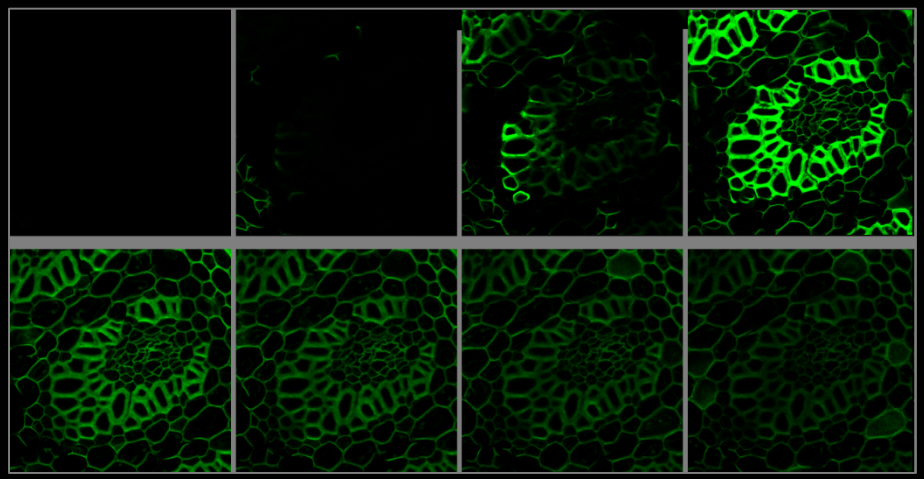
Select the microscope that can optically section your sample



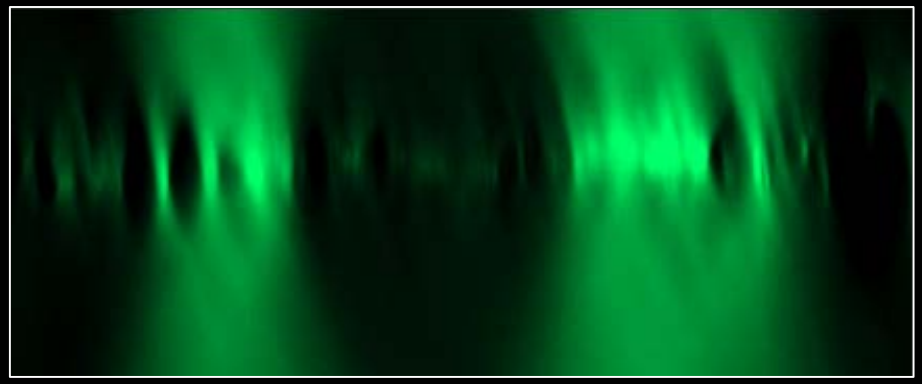
Optical sectioning capability

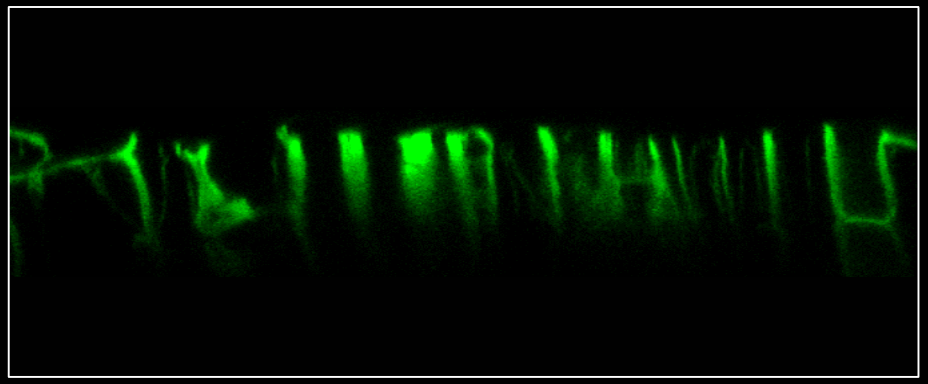
Widefield vs Confocal





XZ view





Z resolution

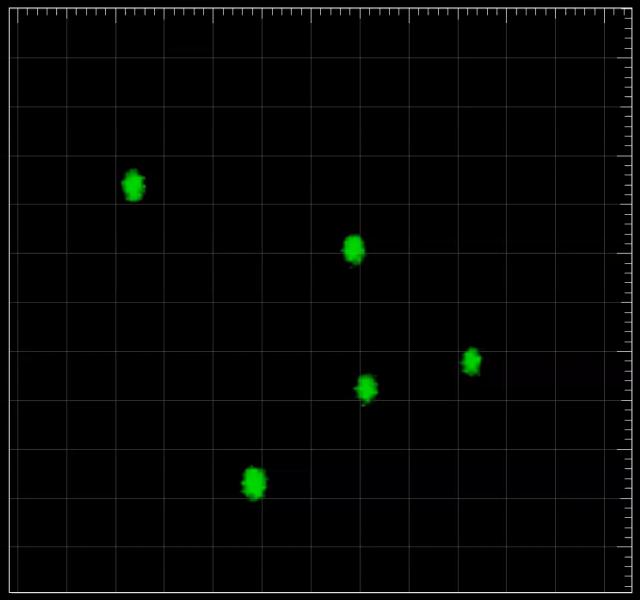
Z resolution is worse than XY resolution.

In confocal microscopy,

$$\text{XY Resolution} = \frac{0.4 \times \lambda}{NA} \qquad \text{Z Resolution} = \frac{1.4 \times \lambda}{RI \times NA}$$

Green fluorophore mounted in glycerol with 63x/1.4 lens

XY Resolution	Z Resolution	xy view	z view
139nm	334nm		



λ: Wavelength NA: Numerical Apeture RI: Refractive Index

Z resolution

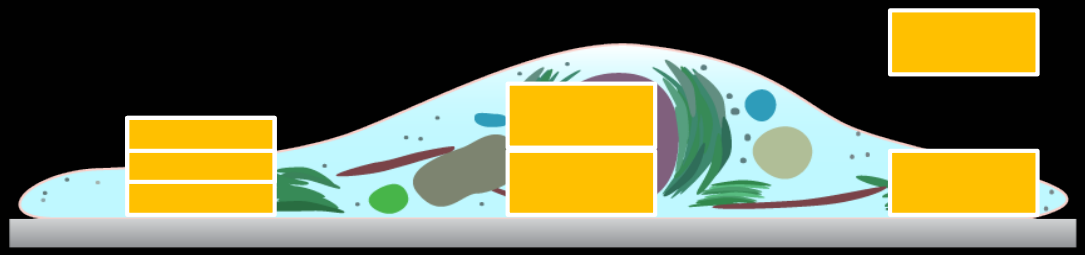
For 3D imaging, consider the Z resolution of the optical system you are using. It is the Z resolution (not just XY resolution) should be smaller than your object of interest.

- Use fluorophore with shorter wavelength
- Use higher refractive index immersion (oil for 1.518)
- Use Higher Numerical Aperture lens

$$\text{Z Resolution} = \frac{\mathbf{1.4} \times \lambda}{\text{RI} \times \text{NA}}$$

Z Sampling rate (stack interval)

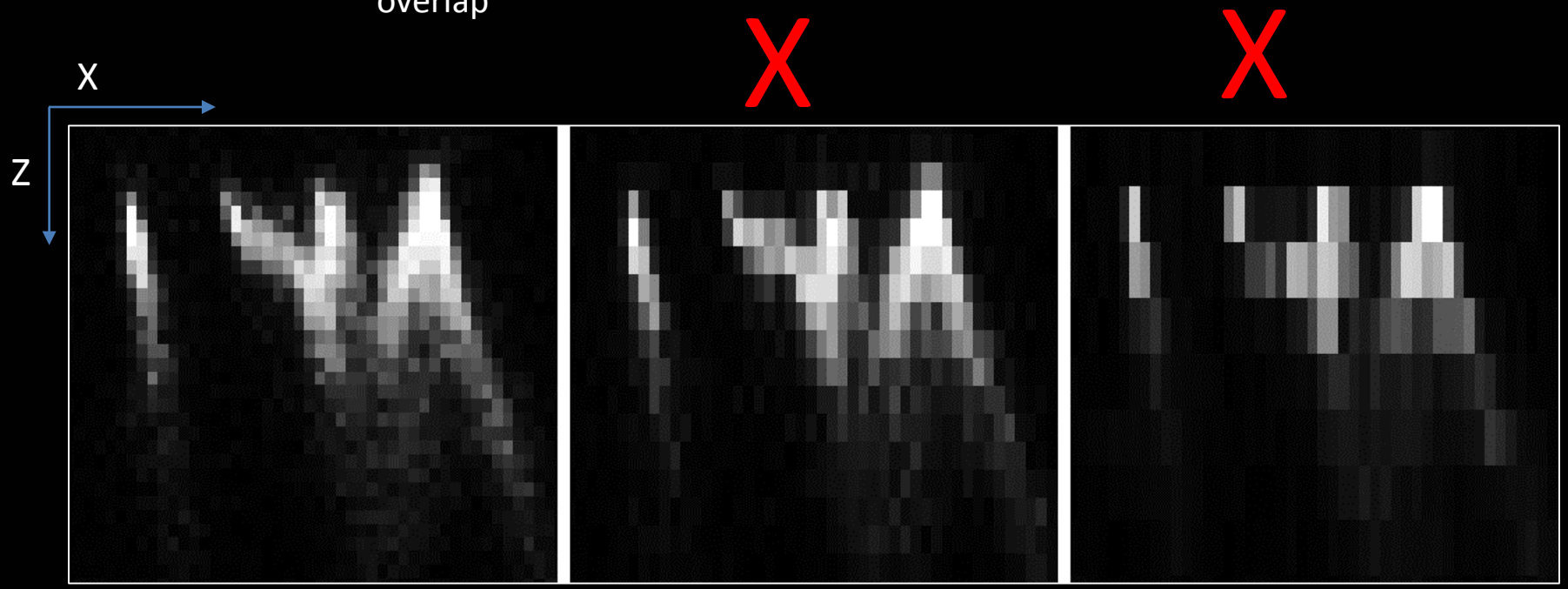
Acquire with at least 50% overlap of optical section for better sampling rate



50% overlap

No overlap

Gap between plane



More considerations

- Practical imaging depth : 20um – 5mm
- Resolution : cell organelles – whole tissue
- Acquisition speed : minutes – hours
- Photobleaching /damaging: stable fluorophore or not. Live or fixed sample.

Table 1. Practical comparison of several 3D microscopy modes

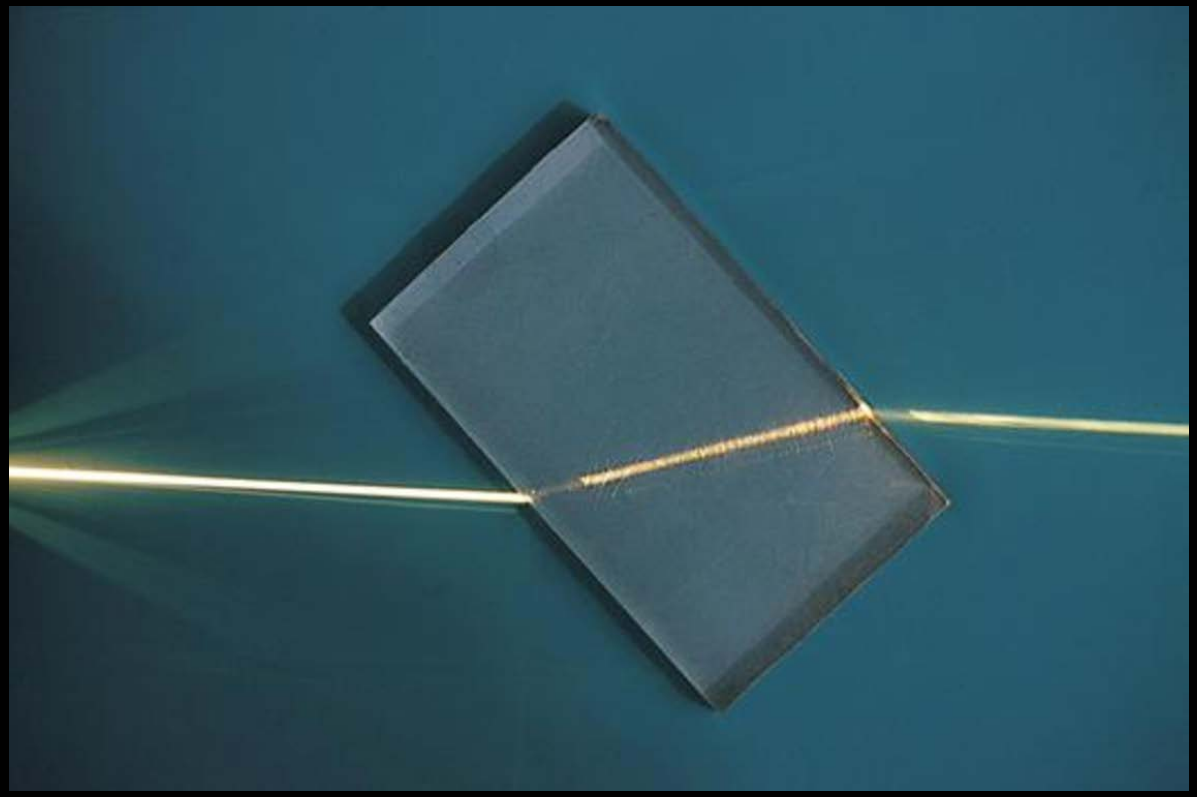
Mode	Best-suited applications	Advantages for 3D biology	Limitations	Practical imaging depth (μm)	Resolution	Commercially available	Refs
CLSM[a]	Cell structure analyses *in vitro* and *in vivo* with bright, fixed samples, live cell FRAP analysis	Widely available, 2D ROI photomanipulation, good axial sectioning performance	High photobleaching and photodamage Slow speed in 4D, poor SNR	200	250 nm XY, >600 nm Z	Yes	[4]
2PM	intravital imaging, SHG imaging of collagen and fluorescence together, bright samples	3D ROI photomanipulation, SHG, deepest sample penetration	Nonlinear photodamage Slow speed in 4D, pulsed lasers expensive, poor SNR	600–800	250 nm XY, >600 nm Z	Yes	[14,15, 72–74]
SDCM	High resolution live cell and organelle dynamics, cyoskeletal and membrane dynamics, dim samples (FSM)	Widely available, high speed, good SNR with good CCD camera, high sensitivity	Pinhole crosstalk, no ROI control No low magnification imaging	150	250 nm XY, >600 nm Z	Yes	[53,56, 75–77]
LSFM	Whole cell dynamics during embryo development or other long time scale dynamics, cell surface dynamics with Bessel beam (e.g. filopodial dynamics on single cells)	Very high speed, very low photodamage, good SNR, higher resolution possible combined with 2P, Bessel, SI, or DLSM excitation	No high NA objectives, poor light collection, lower resolution, sample mounting issues	200–500	500 nm XY, 1–3 μm Z; 300 nm X,Y,Z for Bessel	No	[24,59, 62,64, 66,67]
3D-SIM	Macromolecular assemblies and organelles with relatively slow dynamics (e.g. chromatin remodeling). Low scattering samples are best	Resolution ~2× better than diffraction-limited, light efficient	Slow speed, photodamage possible, limited imaging depth, multiple raw images needed per 3D-SIM image, shot noise not removed, complex optical configuration	20–40	100 nm XY, 250 nm Z. 100 nm XYZ in with 3D-SIM (4Pi configuration)	Yes	[6,28, 29,33, 35]

Fischer et al. Trends Cell Biol. 2011 21(12):682-91

Sample preparation

Refractive Index

How light propagates through the medium



Spherical aberration

In optical system, refractive Index mismatch results spherical aberration

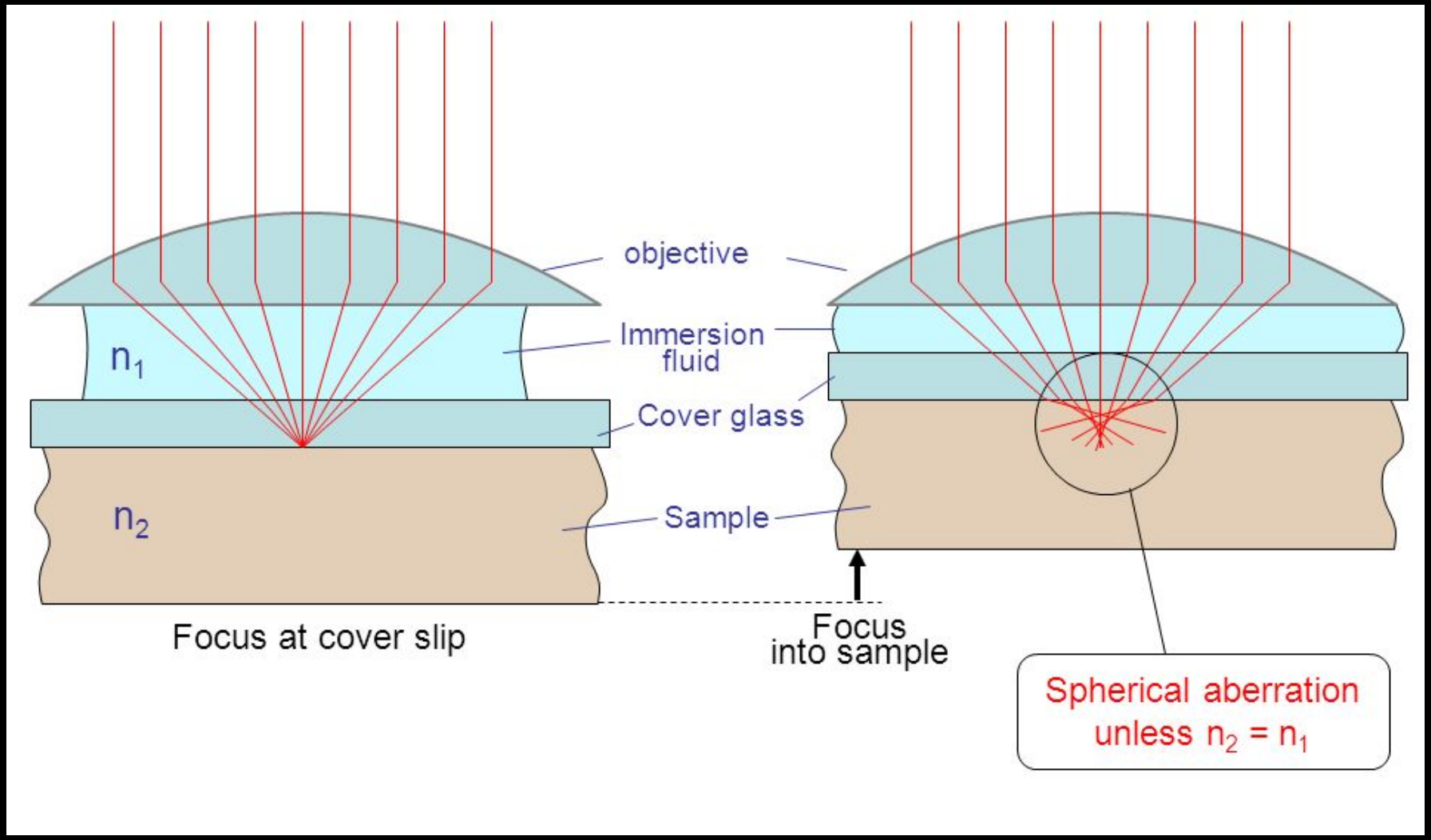


Diagram from Principles & Practice of Light Microscopy: 2 by Solomon Gaines http://slideplayer.com/slide/10577436/

Spherical aberration is worse as it is further from coverslip

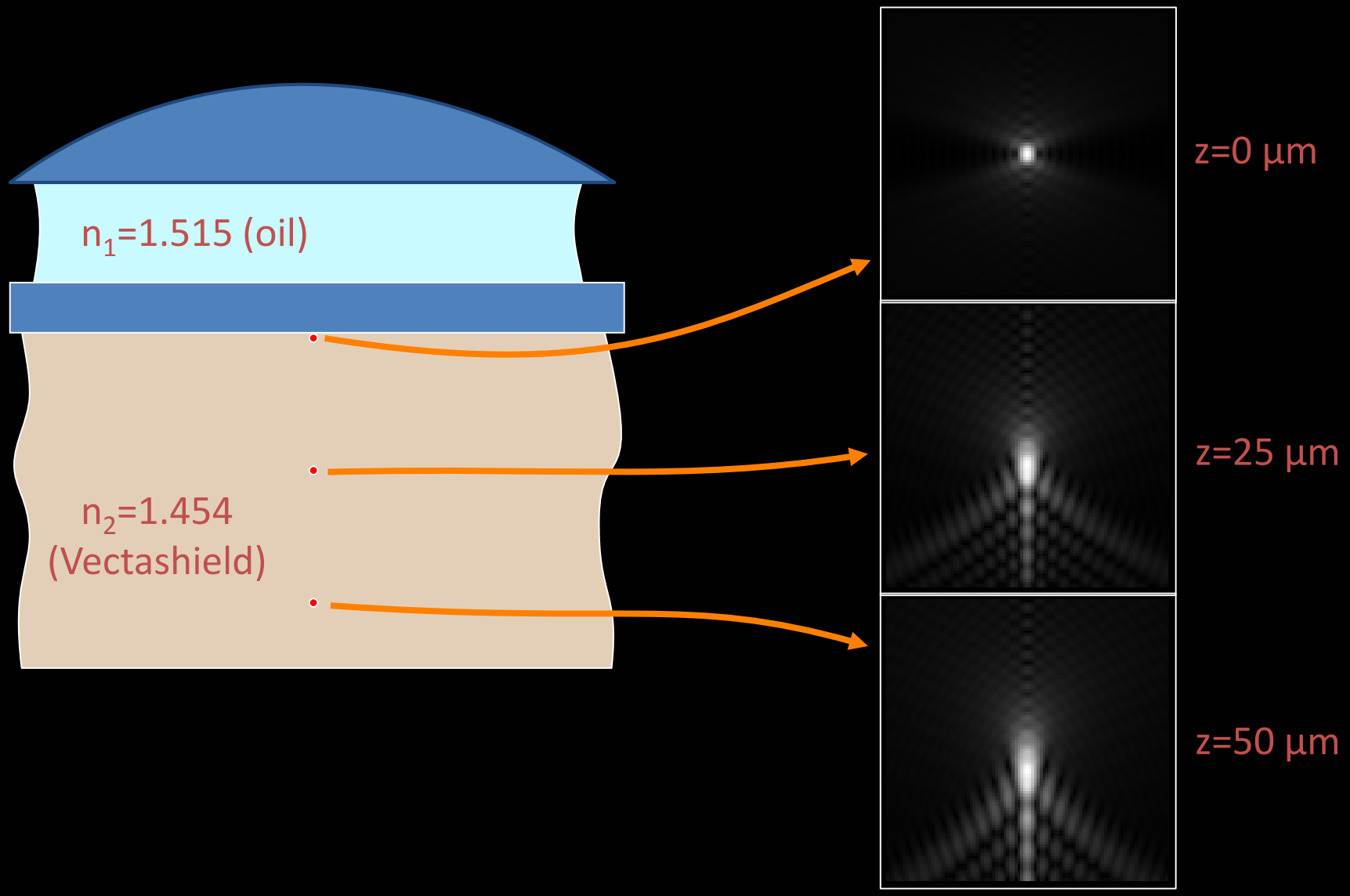


Diagram from Principles & Practice of Light Microscopy: 2 by Solomon Gaines http://slideplayer.com/slide/10577436/

Mounting medium

- **Refractive Index**
- Compatible with your fluorophore (some are also pH dependent)
- If it hardens or not

Mounting medium	Manufacturer	Refractive index	pH	Base compound	Fluorophore compatibility*
2,2'-Thiodiethanol	Sigma-Aldrich, France	1.518	6.5	2,2'-Thiodiethanol	most organic dyes and fluorochromes, RFP, however causes strong GFP quenching (8)
CFM-1	Citifluor Ltd., UK	1.515	7.5	glycerol-PBS-based	nd
CFM-3	Citifluor Ltd., UK	1.518	6.5	glycerol-based	DAPI, Hoechst, Alexa and Cyanine dyes, Venus, GFP#, Tomato, mCherry
Vectashield H-1000	Vector Laboratories Ltd., UK	1.454	nd	glycerol-based	fluorescein, rhodamine, Texas Red, AMCA, DyLight fluorescent dyes and other fluorescent reagents such as Cy3, Cy5, Alexa Fluor 488, and Alexa Fluor 594. GFP, RFP, YFP
Prolong Gold	ProLong Gold	1.390–1.460 depending on curing time	nd	glycerol-based	most organic dyes, however, fluorescent proteins less well preserved
Fluoromount-G	Southern Biotechnology	1.400	7.4	acrylate-PBS	most organic dyes
Mowiol Tris MWL 4–88	Citifluor Ltd., UK	1.410–1.490	9.5	polyvinyl alcohol	most organic dyes

*fluorochrome compatibility was experimentally evaluated only for CFM3, data for other mounting media were taken out of the manufacturers datasheets.

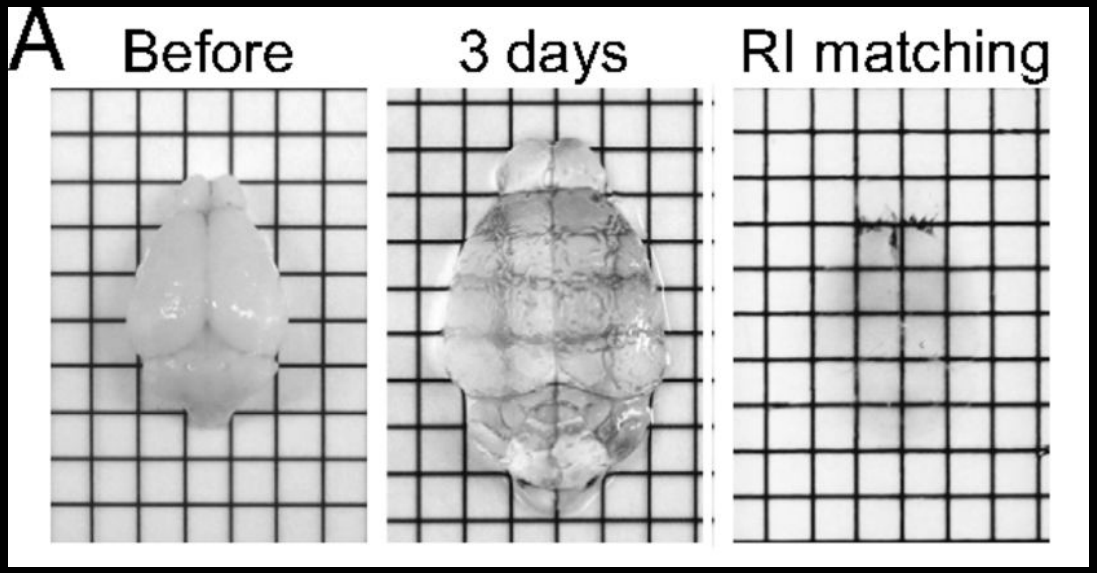
GFP shows enhanced photobleaching in CFM3

doi:10.1371/journal.pone.0121096.t001

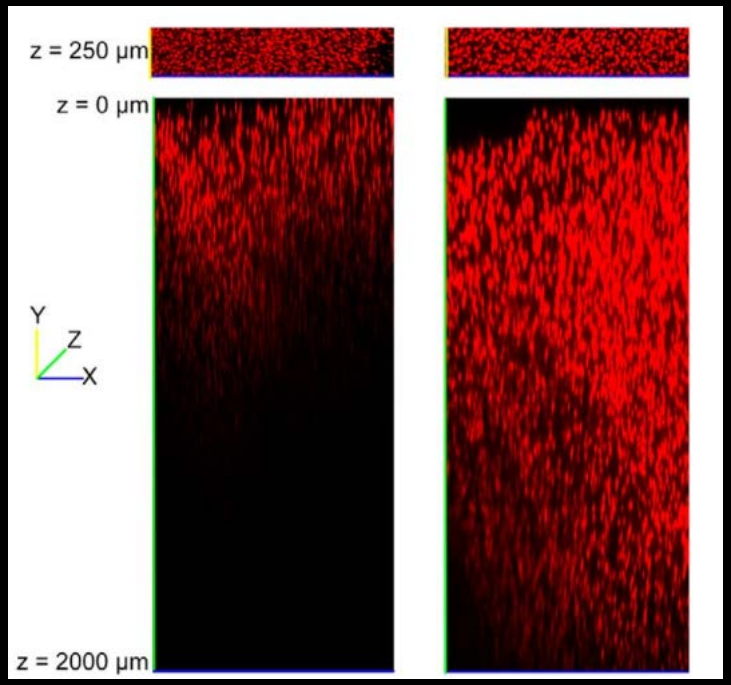
Fouquet C et al. (2015) PLOS ONE 10(3): e0121096

Sample clearing

- For thick sample, sample clearing is necessary
- Refractive Index matching is critical step for clearing

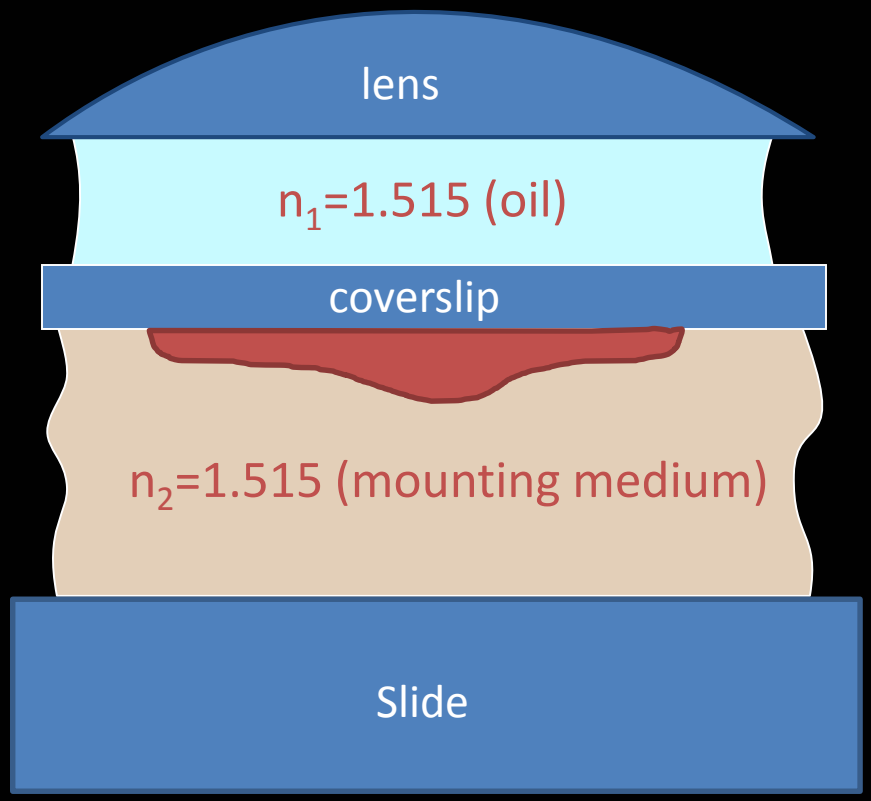


Kim et al. PNAS 2015;112:46:E6274-E6283

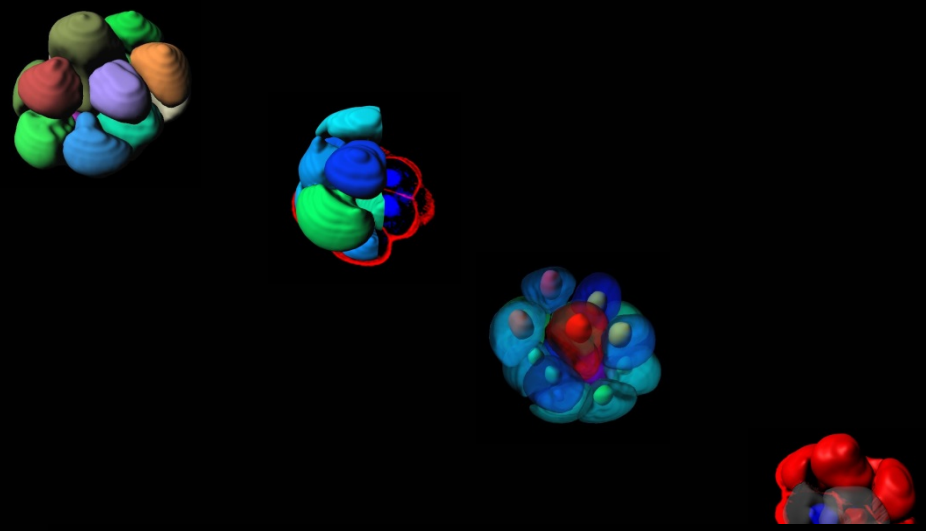


Epp et al. eNeuro 2015May 25;2(3)

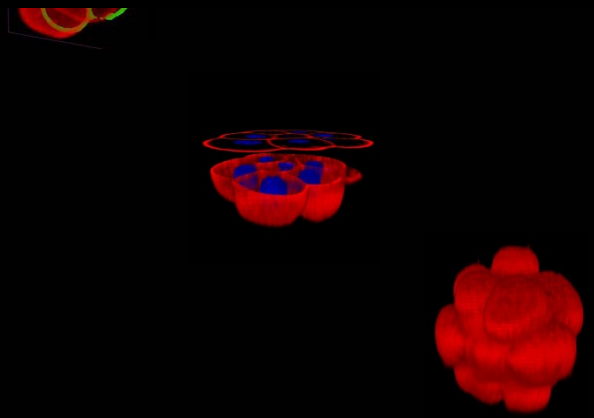
Sample preparation guide

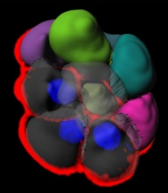


- Use 170um(#1.5) coverslip
- Mount sample to the coverslip not to the slide
- Match refractive index of your samples and lenses
  - For fixed samples and oil immersion lenses, mount your sample in a medium with n = 1.515
  - For live samples, use water immersion / water dipping lenses
  - Adjust objective correction collar when available



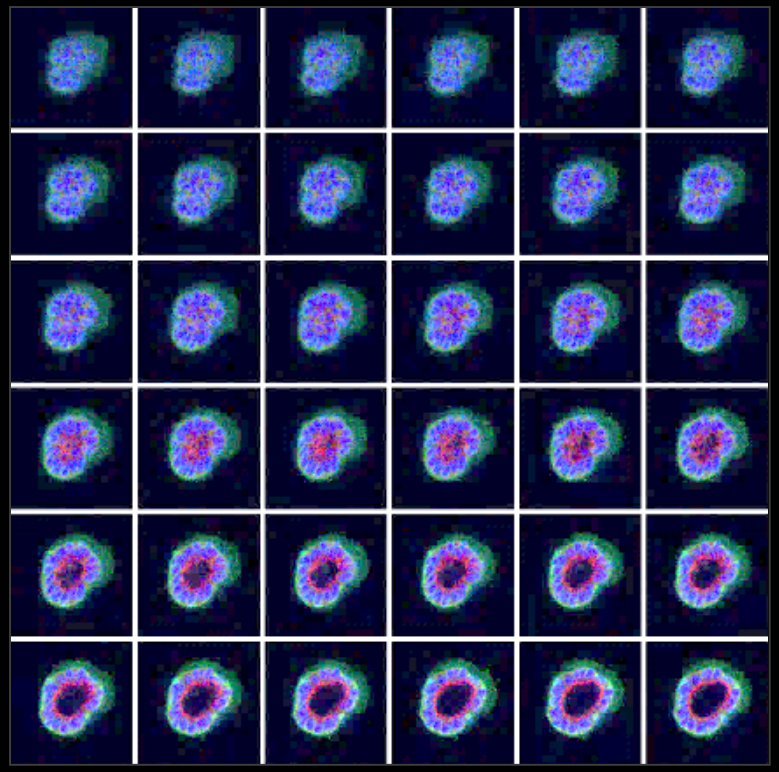
Visualising 3D image



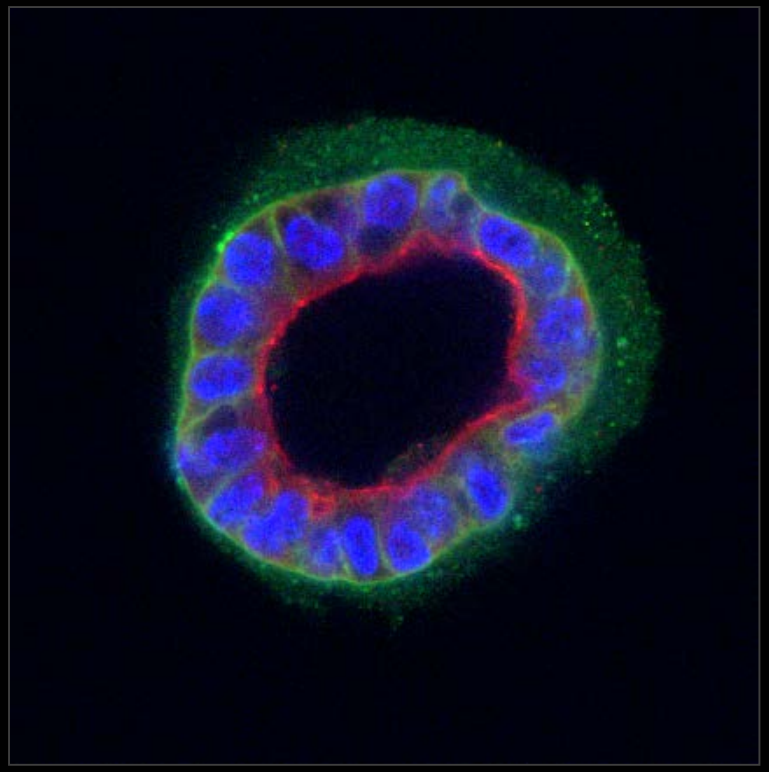


Presenting 3D image dataset without 3D viewer

Multi plane view

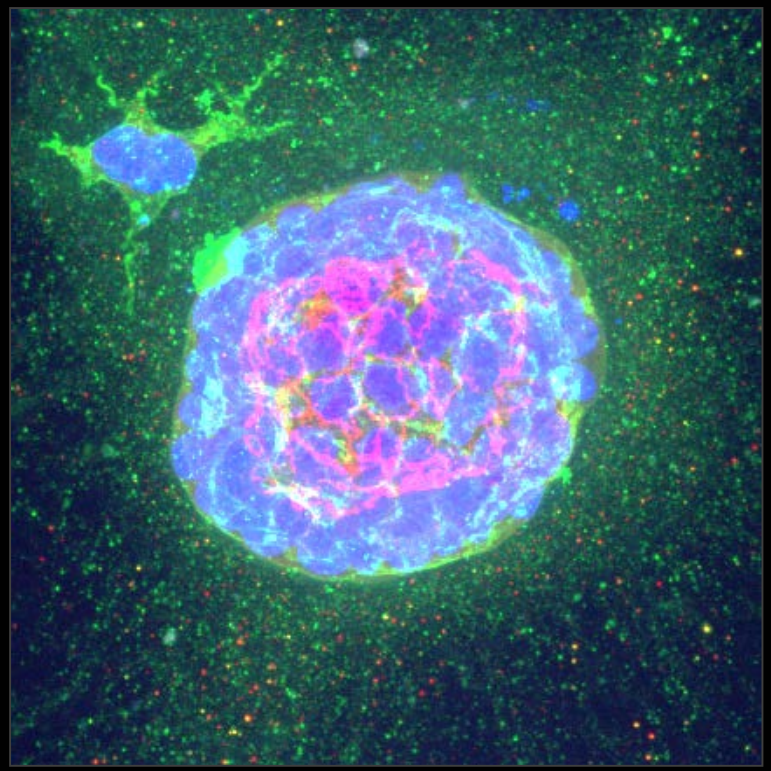


Single XY view of a subset

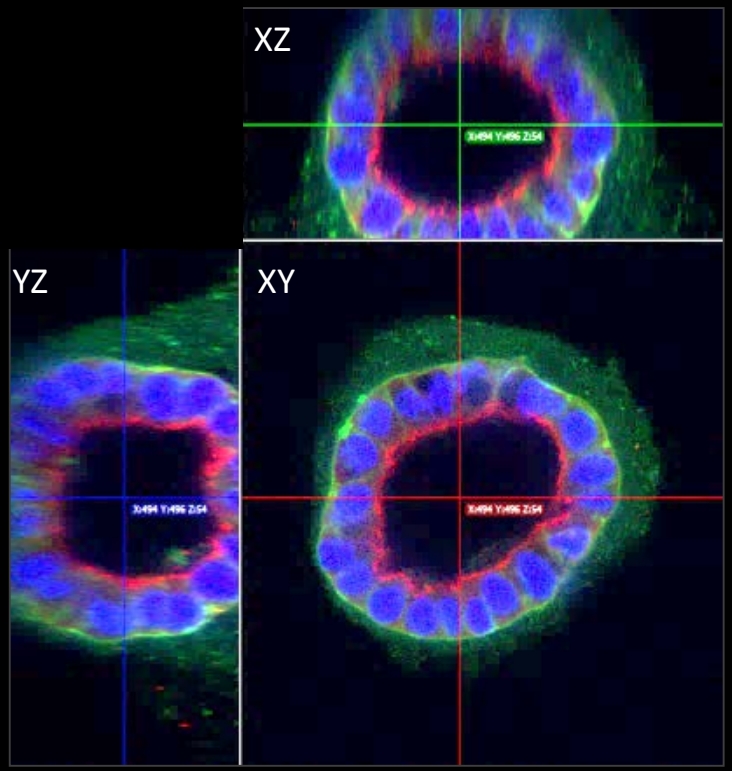


Presenting 3D image dataset without 3D viewer

Maximum Intensity Projection



Orthogonal view



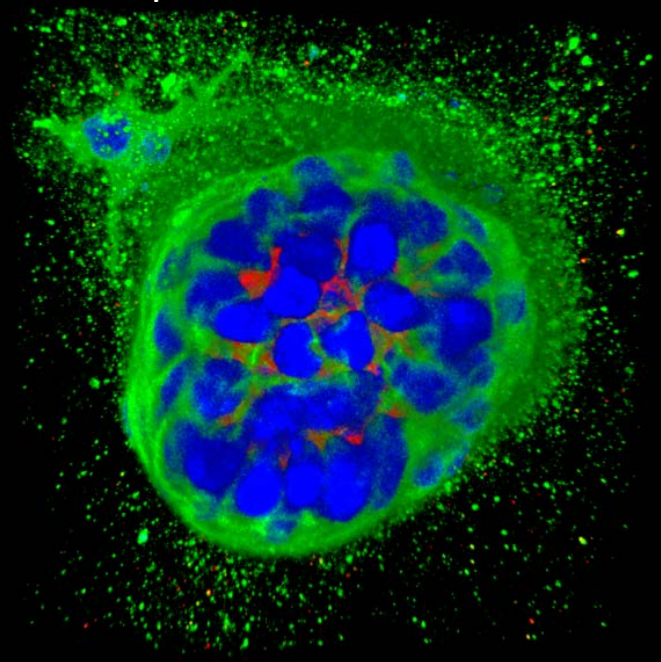
3D image analysis software

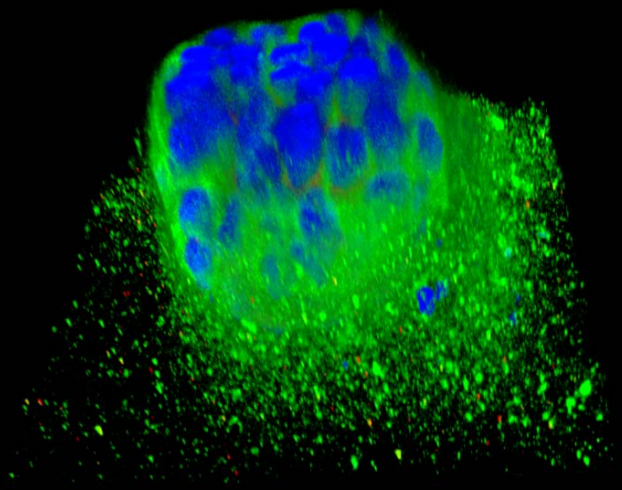
IMARIS (licenced)
Volocity (licenced)
Amira (licenced)
ImagePro (licenced)
Vaa3D(Free)
FIJI/ImageJ (Free)
3D Slicer(Free)
And more…

Try all available software!

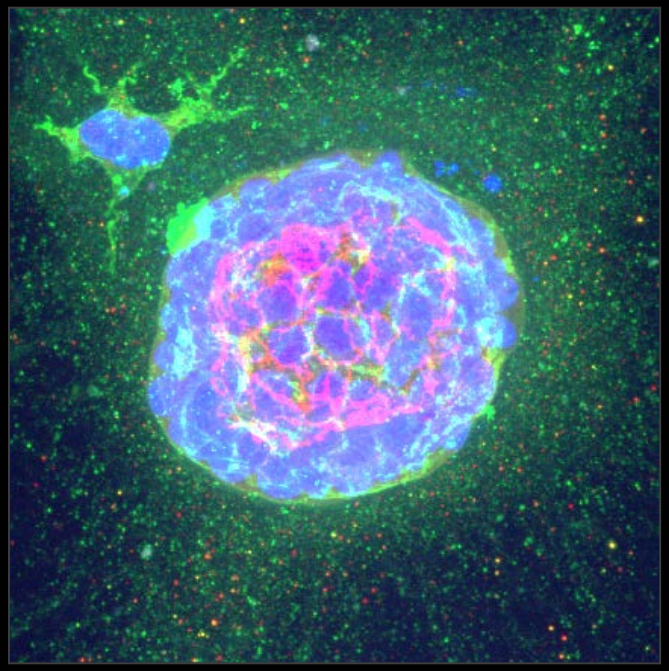
3D viewer - Fluorescence mode with depth effect

3D depth effect

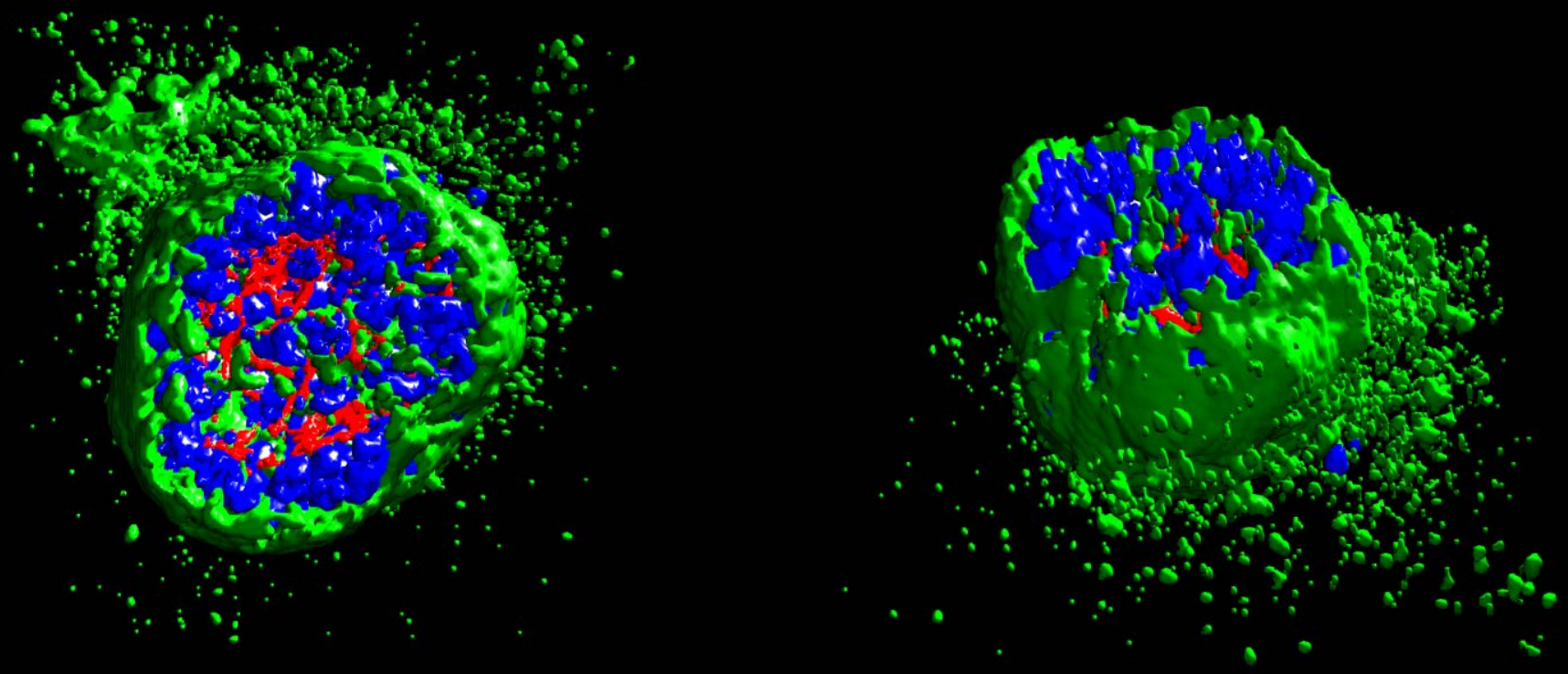




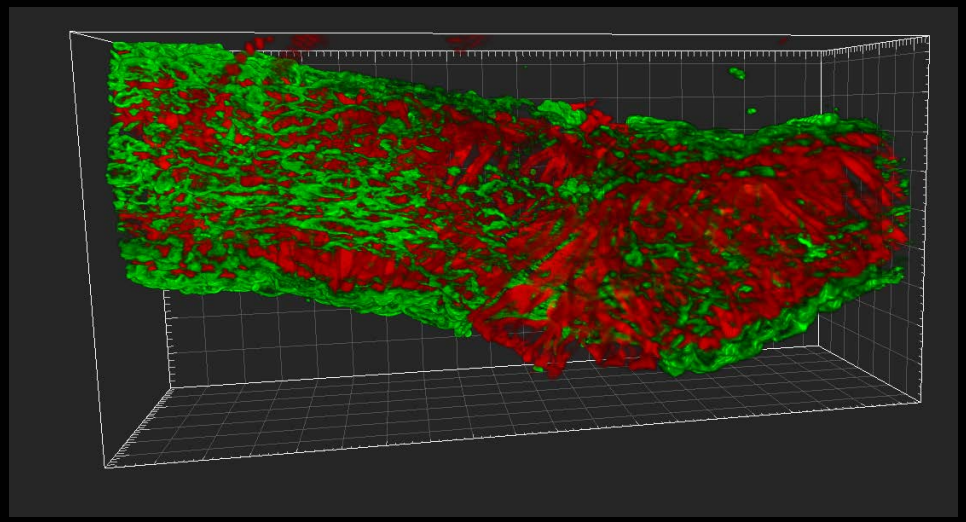
Maximal Intensity Projection

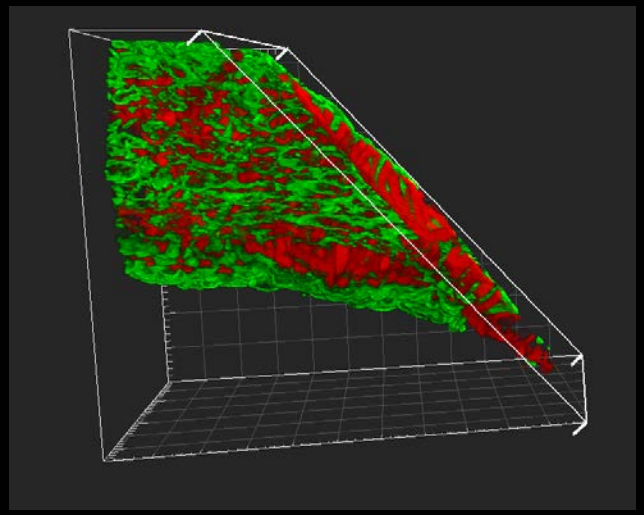


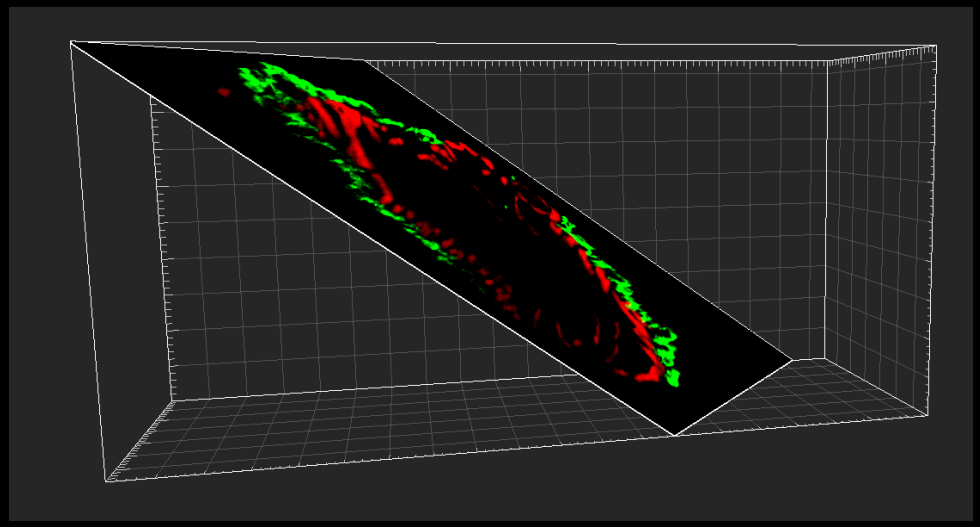
3D viewer – Isosurface mode with depth effect

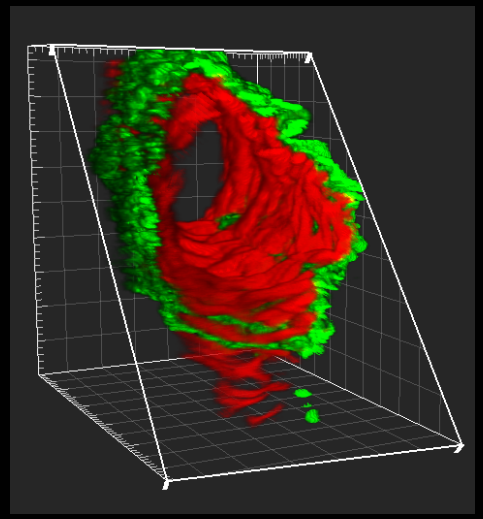


3D viewer - Image plane at different angle can be shown

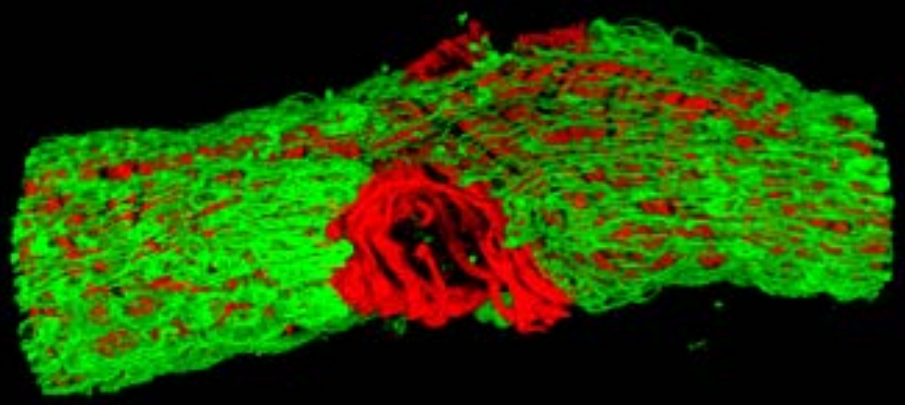




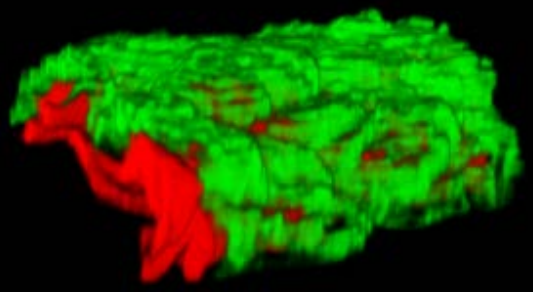


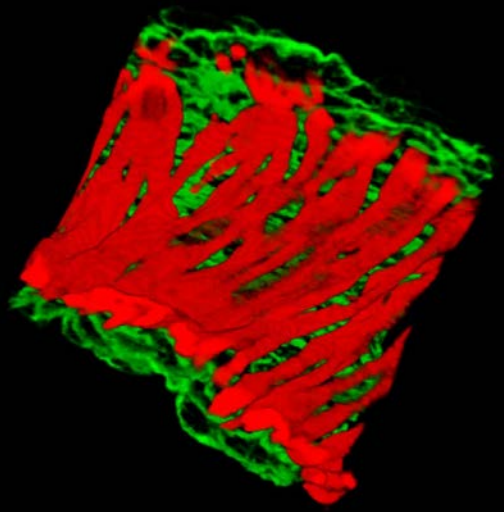


3D viewer - Dataset can be cropped in 3D

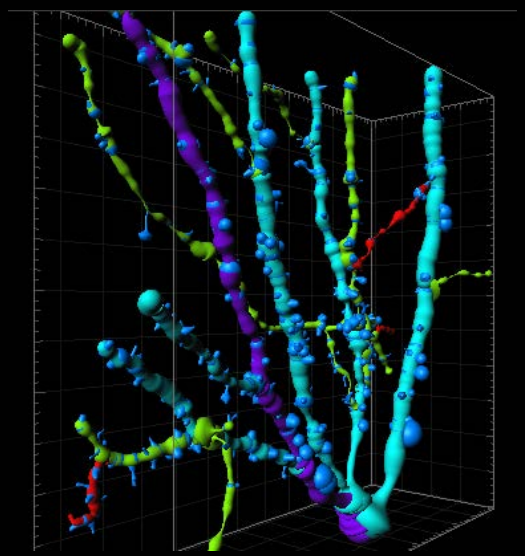


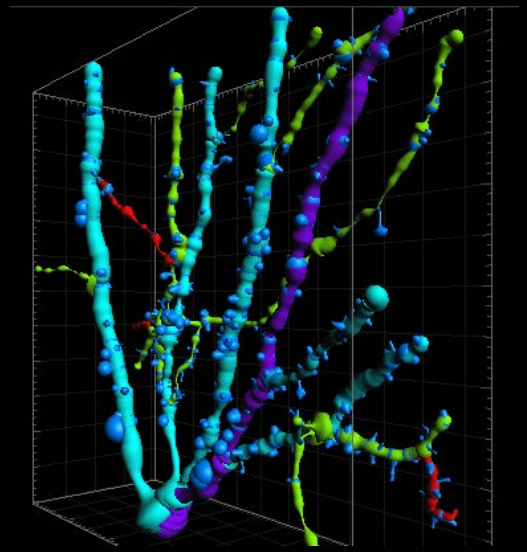
3D crop

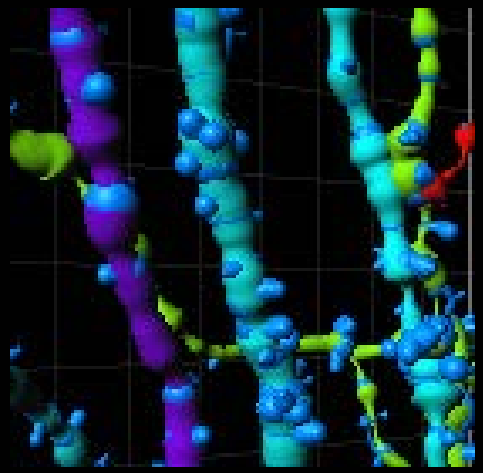


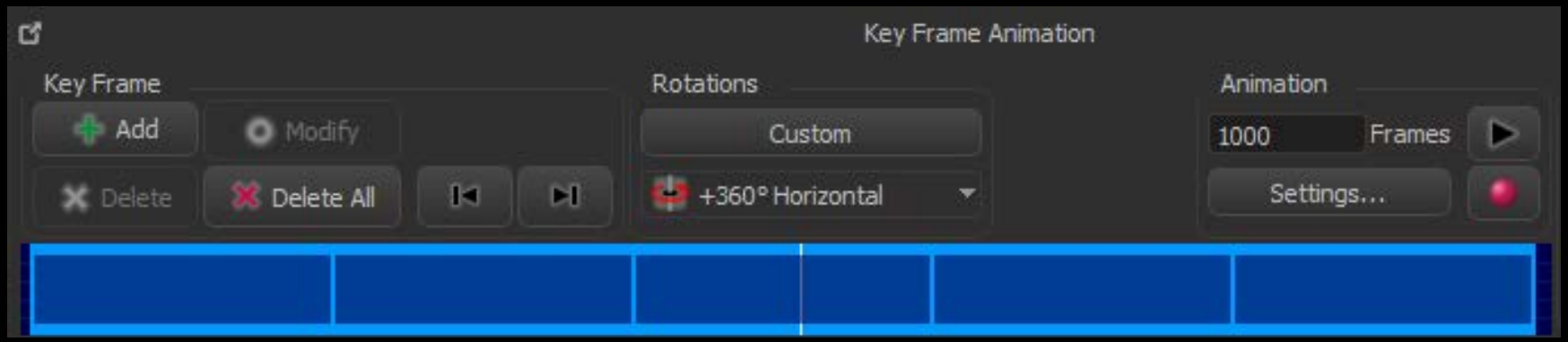


Data Visualisation – Video editing tool

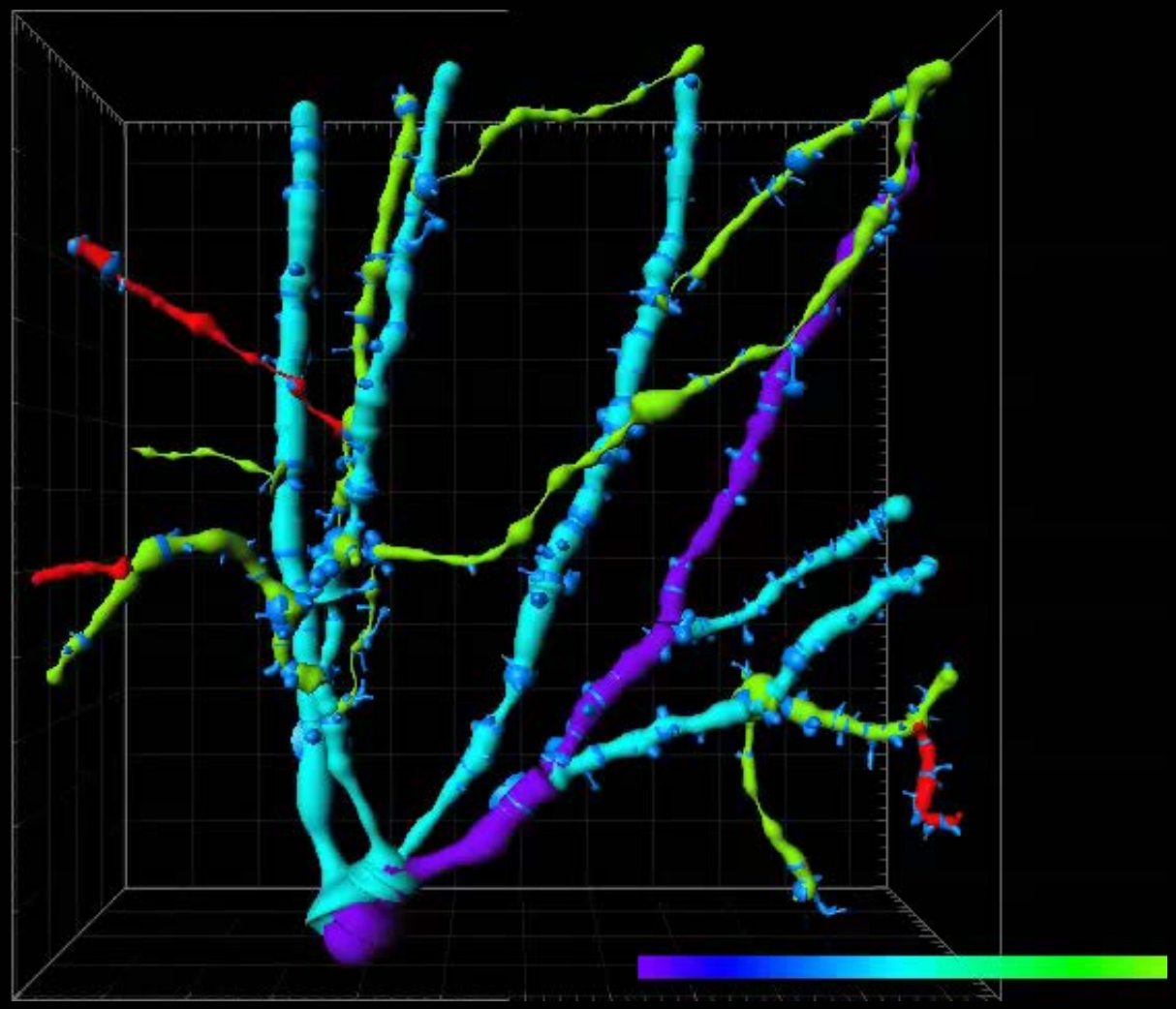






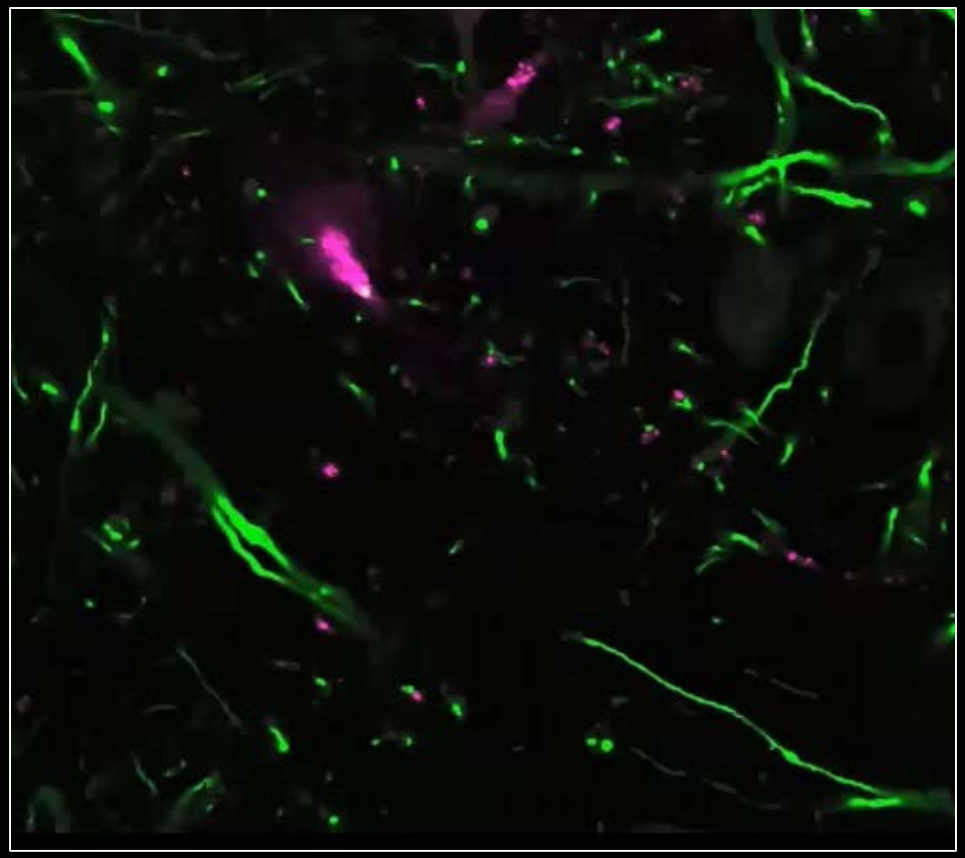


Visualise data set - Animation



Video Rendering – published example

Confocal Z stack. Cleared spinal cord



Soderblom 2015 eNeuro

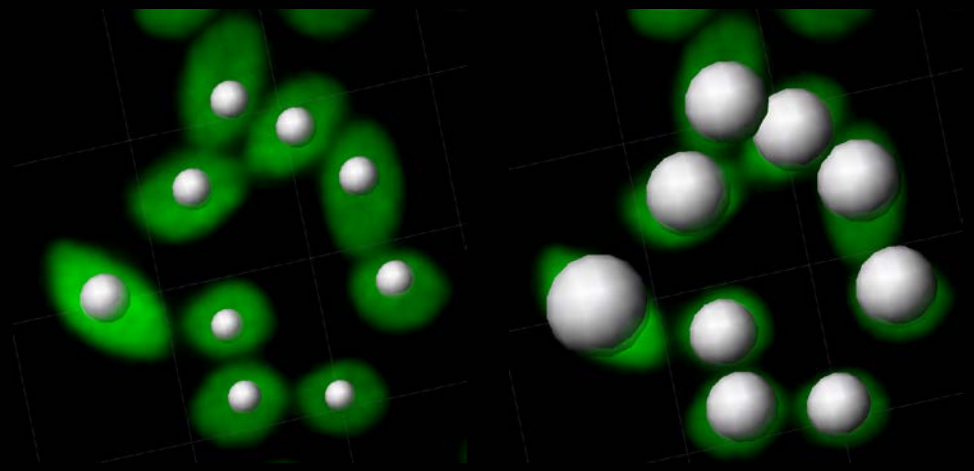
Confocal Z stack. Cleared lung

Li 2017 PNAS

3D image quantification

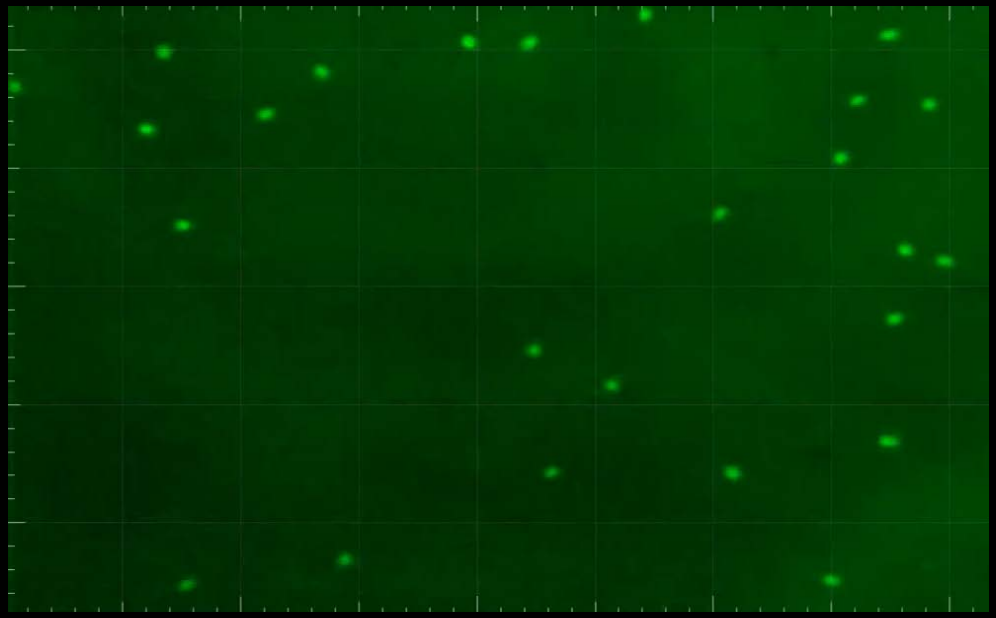
Counting in 3D

Spot detection is useful for simple counting
Does not detect exact volume & shape
simpler & faster than surface rendering

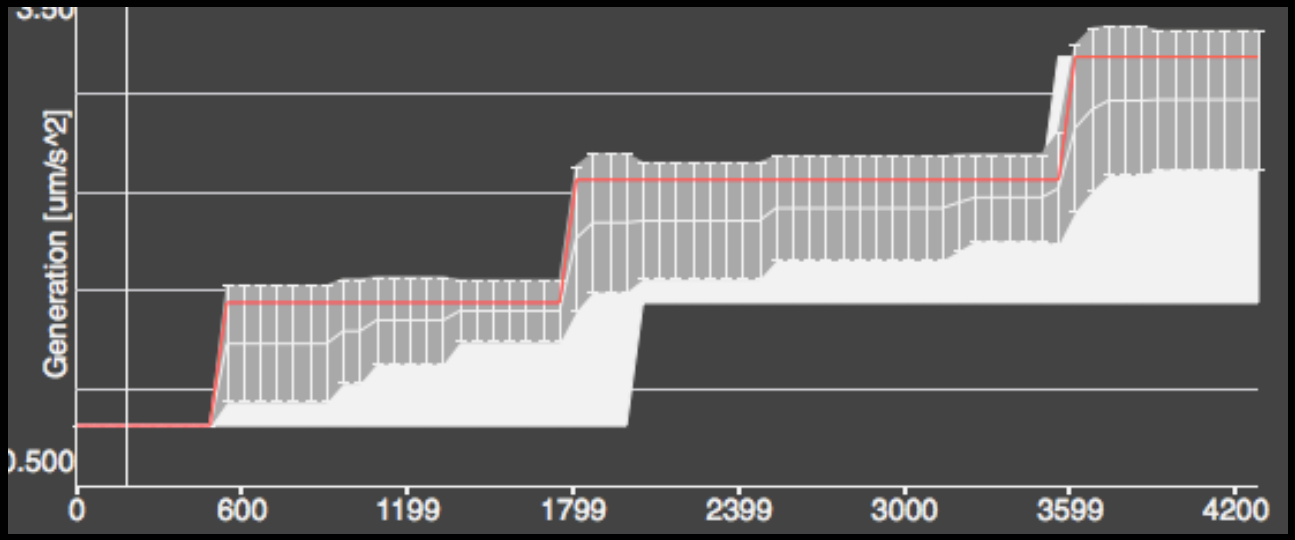


- X,Y,Z Position
- Diameter
- Volume of the sphere

Track – Spot



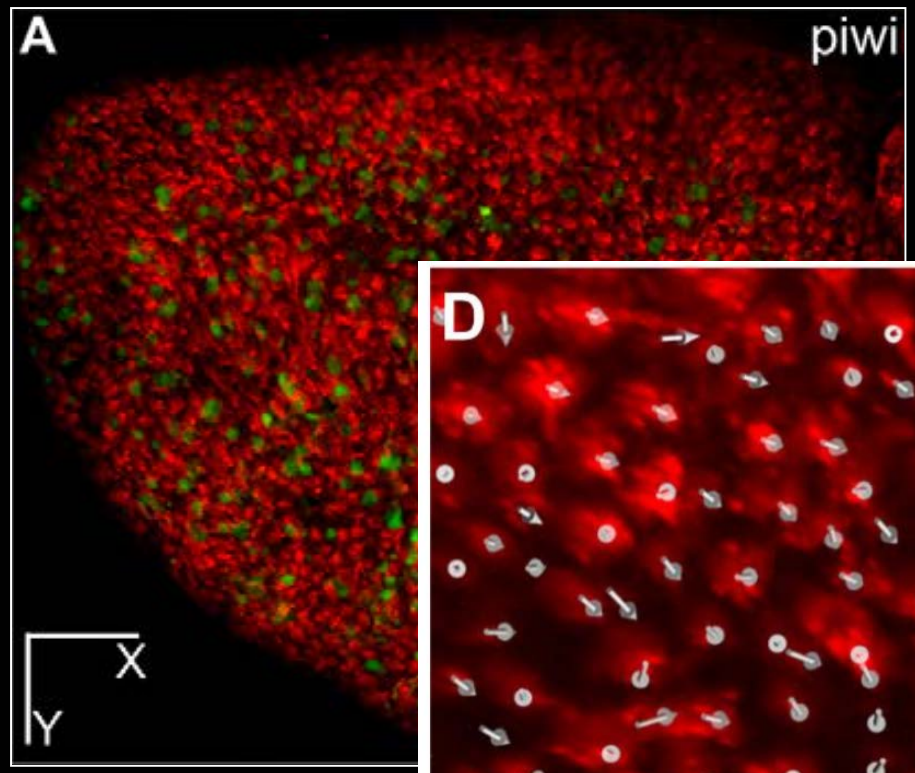
- Displacement
- Straightness
- Speed
- Lineage



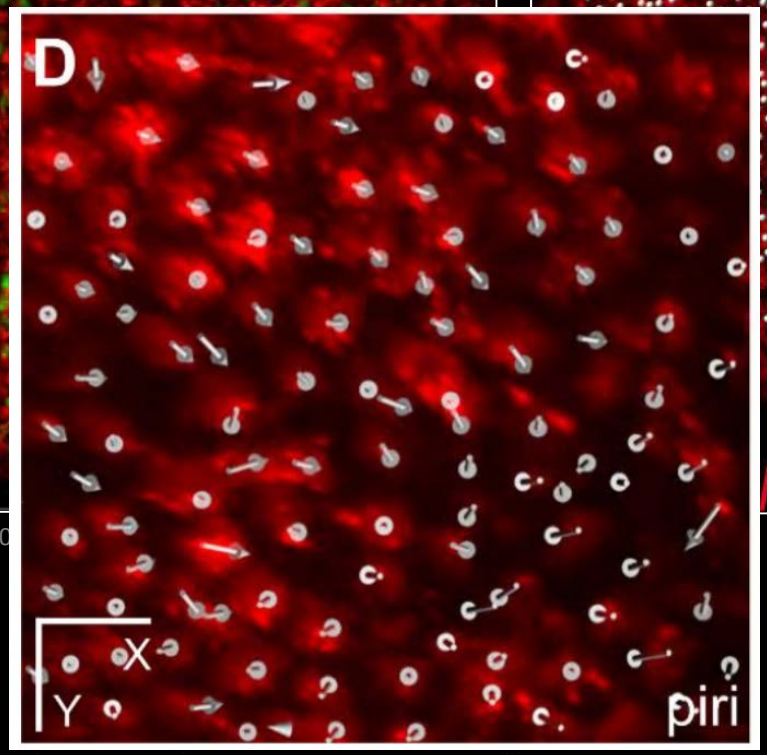
Case study - Counting + Tracking

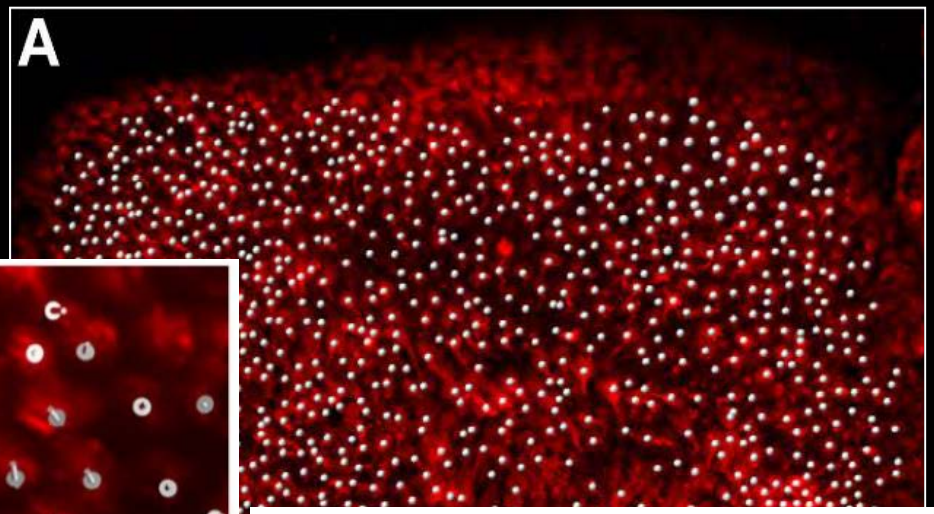
Hemisphere of GFP, RFP transgenic zebrafish image using multiphoton microscopy

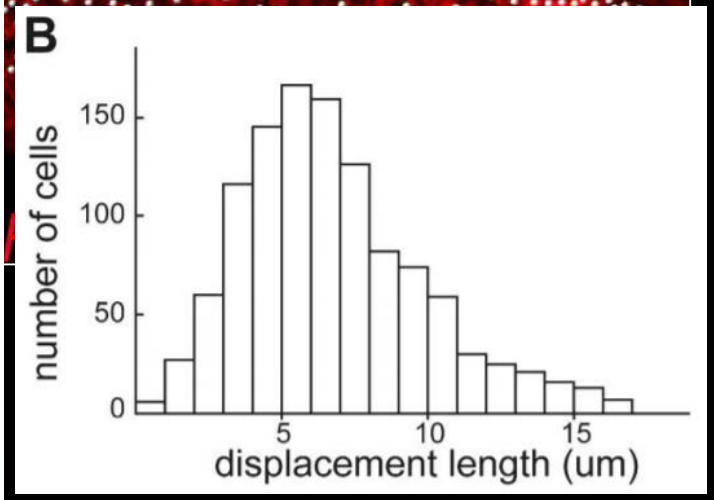
Total displacement length of RFP+ cells(n=1122) over 7 days measured



Dray 2015 Development 142: doi:10

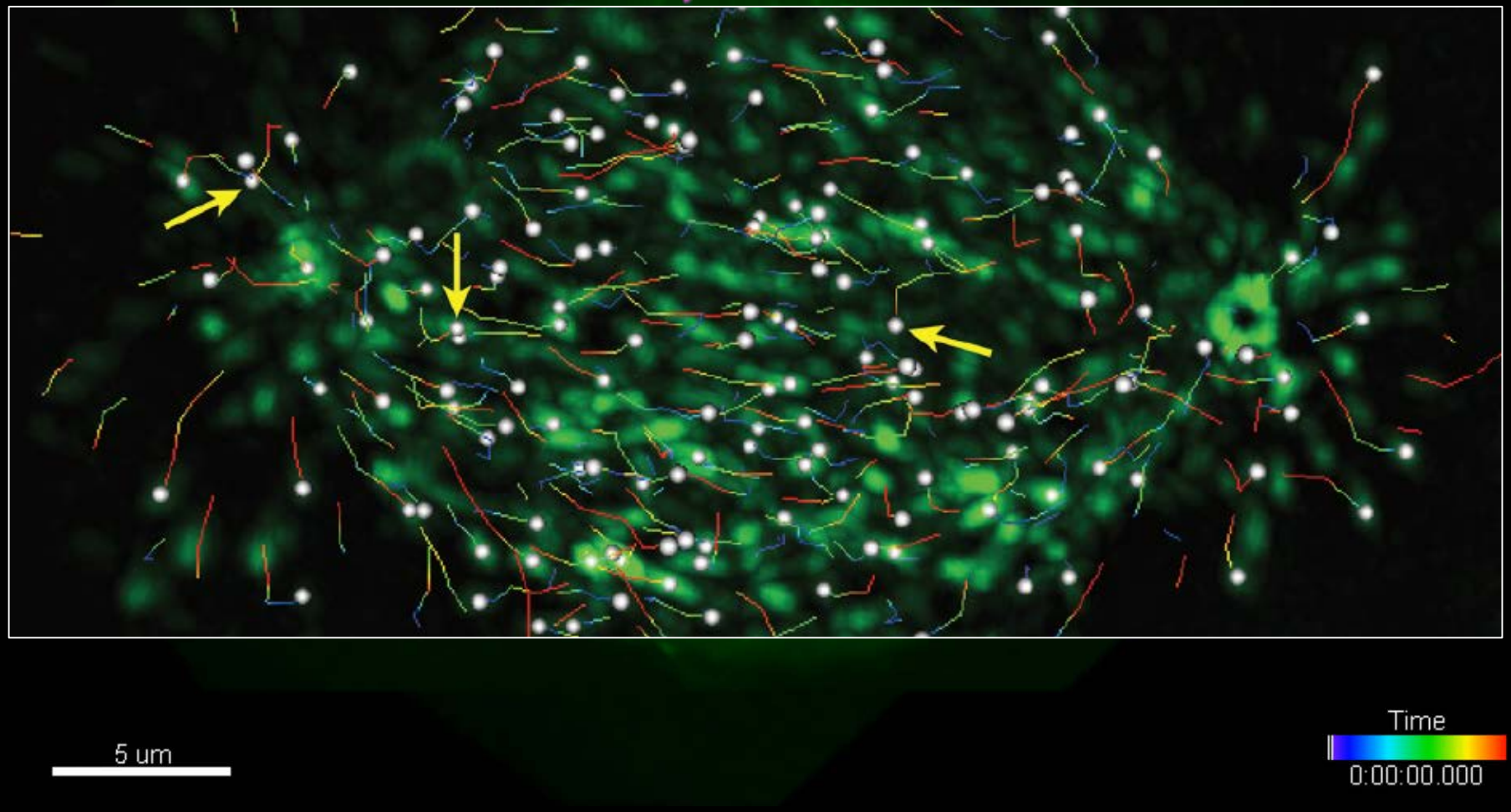






Case study - Tracking Spot

Microtubule growth marker in different phases of dividing cell was tracked in 3D
Using lattice light-sheet microscopy

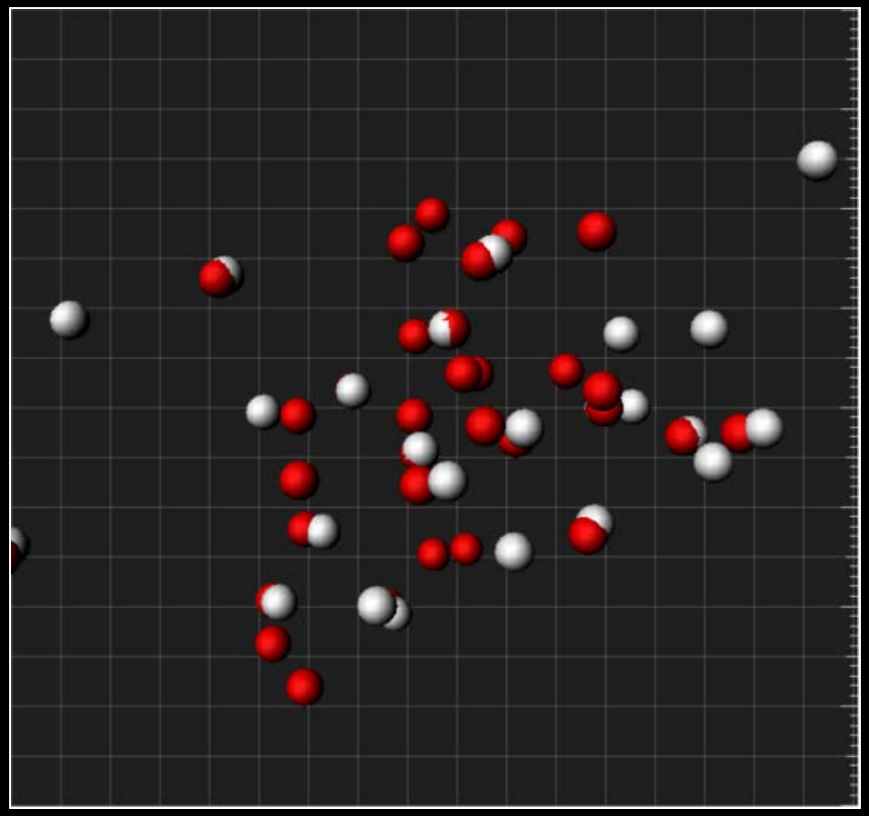


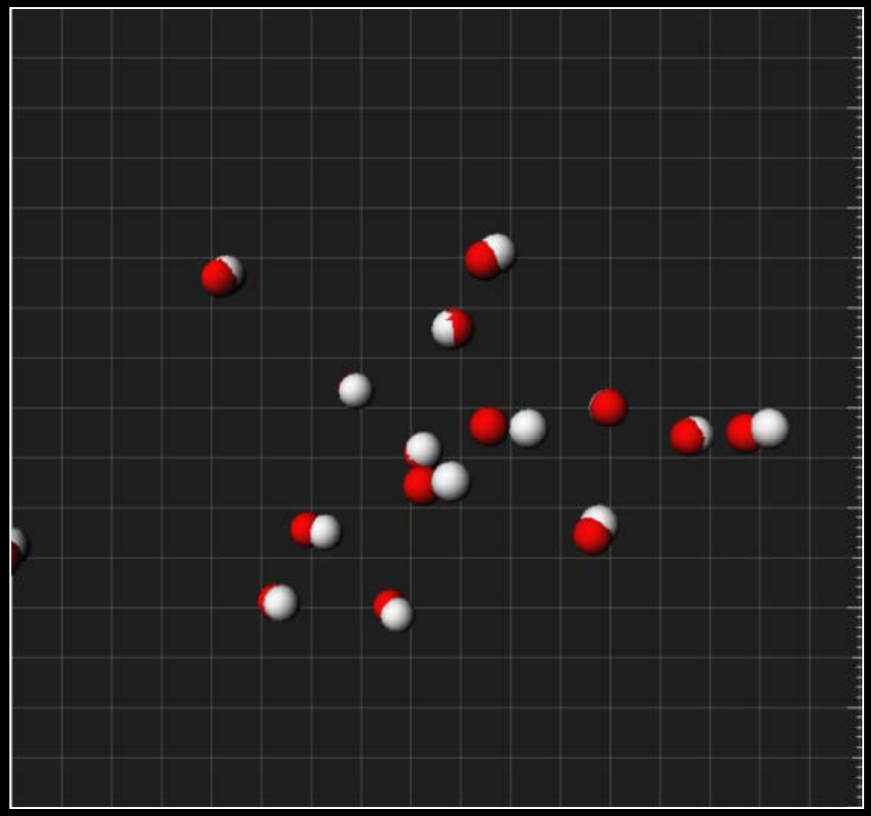
Yamashita 2015 J Biomed Opt

Object based colocalisation

Spot – Spot Colocalisation

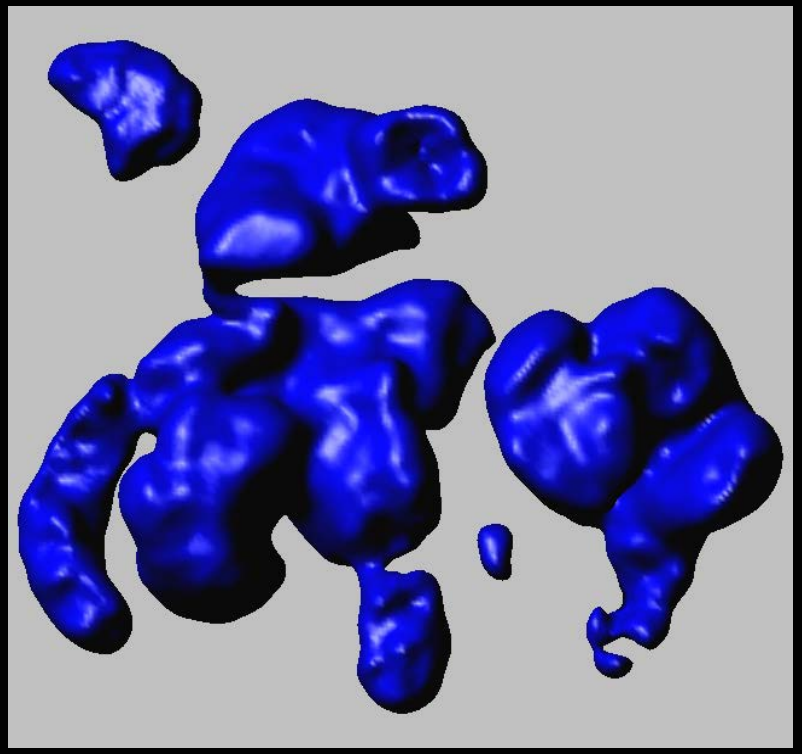
Detect overlapping spots

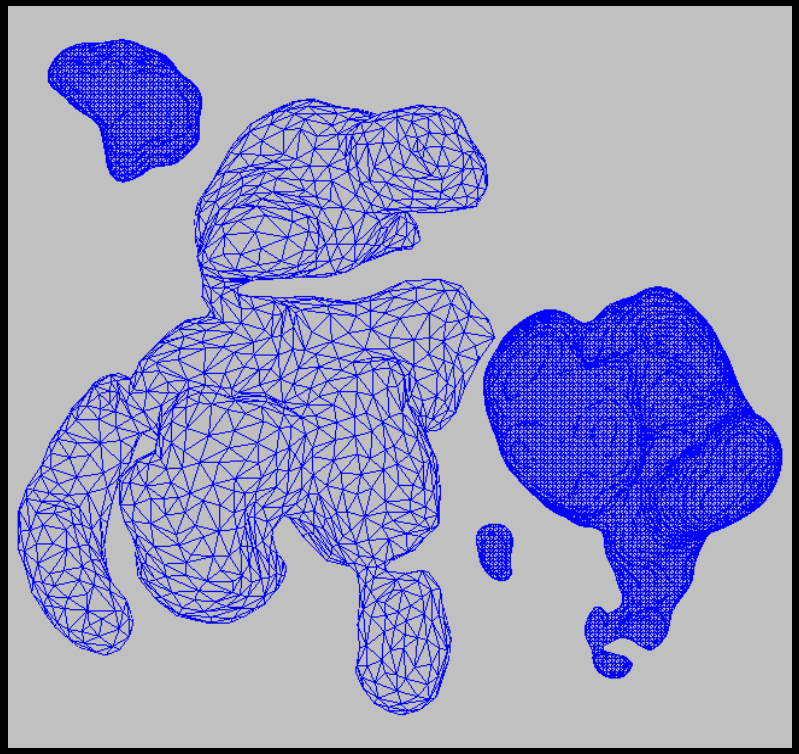




Volume measurement

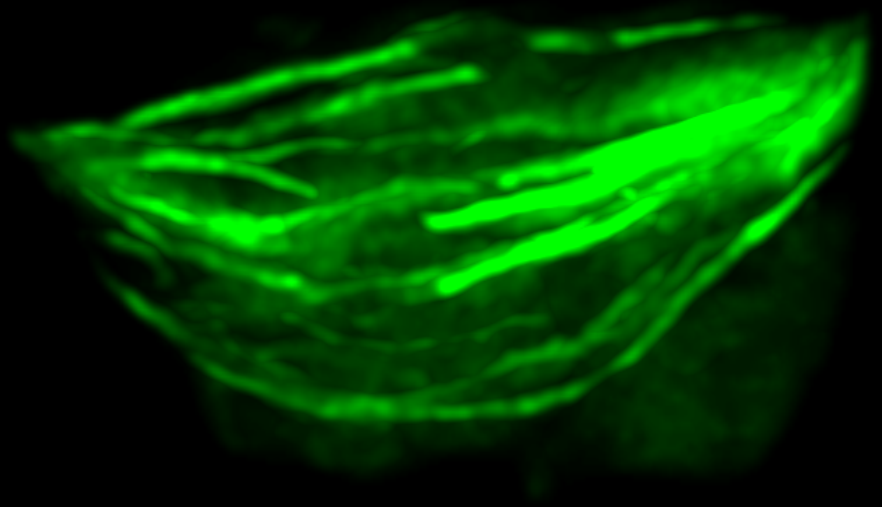
Surface detection finds isoline from the given detail setting and creates 3D surface.



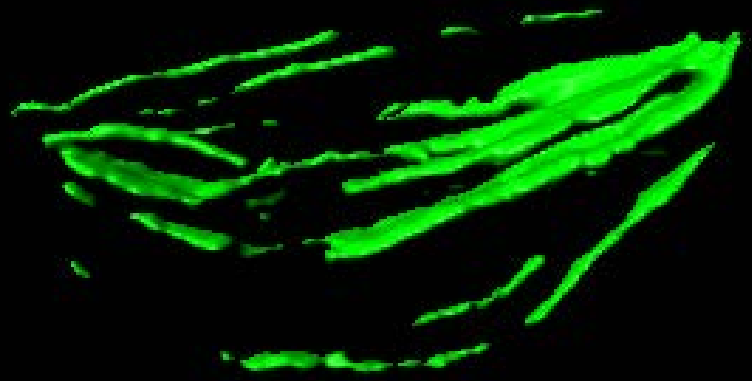


3D Surface detection – Automatic

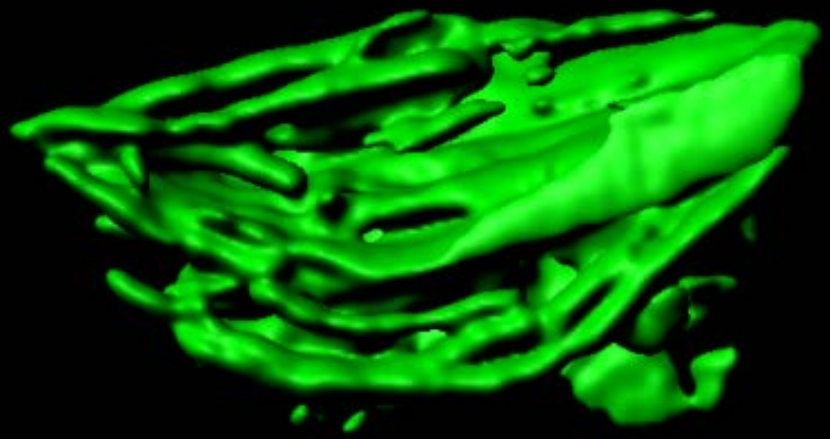
Surface detection is done automatically if using intensity thresholding



High threshold



Low threshold

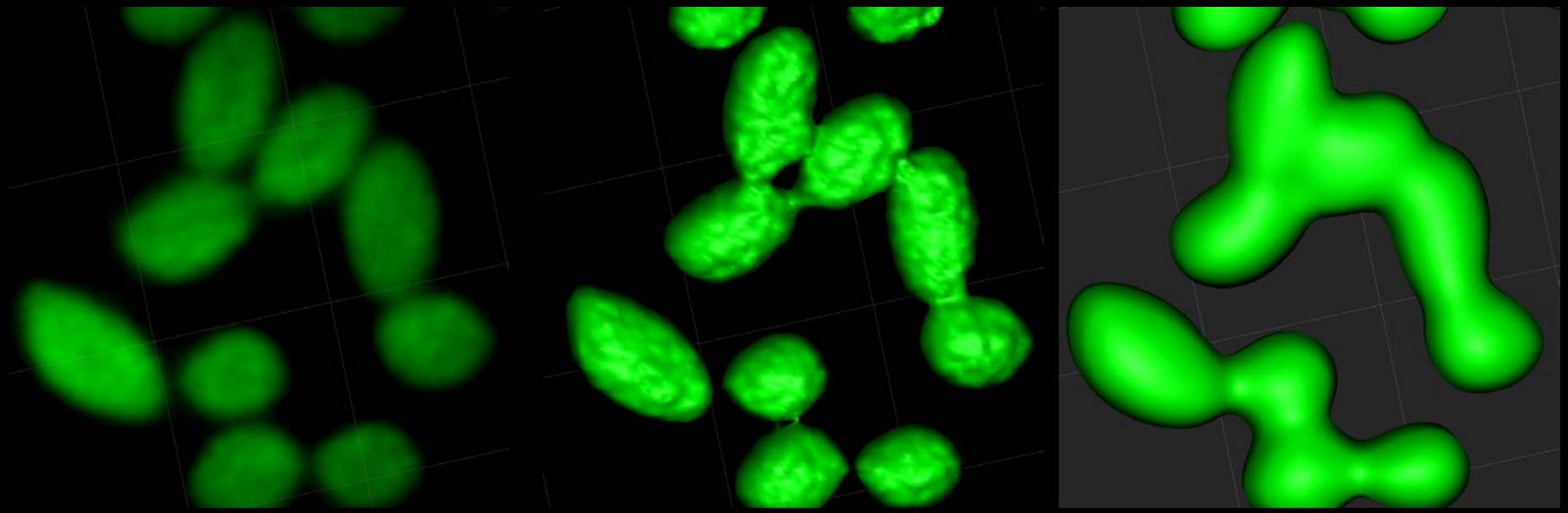


3D Surface detection – Automatic

Surface detail can be adjusted during detection

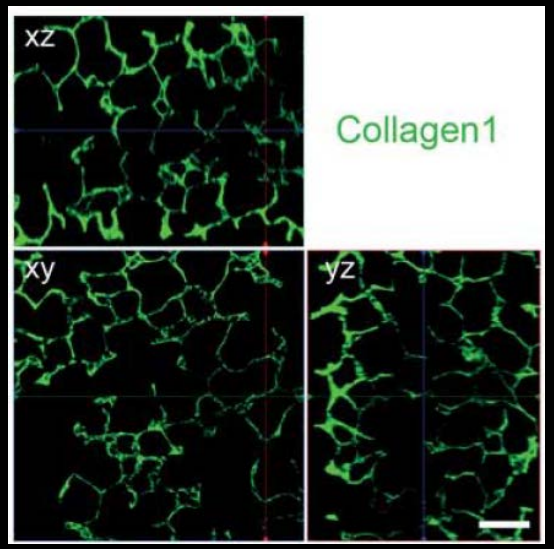
High detail
Slower process

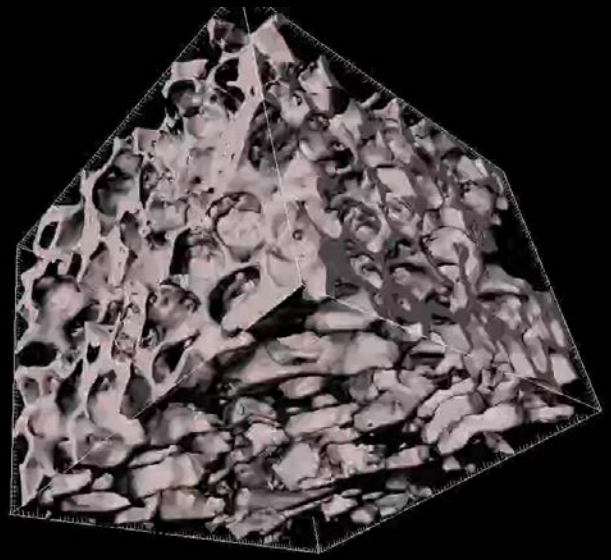
Less detail
Faster process



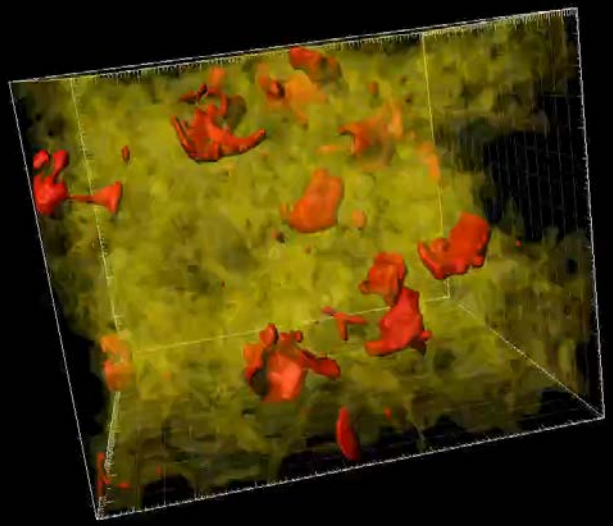
Case study - Surface detection

3D cell culture extracellular matrix model
decellularized *ex vivo* lung tissue scaffolds, Reseed the fibroblast cells





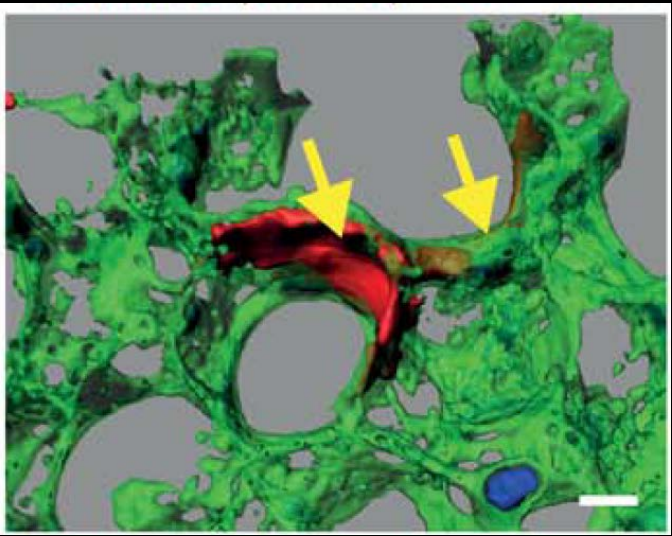
3D confocal z-stack of Collagen1



3D confocal z-stack of
- Lung fibroblasts (red)
- Collagen 1 (yellow)

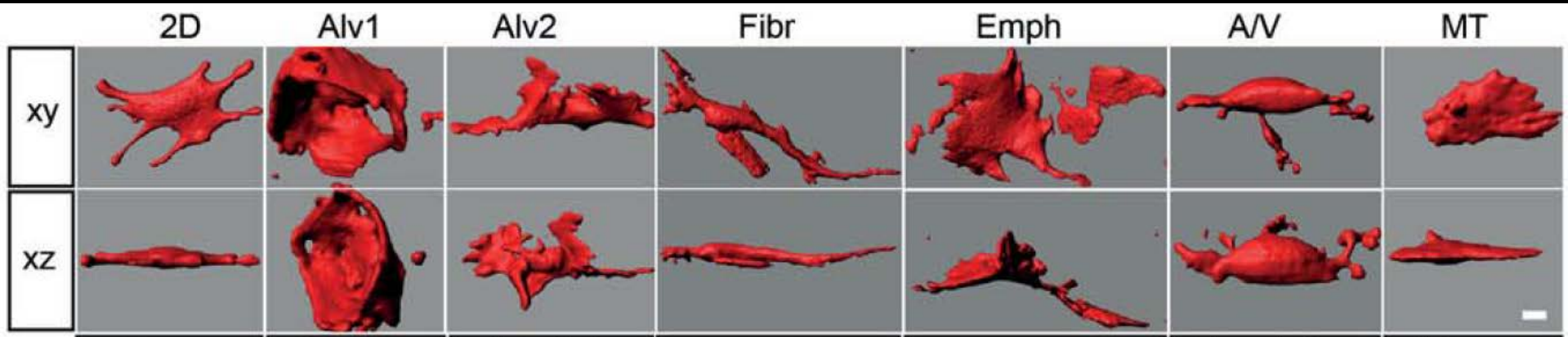
Burgstaller 2018 Am J Physiol Lung Cell Mol Physiol. doi: 10.1152/ajplung.00408.2017

Case study - Surface detection



3D cell shapes of mouse lung fibroblasts

- cell volume (µm3)
- cell surface area (µm2)
- Shape descriptor (prolate, oblate)

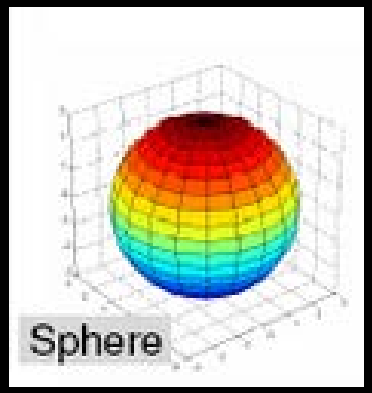


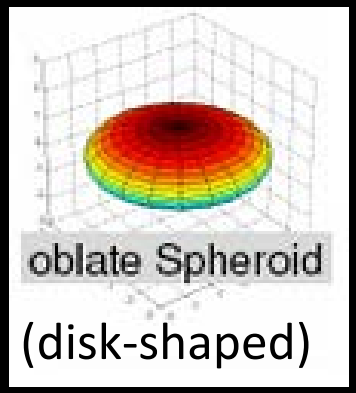
2D	Alv1	Alv2	Fibr	Emph	A/V	MT
- flat - multipolar - huge cell body	- flat - dome like - moderate cell body	- elongated - multipolar	- elongated - spindle shaped - bipolar	- flat - sheet like - multipolar - huge cell body	- elongated - bulbous - bipolar - small cell body	- flat - small cell body

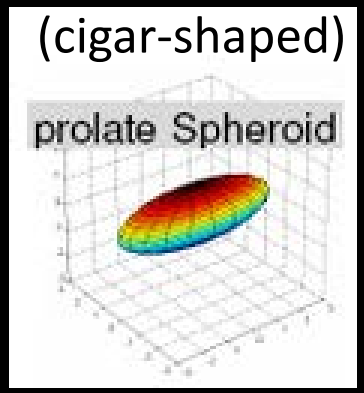
Burgstaller 2018 Am J Physiol Lung Cell Mol Physiol. doi: 10.1152/ajplung.00408.2017

Measurement - 3D Shape descriptors

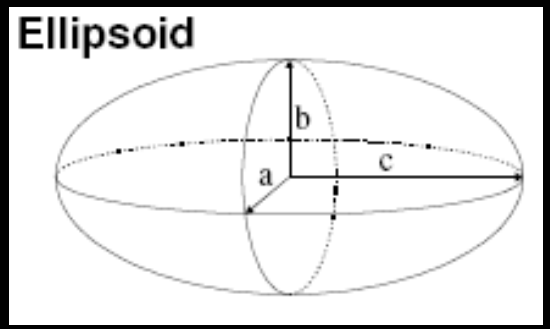
Sphericity



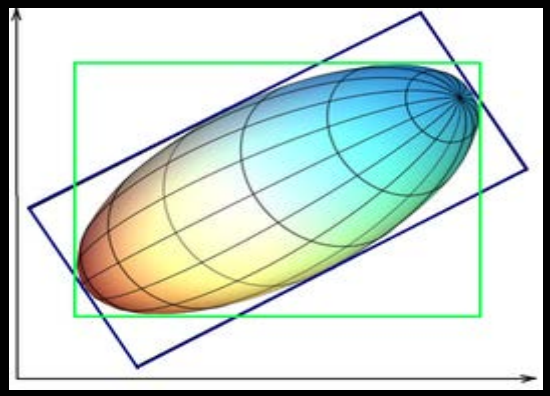




Ellipticity in 3D



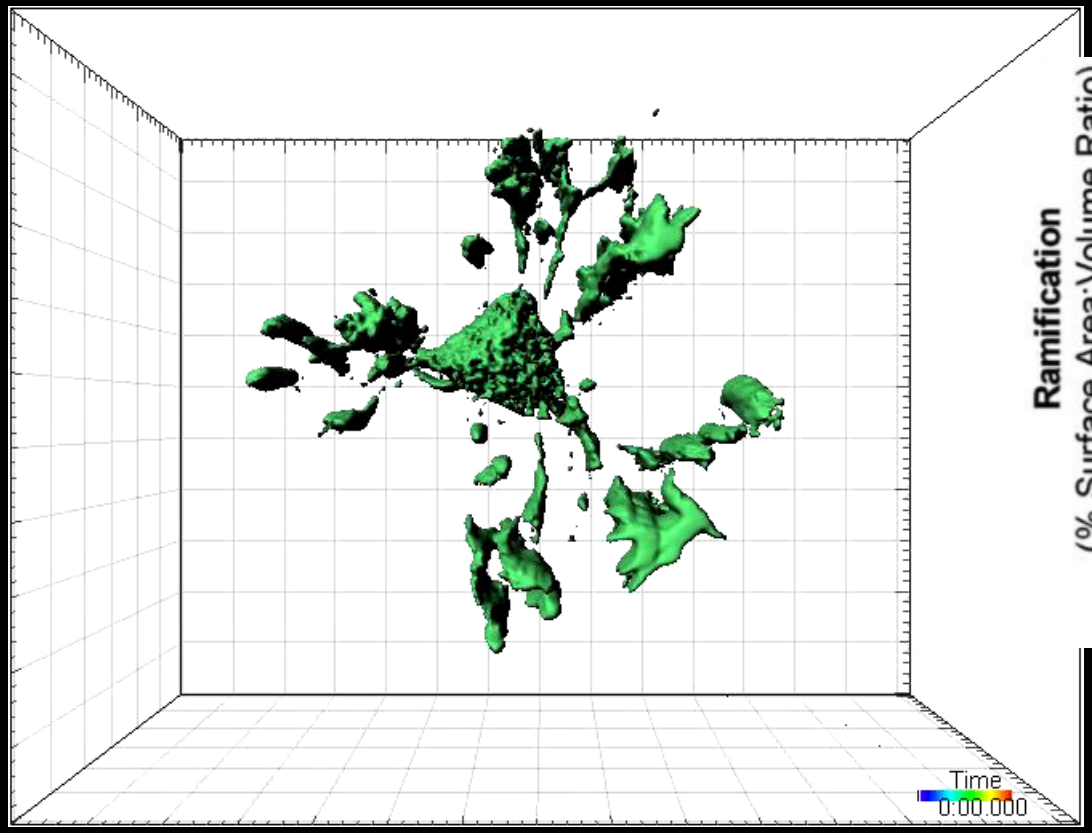
Bounding box (x,y,z)
(axis-aligned, object oriented)

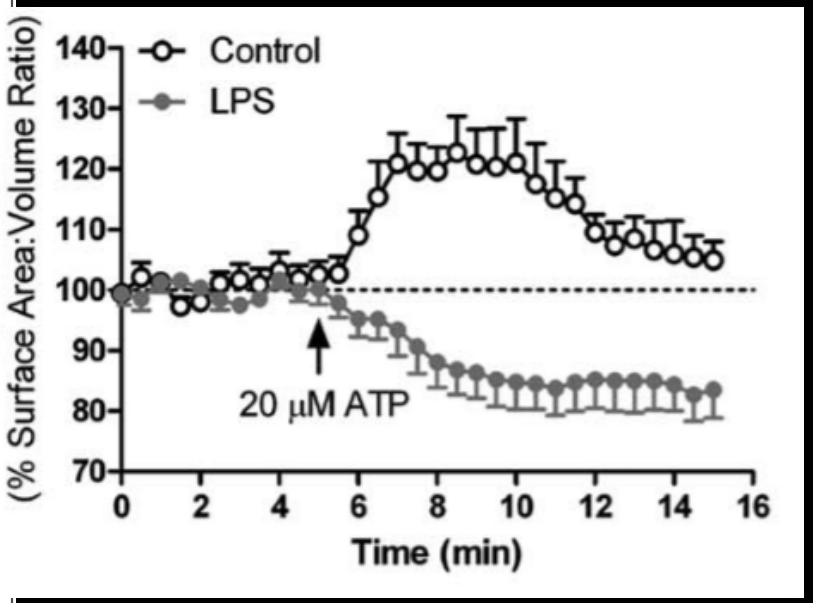


Case study - Tracking surface

Ramification of microglial cell were analysed in time series.

Area and volume of surface object were measured and the ratio was calculated at each time point.

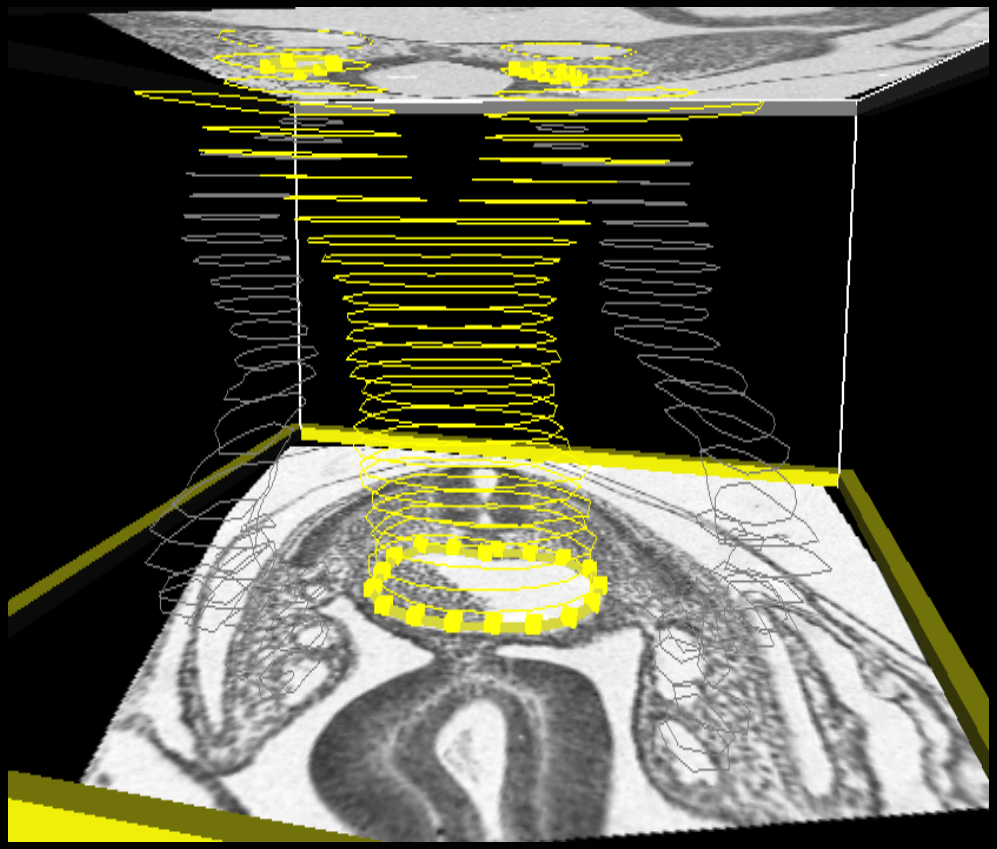


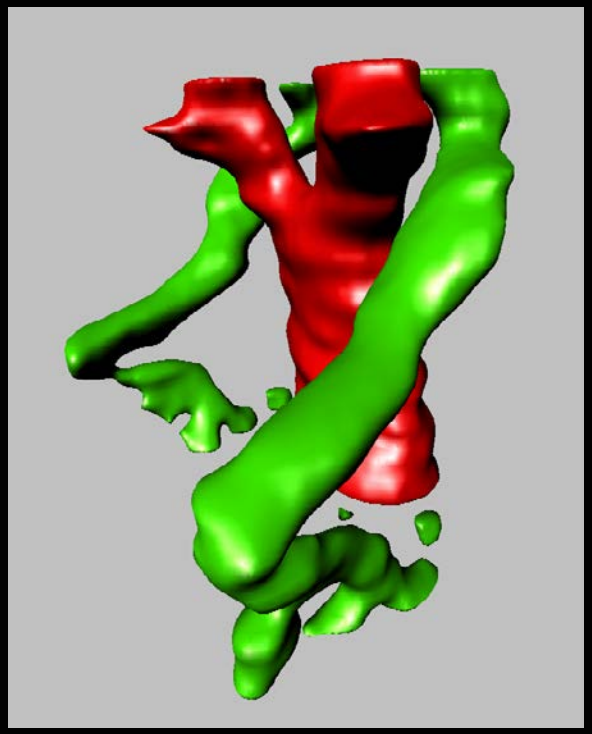


Gyoneva 2014 Glia

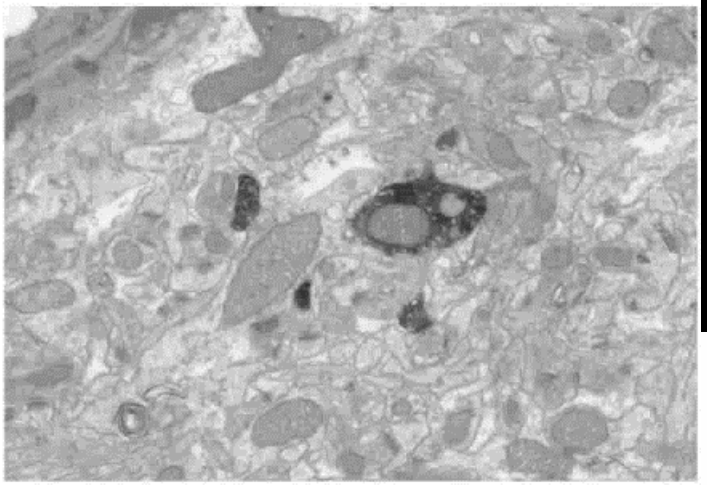
3D Surface detection – Manual

Manual Surface detection may be necessary if the signal cannot be segmented from background or neighbouring structures





Case study - Manual 3D Surface detection



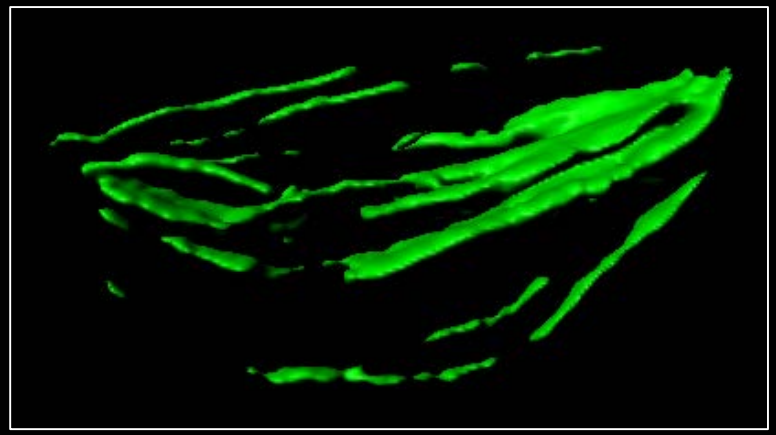
Bosch et al 2015 Frontiers in Neuroanatomy

1000 nm

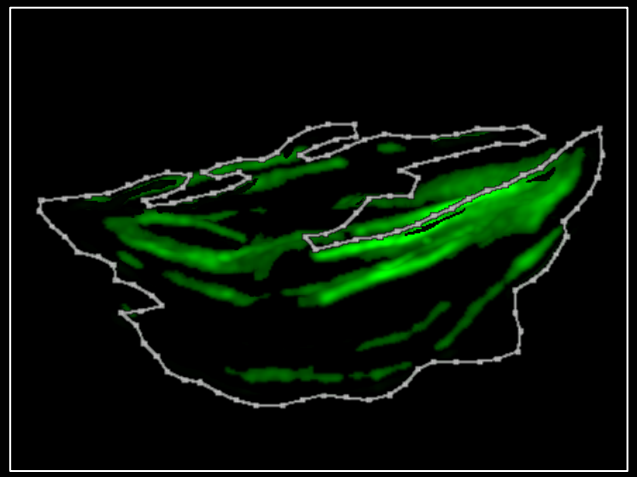
Colocalisation
Coexistance
Relationship

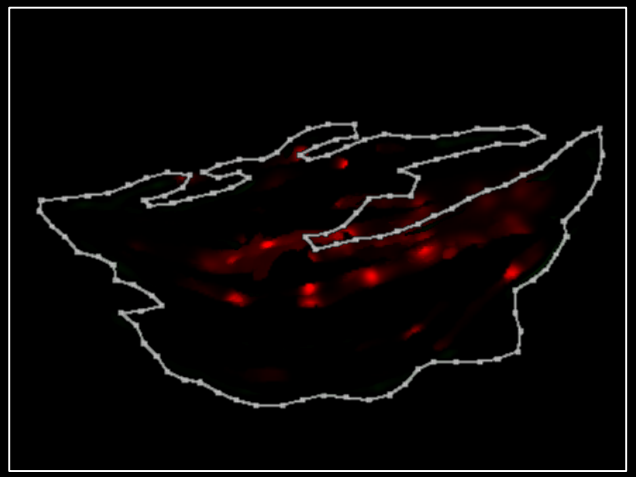
Measurement from segmented objects

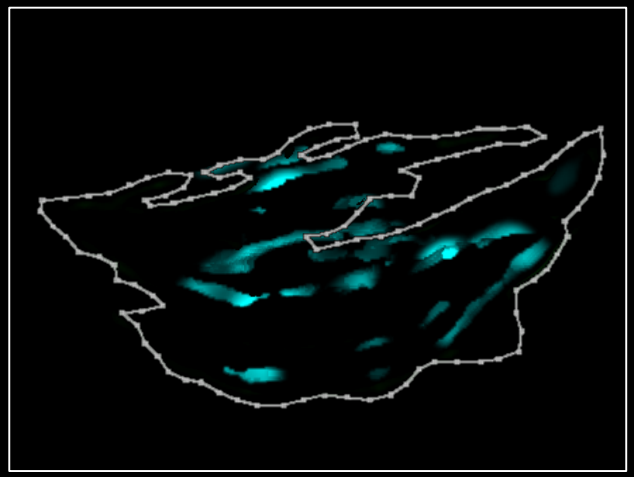
Surface created from green channel



Segmented objects can be used as a mask
Intensity of all other channels can be measured

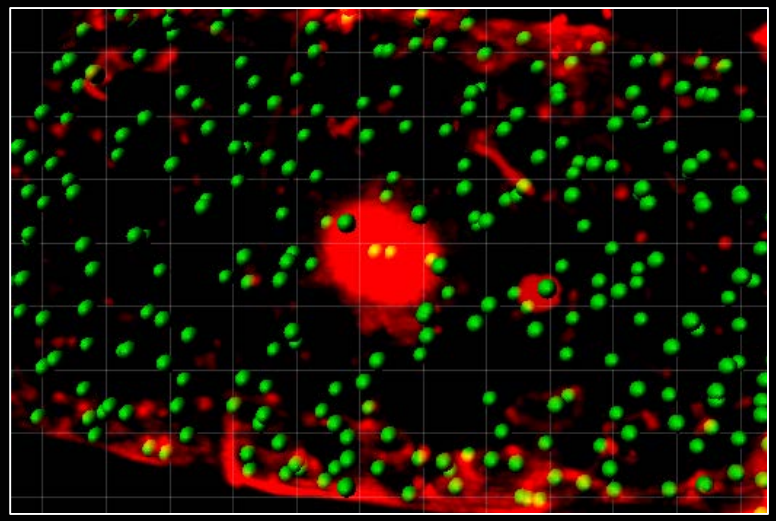






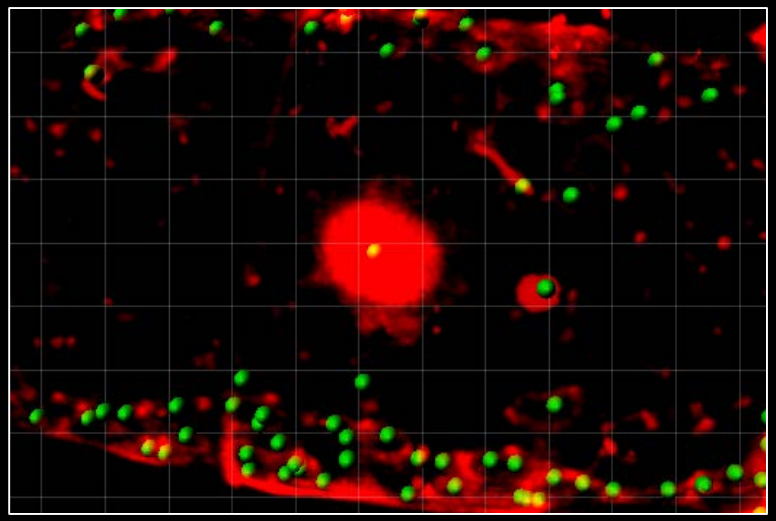
Select based on the intensity of other channel

e.g. Counting green cells that are also Red positive



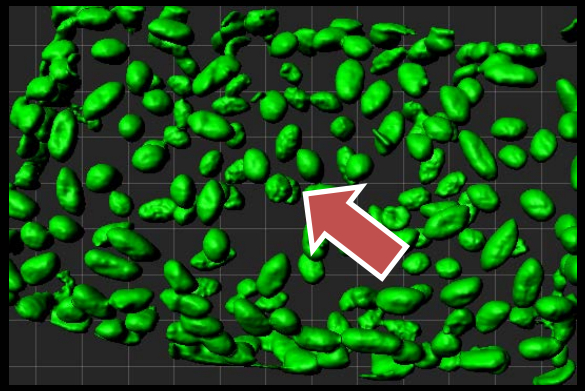
Detect Green cells

Measure the red intensity inside the green spots

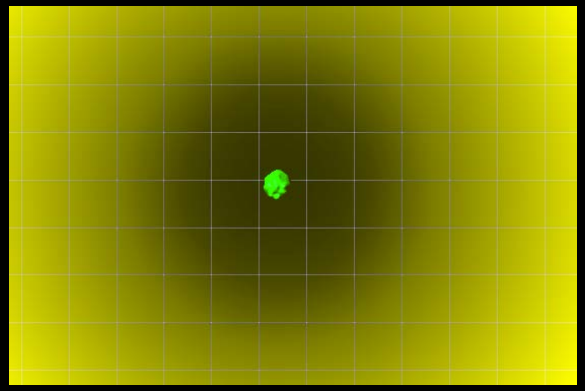


Select green cells with red intensity above threshold

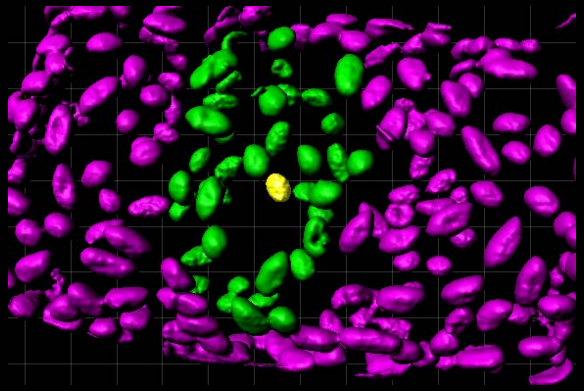
Select based on the distance from other object



e.g. Select the Green cells that are within 30um from the center cell (arrow)

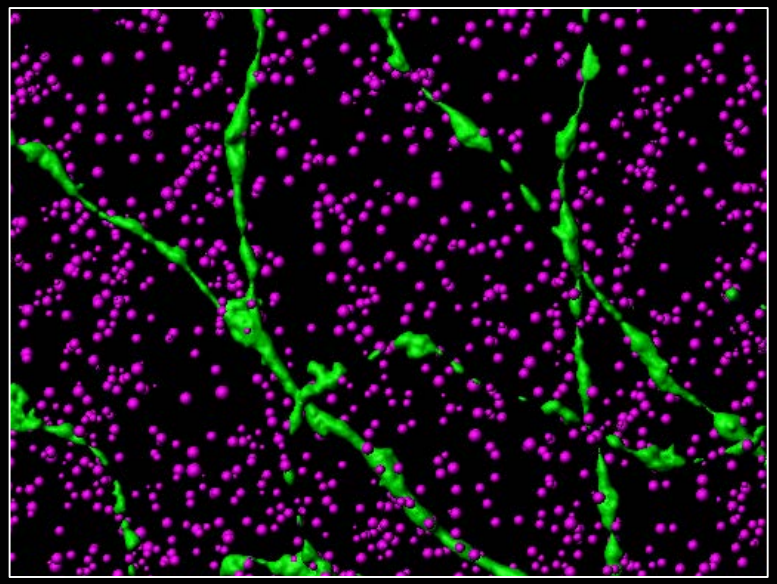


Create a distance map from the center cell



Select green cells based on the distance map

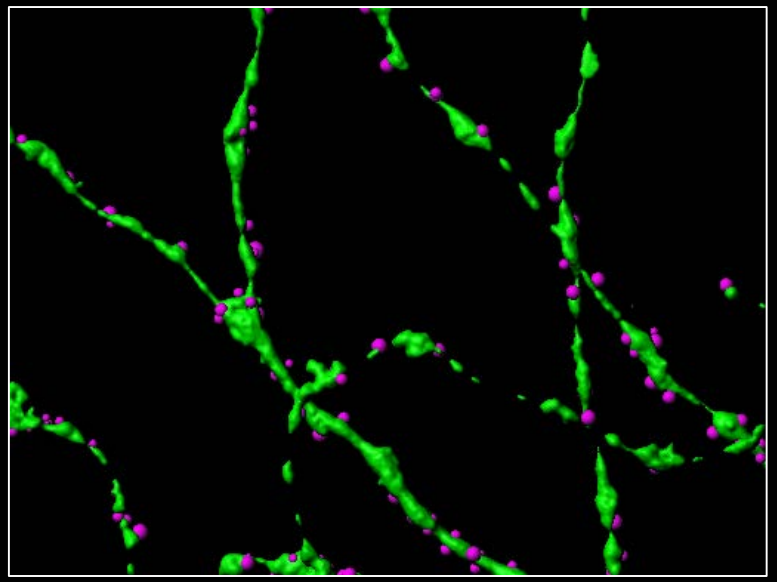
Case study – touching object detection



Select the synaptic marker that are in contact with the neurons

Synaptic marker : Spot detection (Magenta)
Neuron : Surface detection (Green)

Distance map for Green
Select spot based on the map (within 1um)



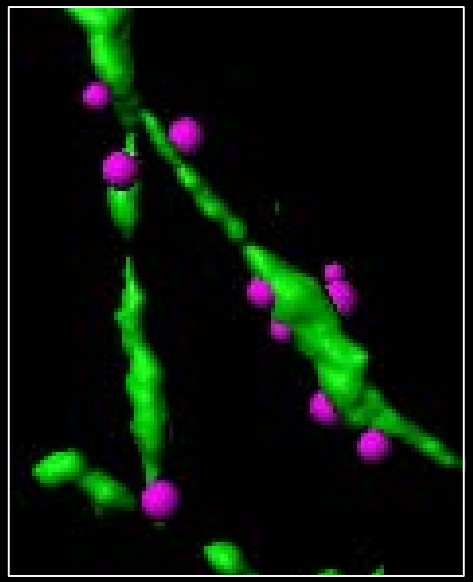
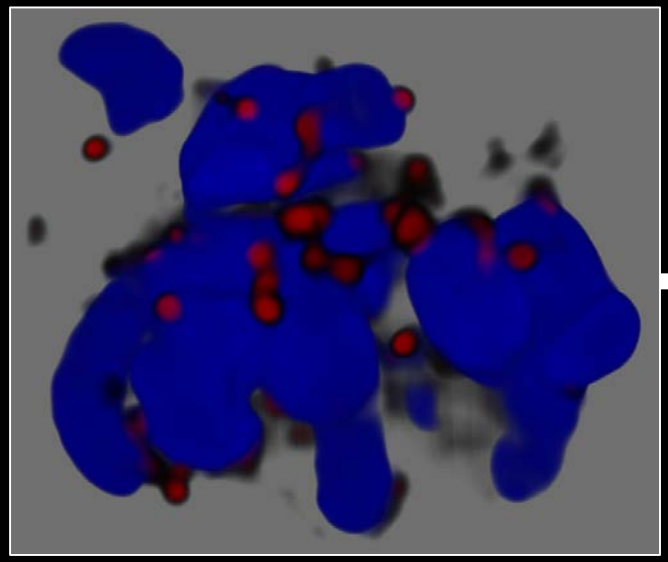
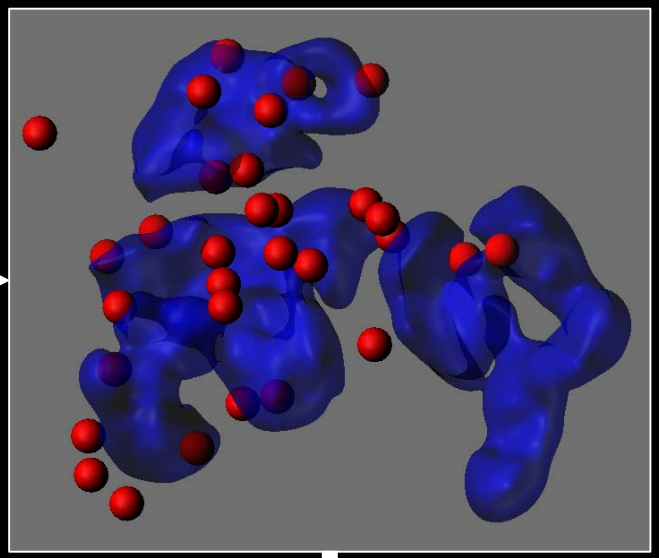


Image courtesy of Susmita Saha

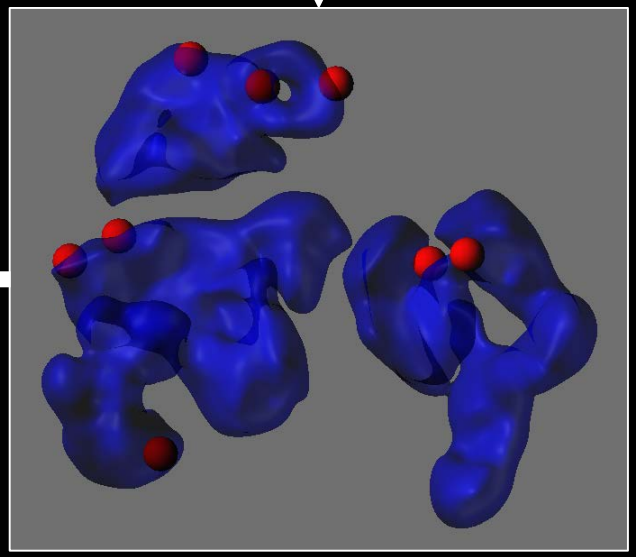
Categorise objects by other object



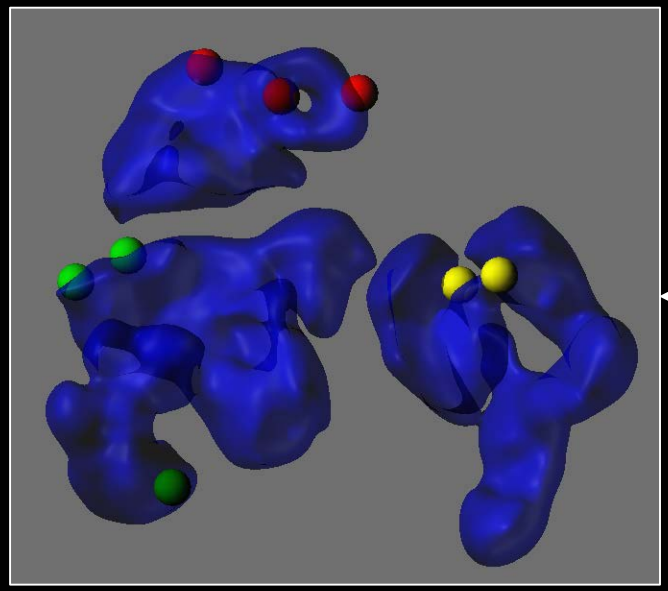
Detect 3D objects (red, blue)



Detect touching object

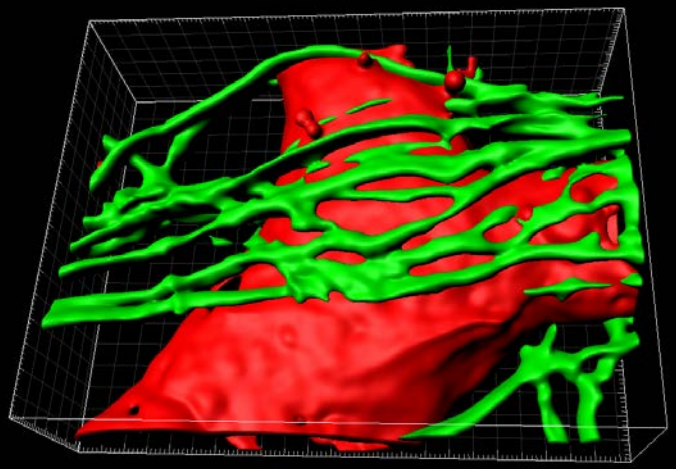


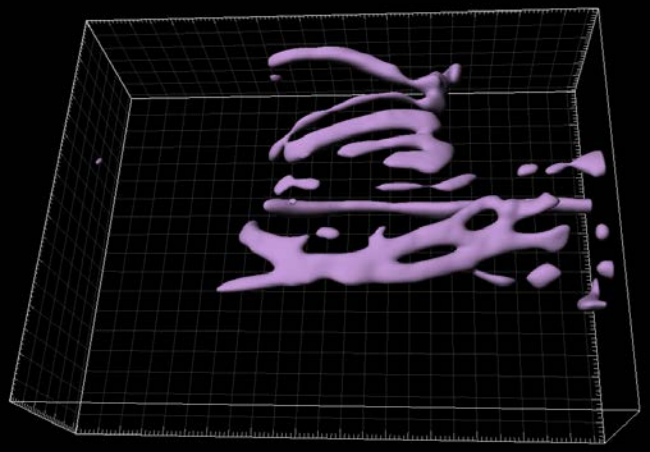
Categorise object By blue surface



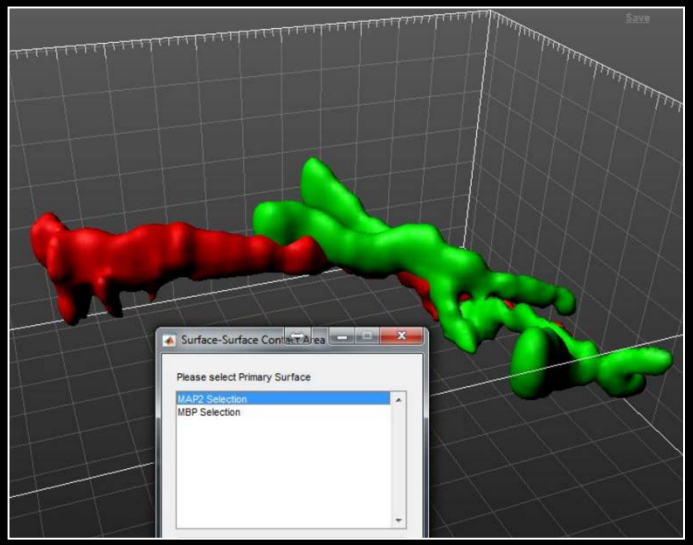
Object based colocalisation

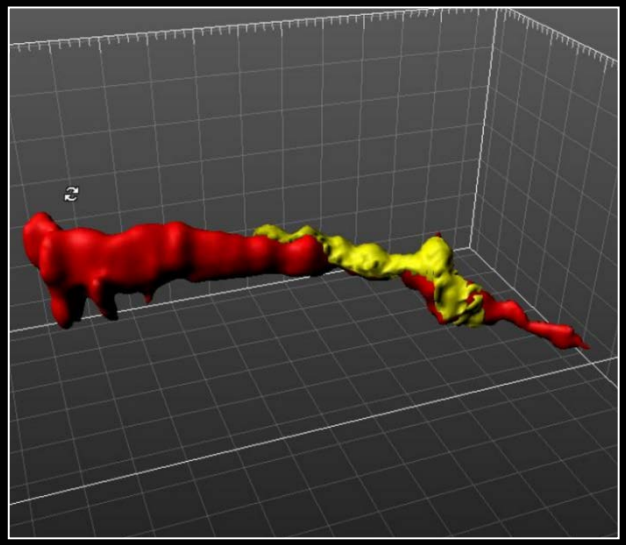
Volume Colocalisation - create a new surface on overlapping area





Surface-surface Contact Area

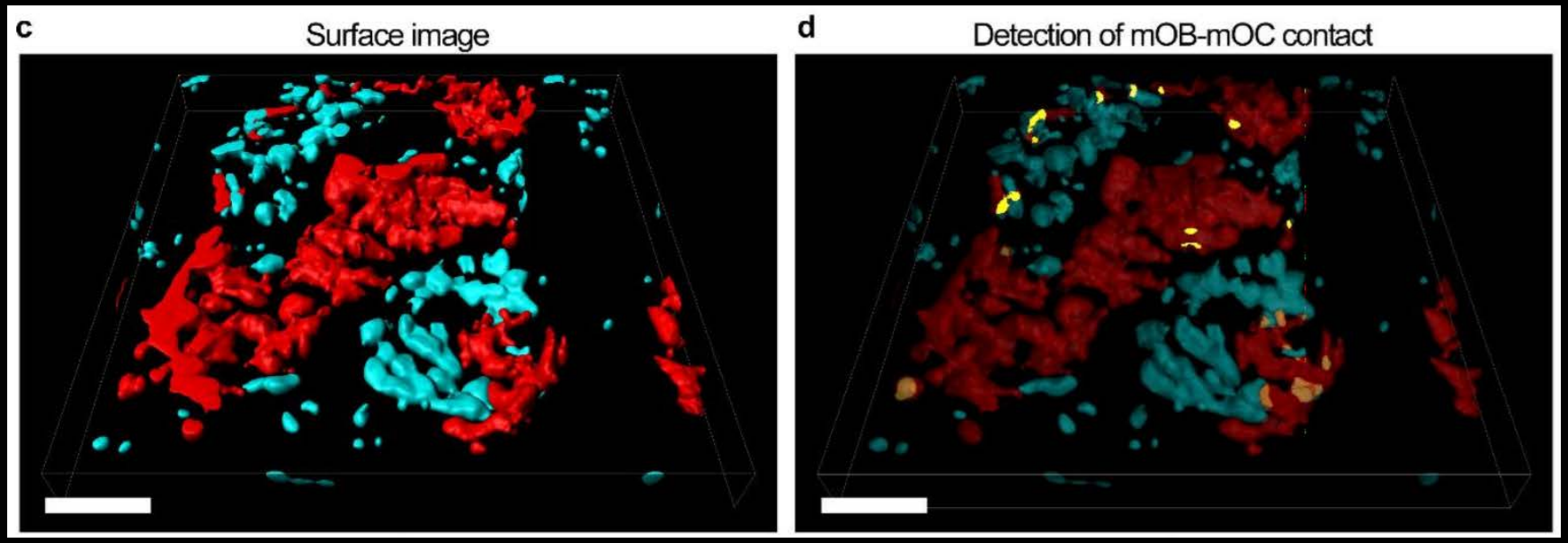




Case study - Surface-surface Contact Area

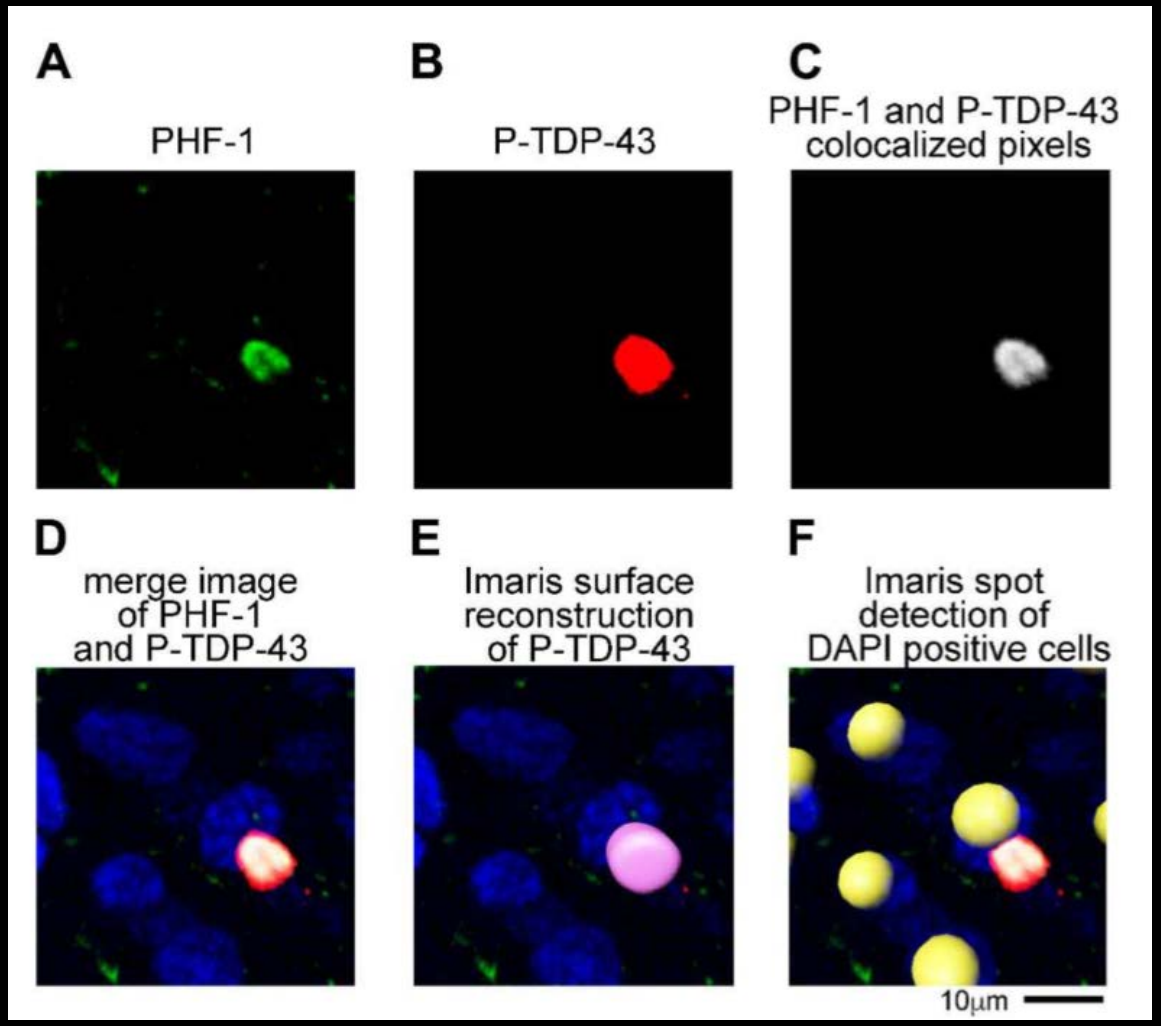
Mouse skull bone tissue. Zstack from two-photon microscopy

Direct cell–cell contact between mature osteoblasts and osteoclasts
Cell surface was created (cyan, red) then the surface contact area (yellow) was detected



Furuya et al. 2018 Nature Communications 9:300

Utilising 'Colocalisation channel'



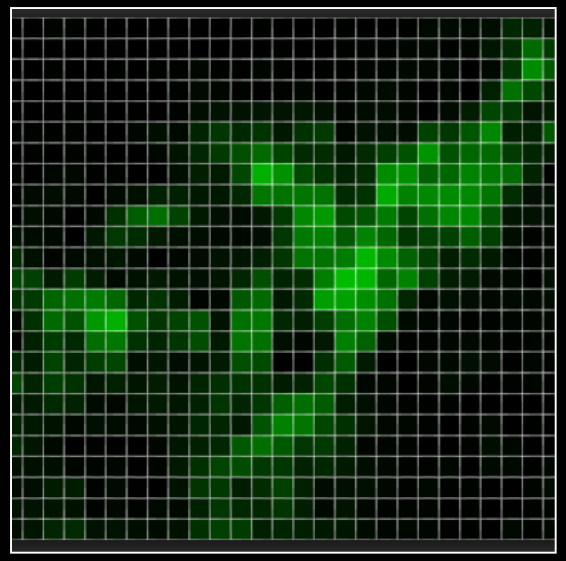
In Alzheimer's disease study, coexistence of 2 markers, PHF-1 and TDP-43, were quantified.

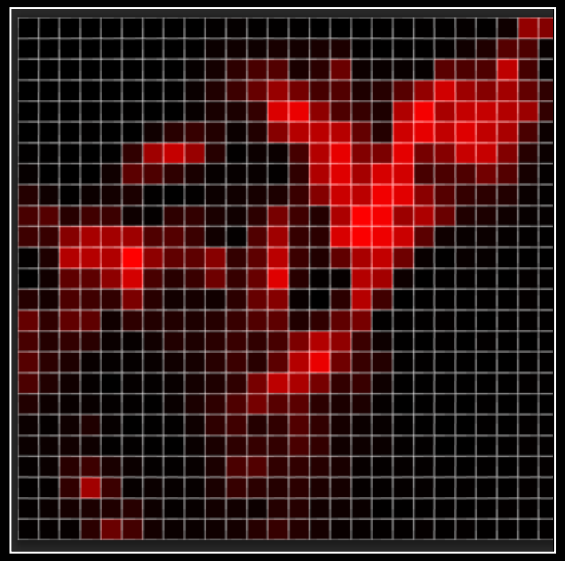
<u>Colocalization channel</u> was created

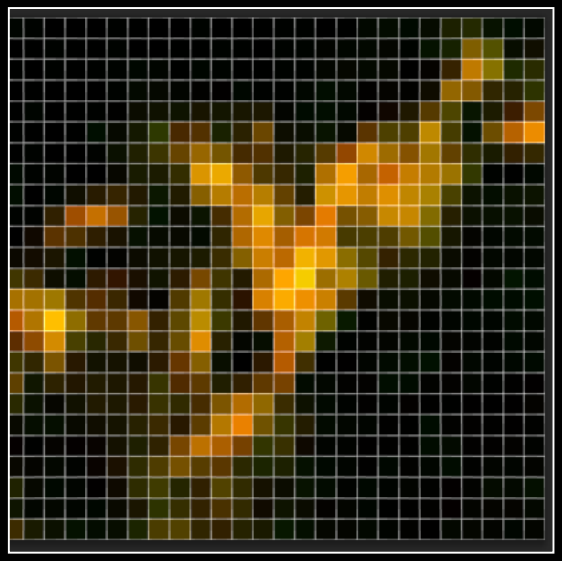
Surface was created from colocalization channel

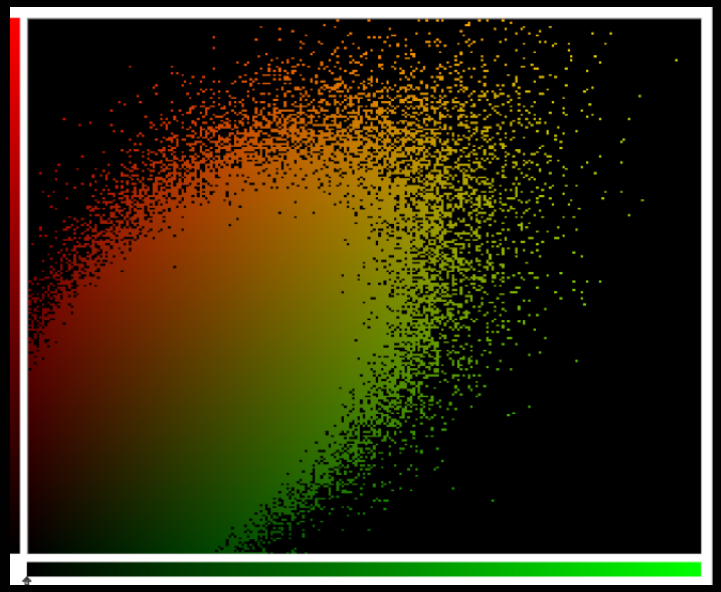
Smith et al. 2018 Brain Pathology

Pixel intensity based colocalisation









Measure pixel colocalisation in 3D

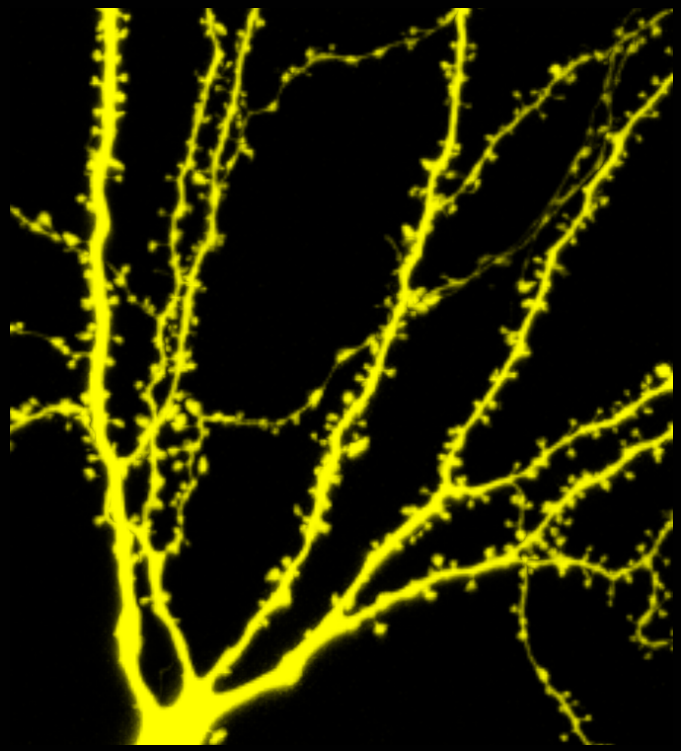
- Pearson`s coefficient
- Mander`s coefficient

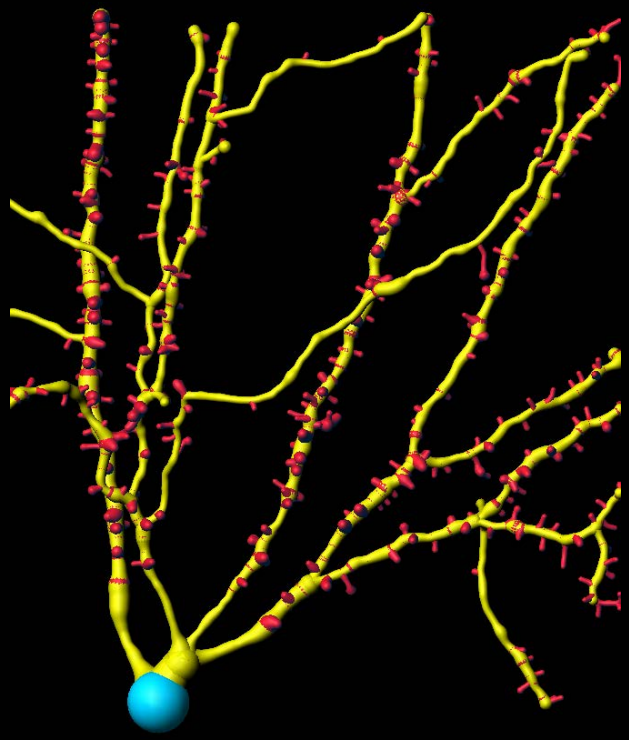
Colocalisation seminar
9th July 2018

Filament tracing

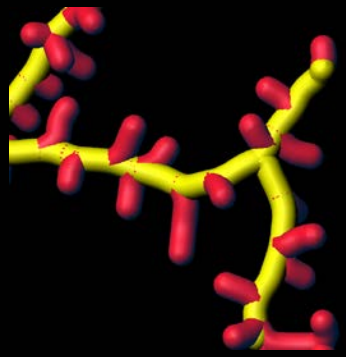
Filament detection for neuron

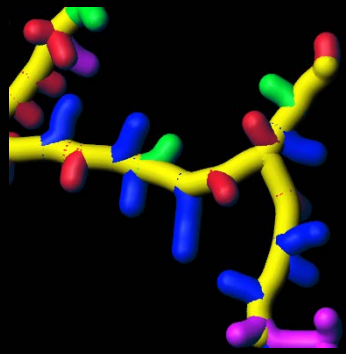
Filament tracing detect structure as "**Filament**", "**Dendrite**", "**Spine**"





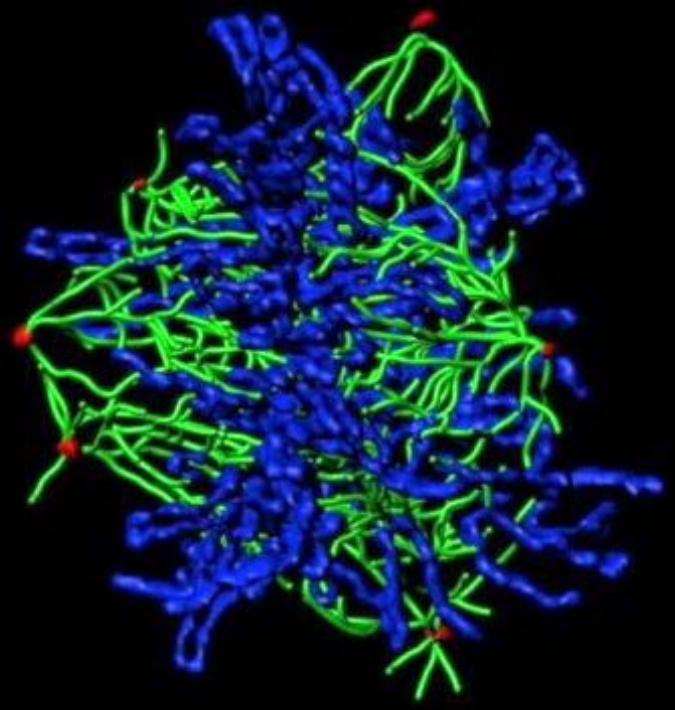
Spines can be further classified
- Stubby / Mushroom / Long Thin / Filopodia



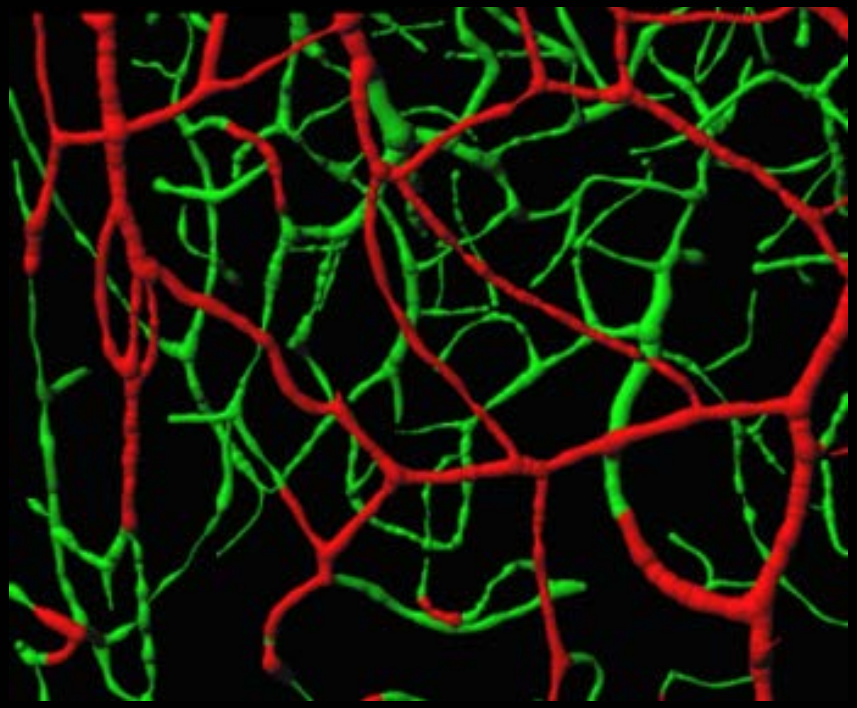


Filament detection for any line structure

- Detect filamentous structures by defining start & end point
- Tracing the meshwork of filaments is possible
- Manual tracing is also available



Courtesy of Dr Zhu, Dr Fitzpatrick,
Waitt Advanced Biophotonics Center, Salk Institute

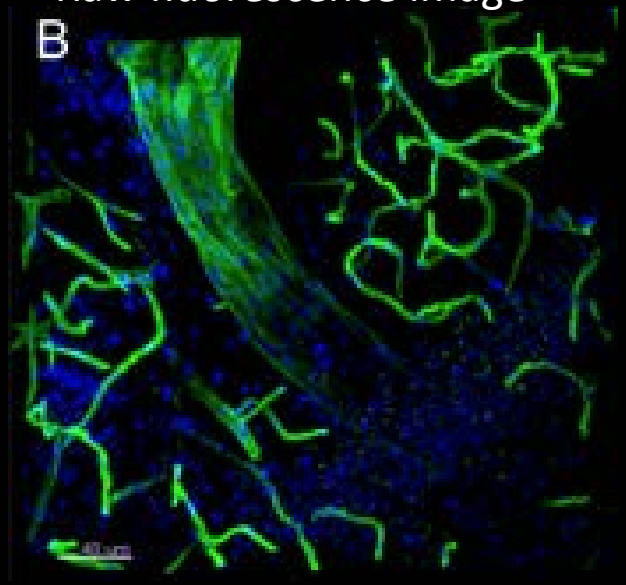


Culver JC (2013) PLoS ONE

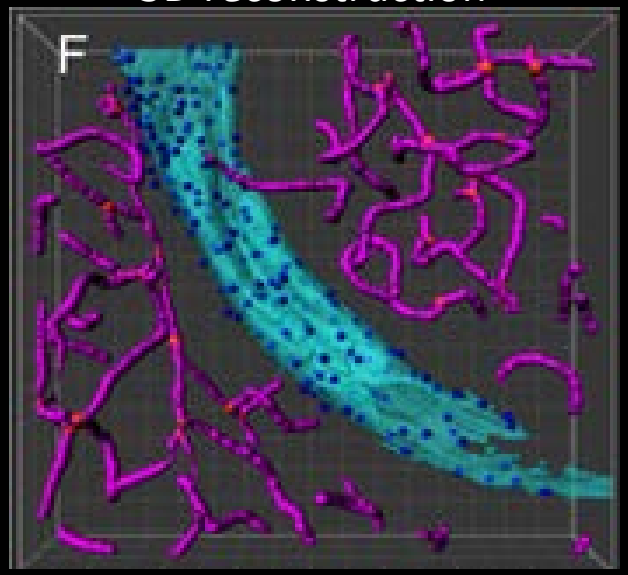
Case study - Filament analysis

microvasculature of an irradiated and unirradiated rodent hippocampus

Raw fluorescence image



3D reconstruction



Vessel (cyan) : surface detection
Nuclei (blue) : spot detection. Only the cells attached to the vessels were isolated
Microvessel : filament detection

- Total volume
- No. of branch points
- Mean diameter
- Surface area
- Total length

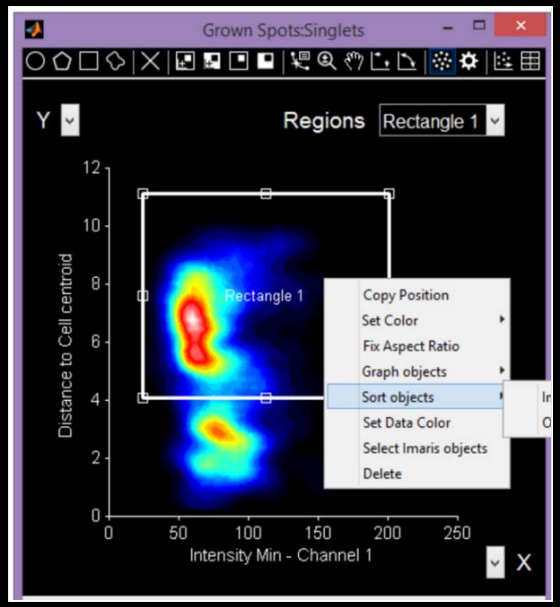
Craver BM et al. Environ Mol Mutagen. 2016 Jun;57(5):341-9.

More applications

More applications

Imaris XTension

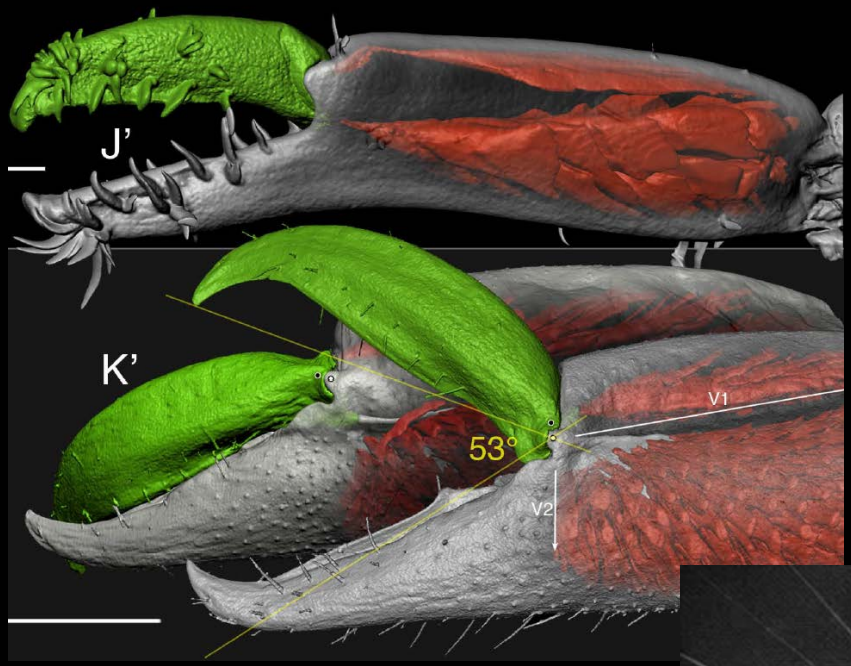
Plug-in can be written using Matlab, Python, ImageJ



Sortomato

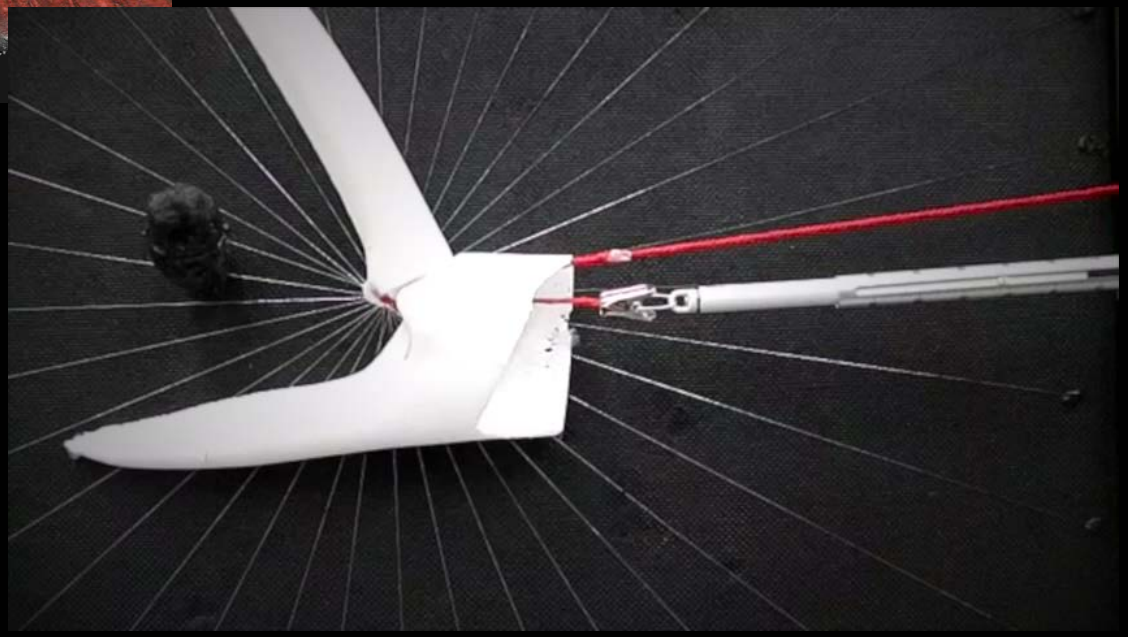
Sorting the objects based on statistical properties in interactive window

More applications – 3D printing



Snapping claw evolution

Shrimp claws were imaged using microCT
Each morphological unit (exoskeleton, muscles, etc) were segmented using Imaris
Isosurfaces were exported to STL files for 3D printing

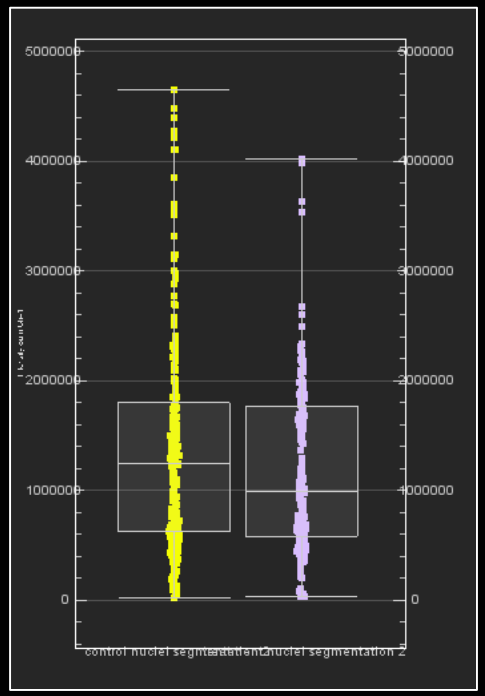


Kaji et al. Curr Biol. 2018 Jan 8;28(1):106-113.e4.

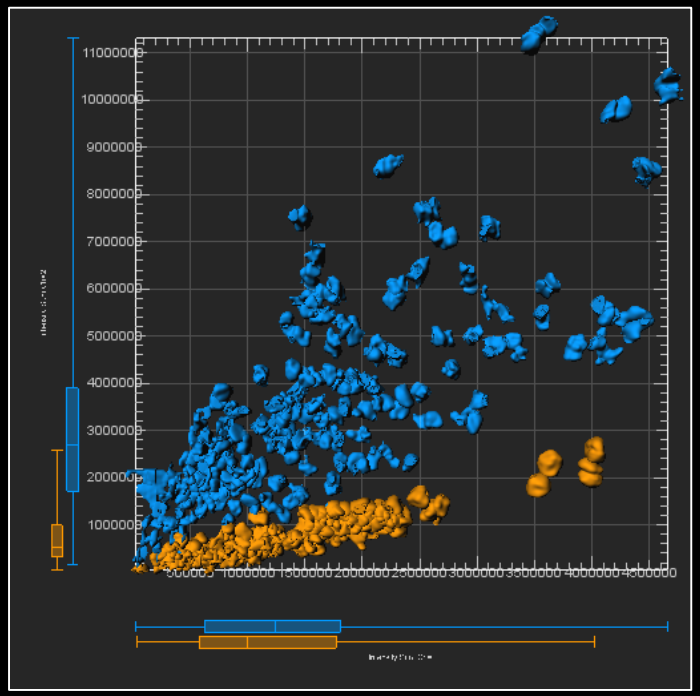
Data management / Plot

Presenting 3D objects in the graph with statistics

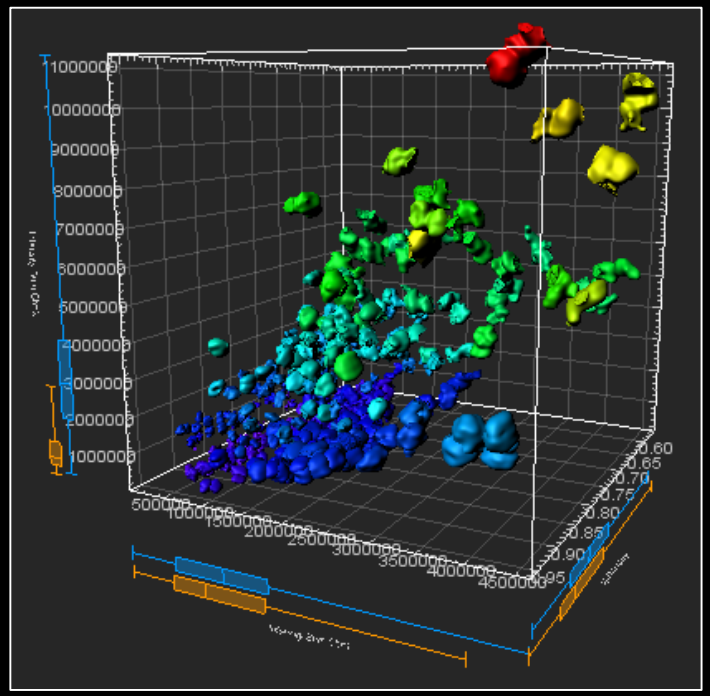
1D



2D

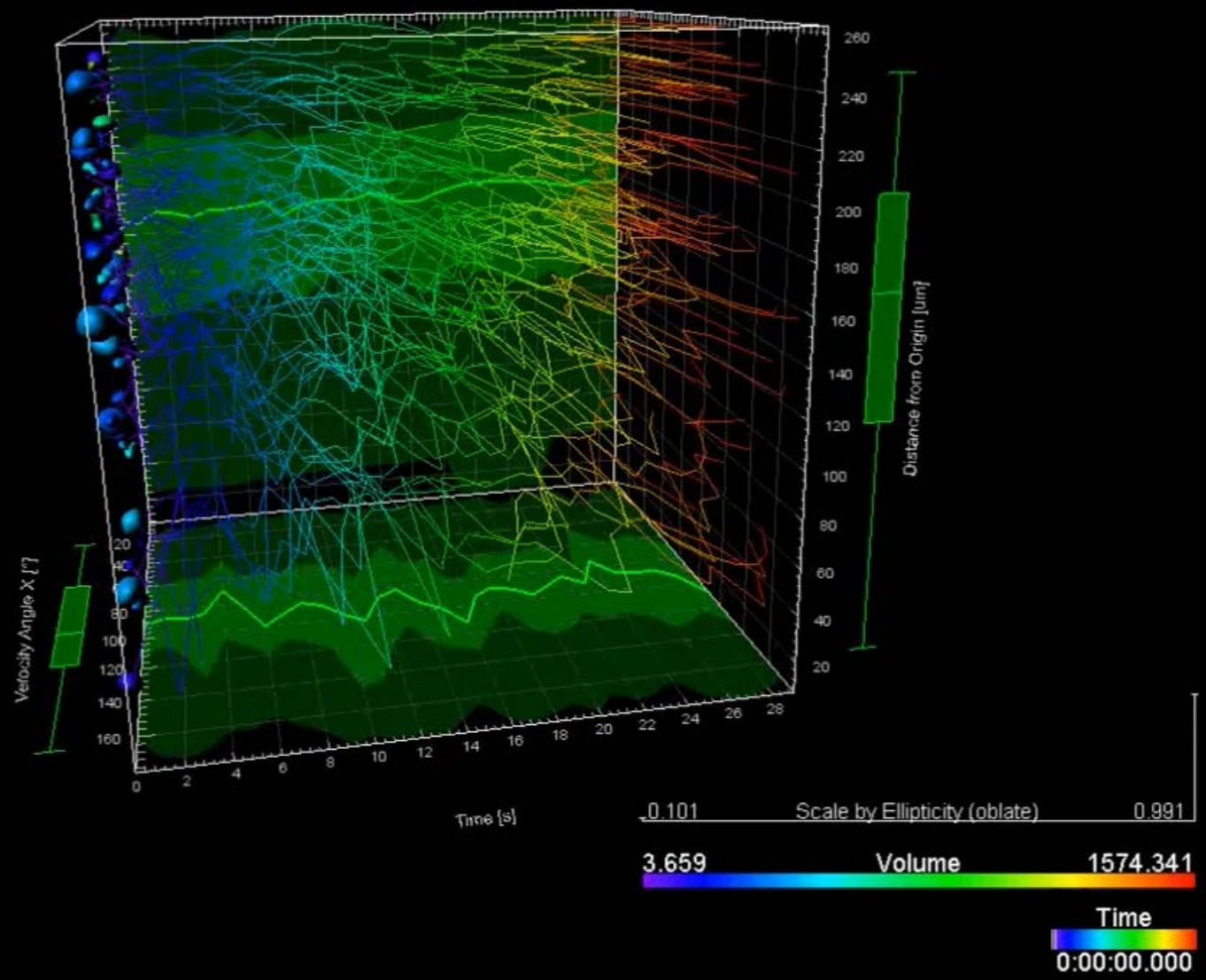


3D



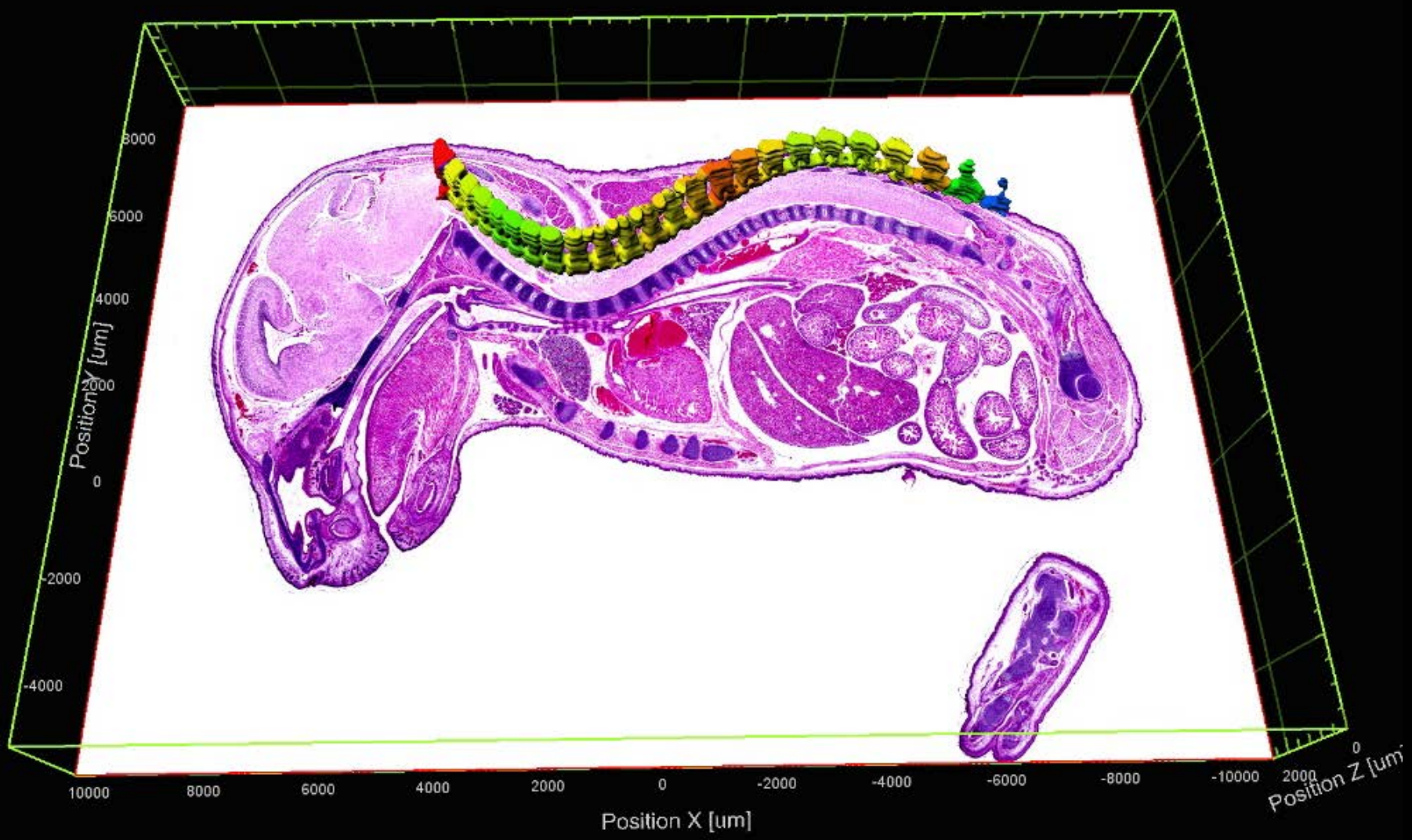
Presenting 3D objects in the graph with statistics

5D with Colour & object display scale



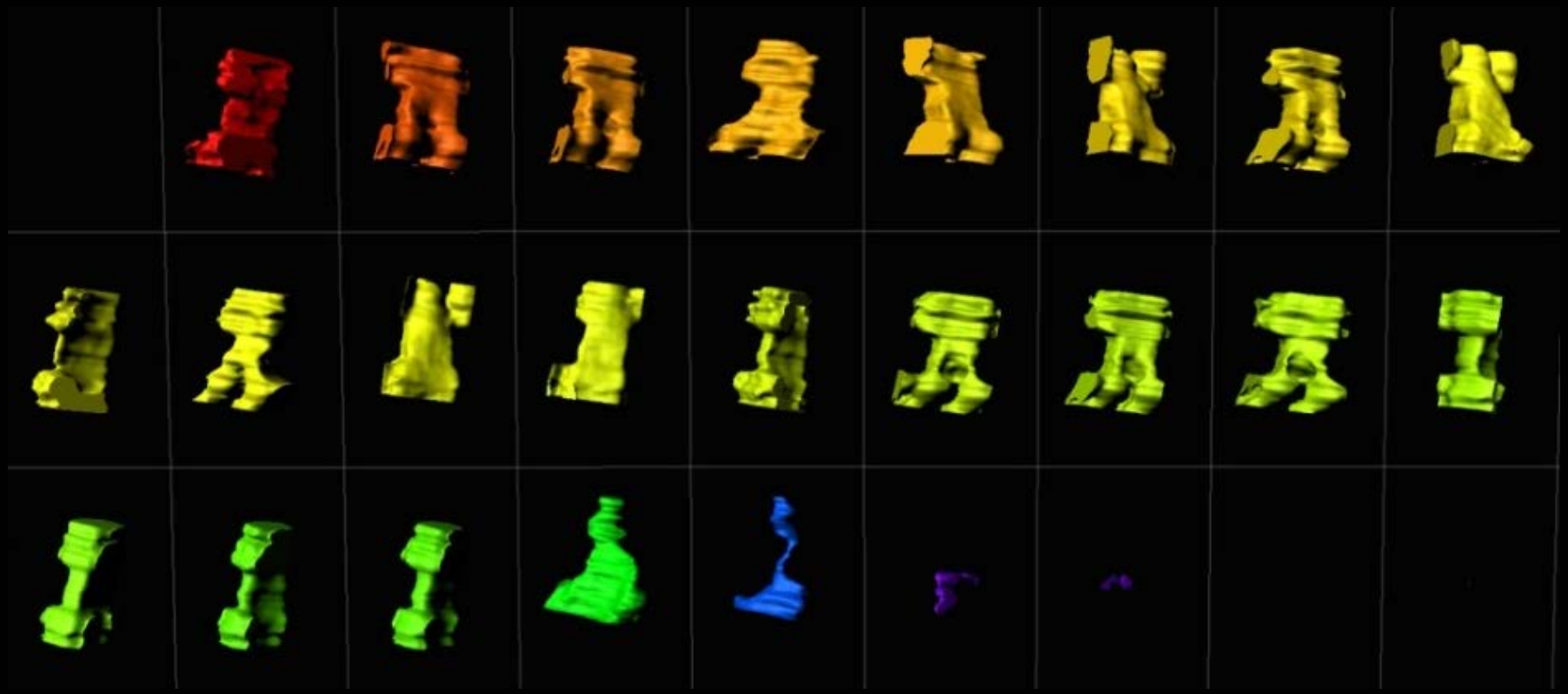
Data Plot – 3D plot example

3D rendering of mouse spine



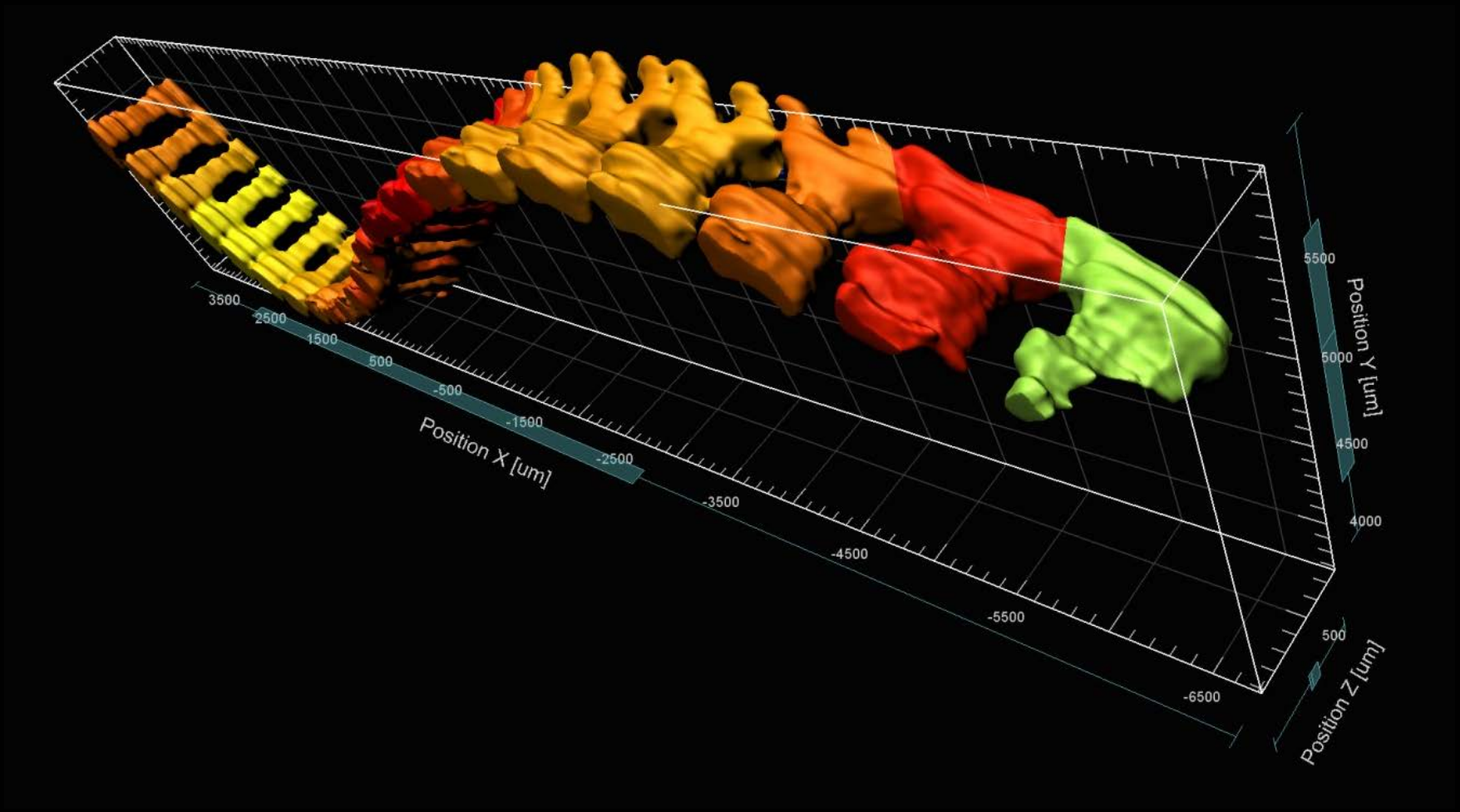
Data Plot – 3D plot example

Gallery mode

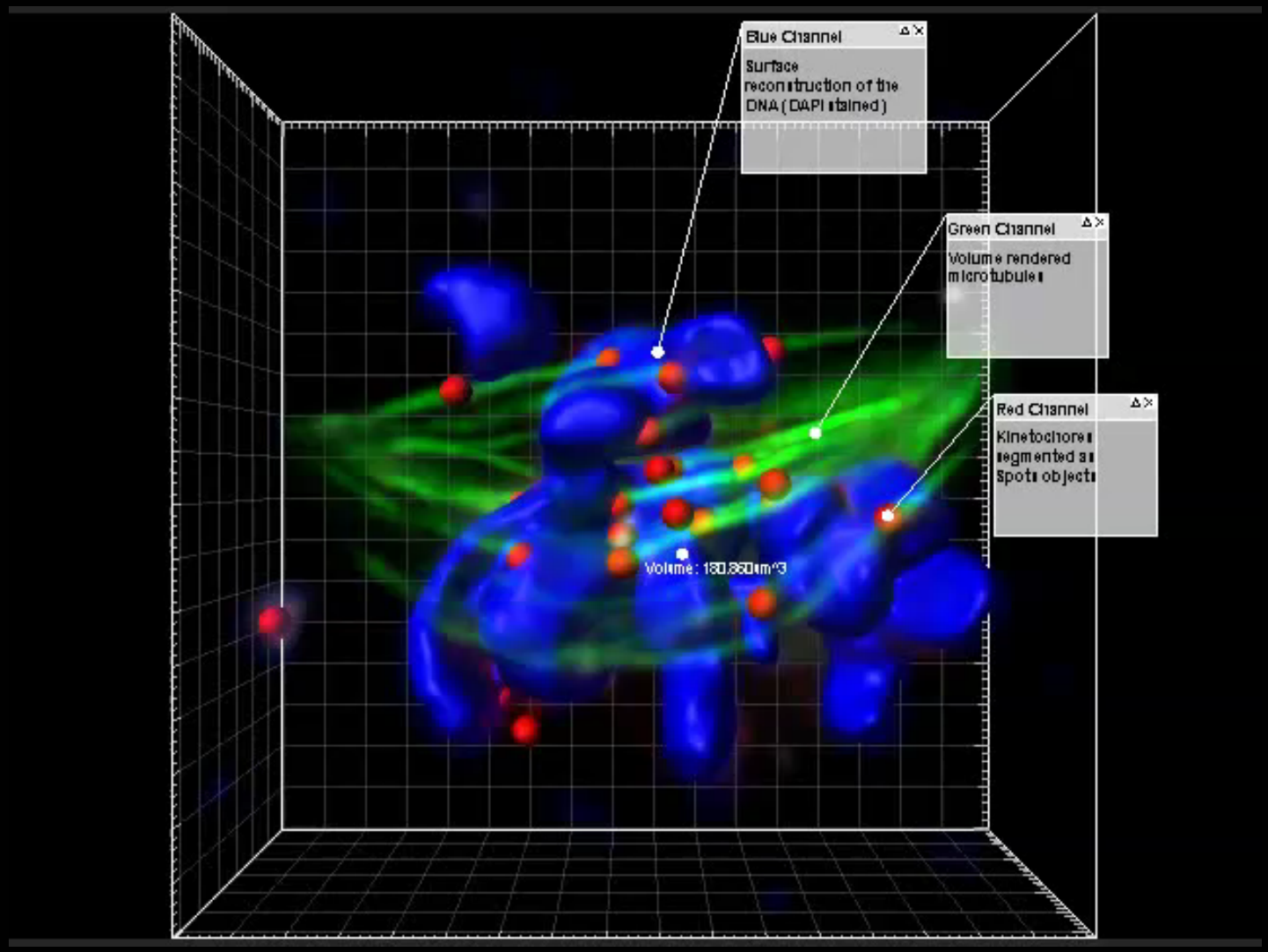


Data Plot – 3D plot example

3D Scattered Plot with statistics

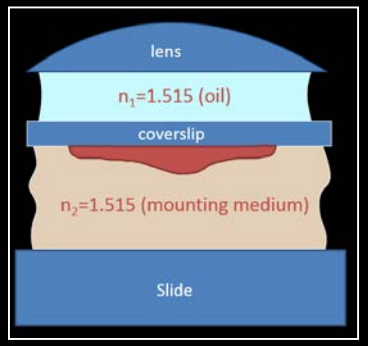


Annotation

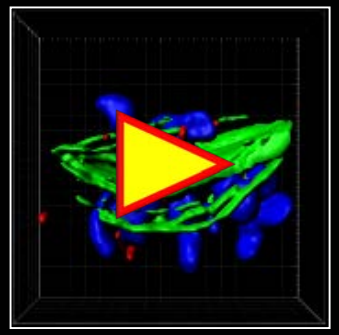

Blue Channel
Surface reconstruction of the DNA (DAPI stained)
Green Channel
Volume rendered microtubules
Red Channel
Kinetochores segmented as Spots objects
Volume: 180.860um^3

What we covered today..

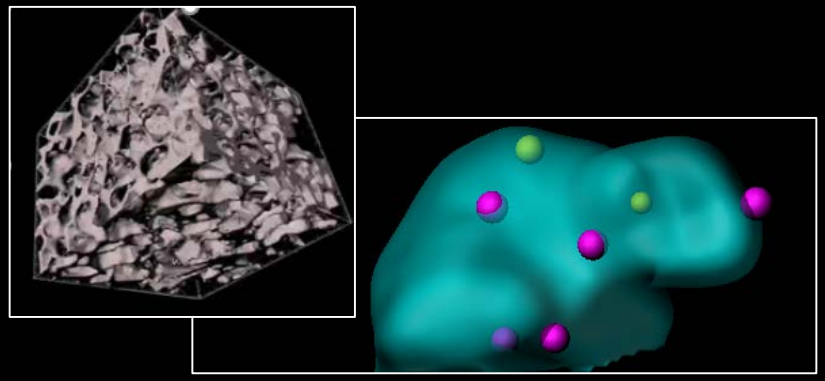
3D sample preparation



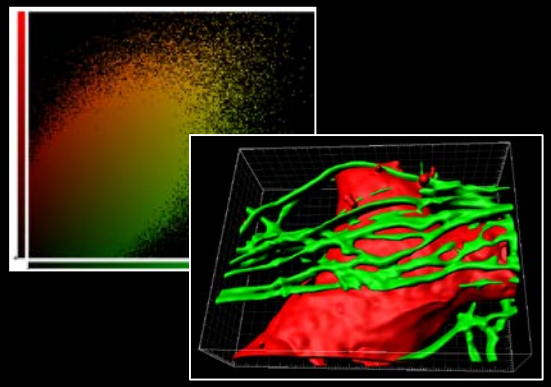
3D visualisation Animation



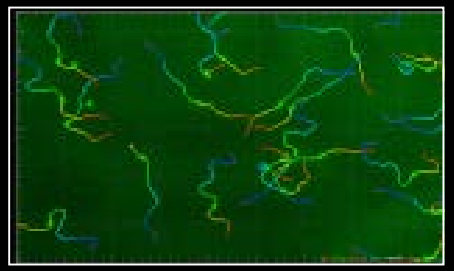
Segmentation / Quantification (Batch)



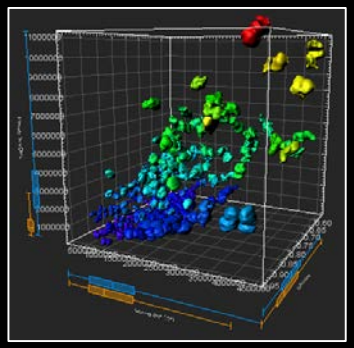
Colocalisation



Object Tracking



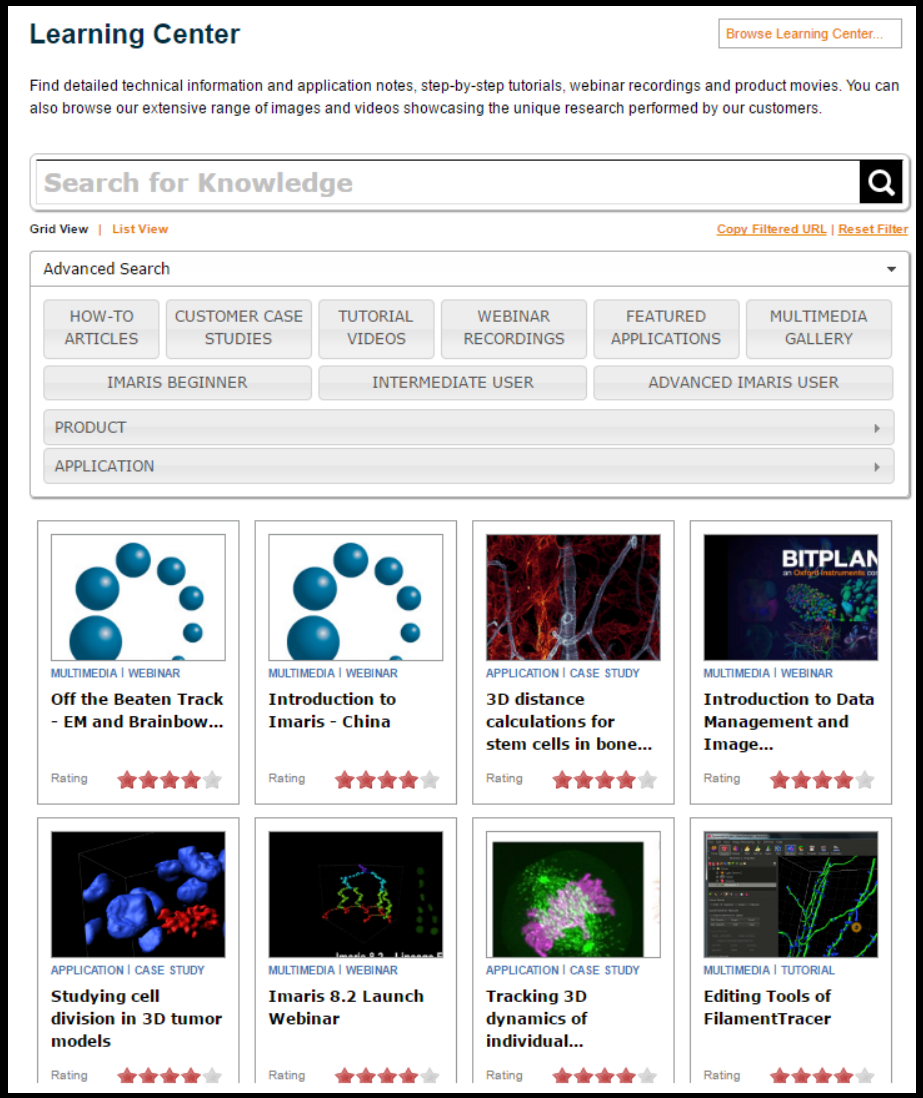
Data Plotting



Online Tutorials

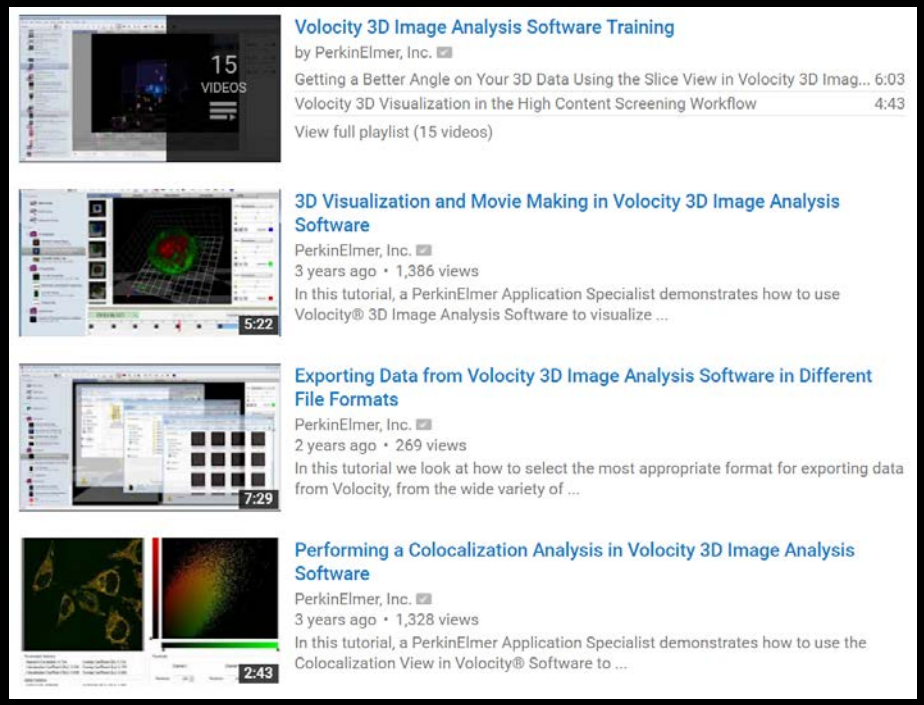
Imaris

Learning Center http://www.bitplane.com/learning



Volocity

Playlists in YouTube 'Volocity 3D Image Analysis Software Training'



Where & how to access - Imaris

10 days evaluation trial

- ✓Ultimate version
- ✓Can be used for 10 non-consecutive days
- ✓Need high spec computer (http://www.bitplane.com/systemrequirements.aspx)

Image Analysis workstations @ Anatomy and Neuroscience

Choose Imaris for

- Large size data
- Filament tracing
- 3D data plot
- Flexibility with plugins

Where & how to access - Volocity

No demo version available but BOMP can install Volocity on your lab computer If

- computer specification meets http://cellularimaging.perkinelmer.com/pdfs/technotes/TN263.pdf
- Computer can be also accessed to other users

Image Analysis workstation @Bio21, BMB, ANS, Bioscience (via license server)

Choose Volocity for

- Simple measurement with Batch process
- Pixel intensity based colocalisation
- Accessibility

Any Question?

bomp-enquiries@unimelb.edu.au