SPIM, fancy SPIM

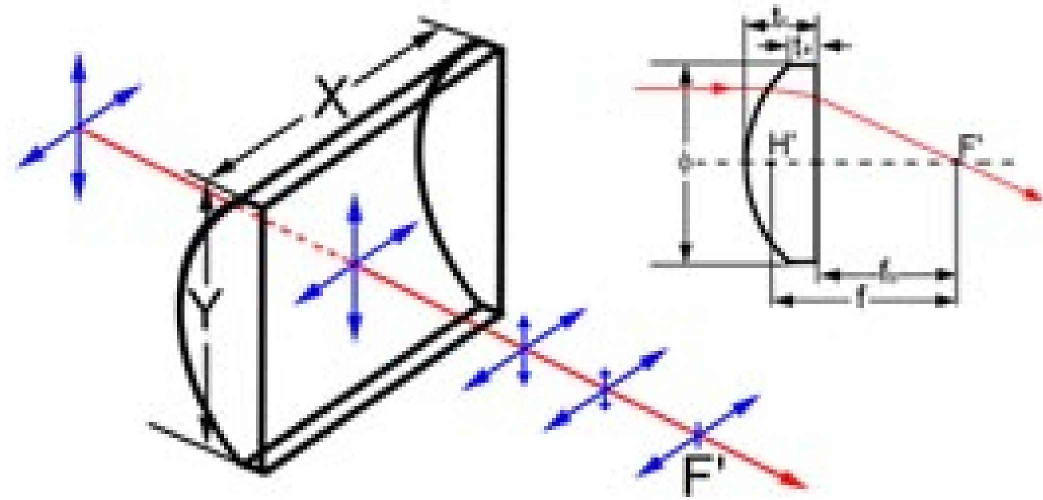
- Last class
    - More biosensors
    - Actuators
- This class
    - Intro to SPIM
    - Advantages and uses
    - Fancy SPIM

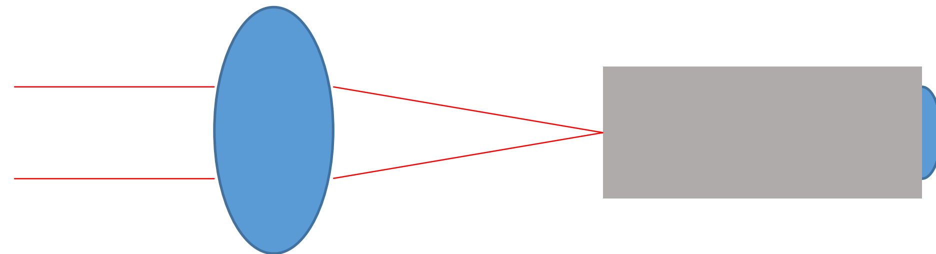
Light sheet microscopy

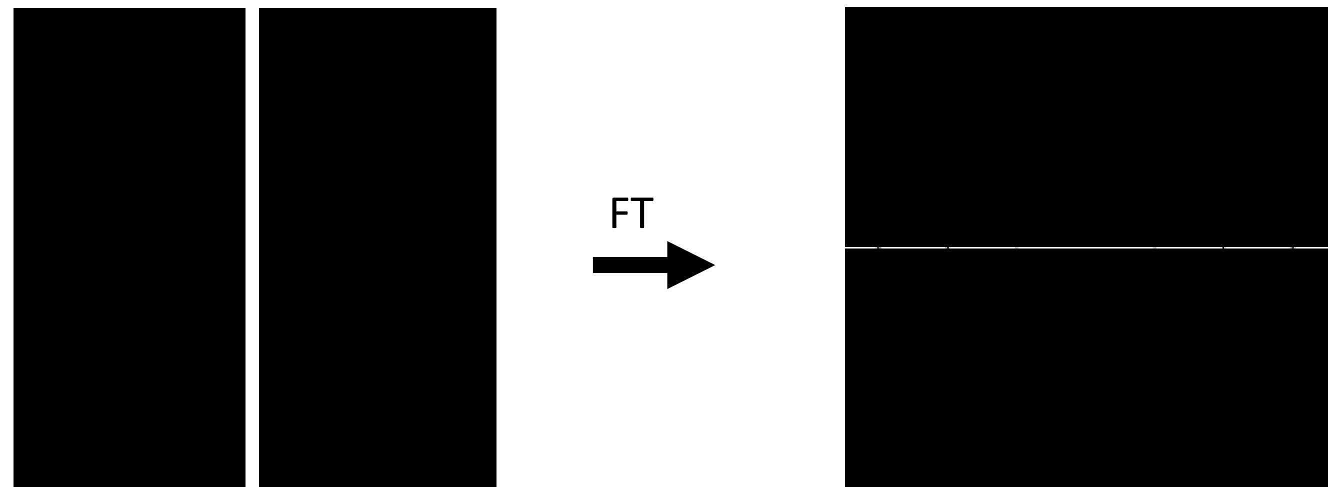
- Also called Single Plane Illumination Microscopy (SPIM)
- Not super resolution
- Used to rapidly section 3D samples
- Useful for imaging deep within transparent tissues or organisms

Sheet focusing

- Spherical lens focuses light to a spot
- Cylindrical lens focuses light only along one axis, sheet
- Illuminating the back aperture of an objective with a line -> another line

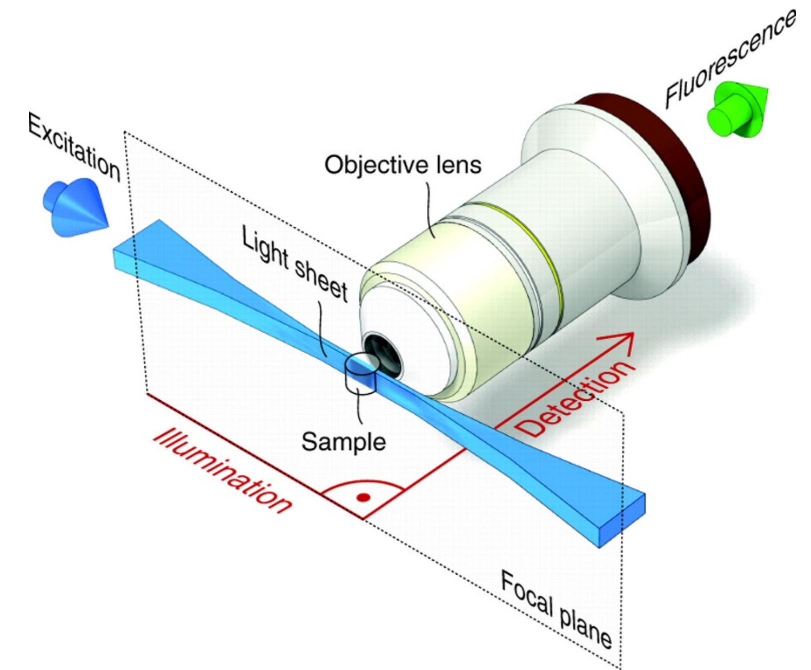


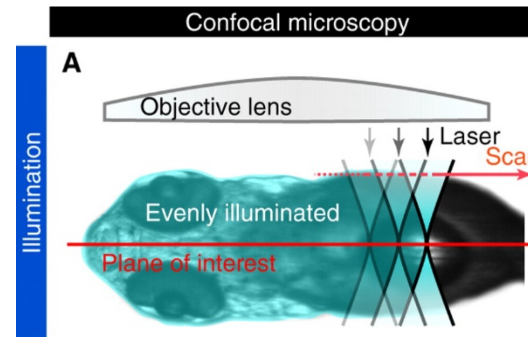




SPIM illuminates one axis only

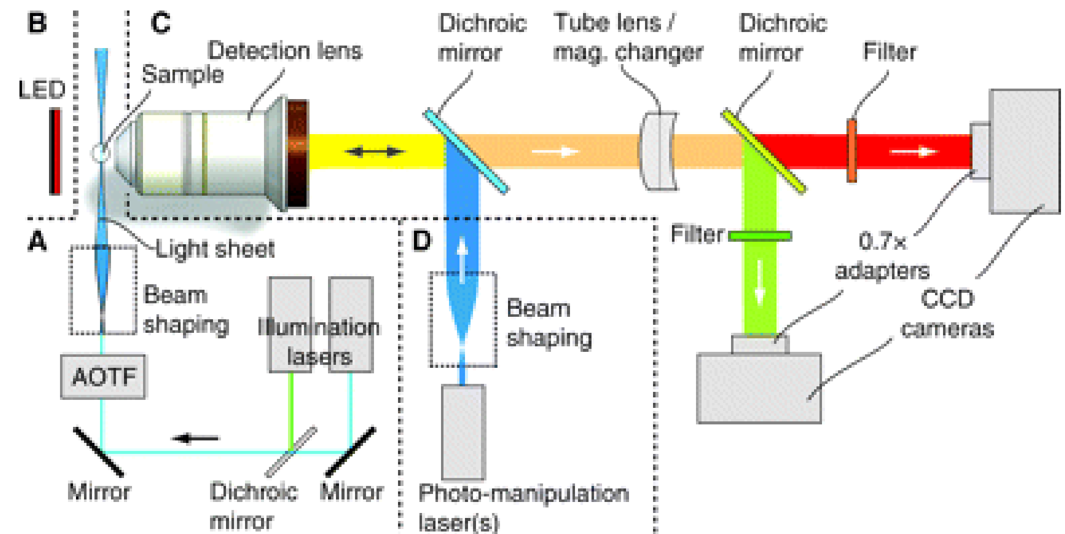
- By confining excitation in z, we know exactly where the fluorescent photons were generated
- Still limited to normal widefield diffraction limit in lateral dimensions
- Limiting excitation also limits photobleaching and phototoxicicty
- Detection is orthogonal to illumination, unlike everything else we've seen so far
- Confocal rejects scattered light, SPIM doesn't care

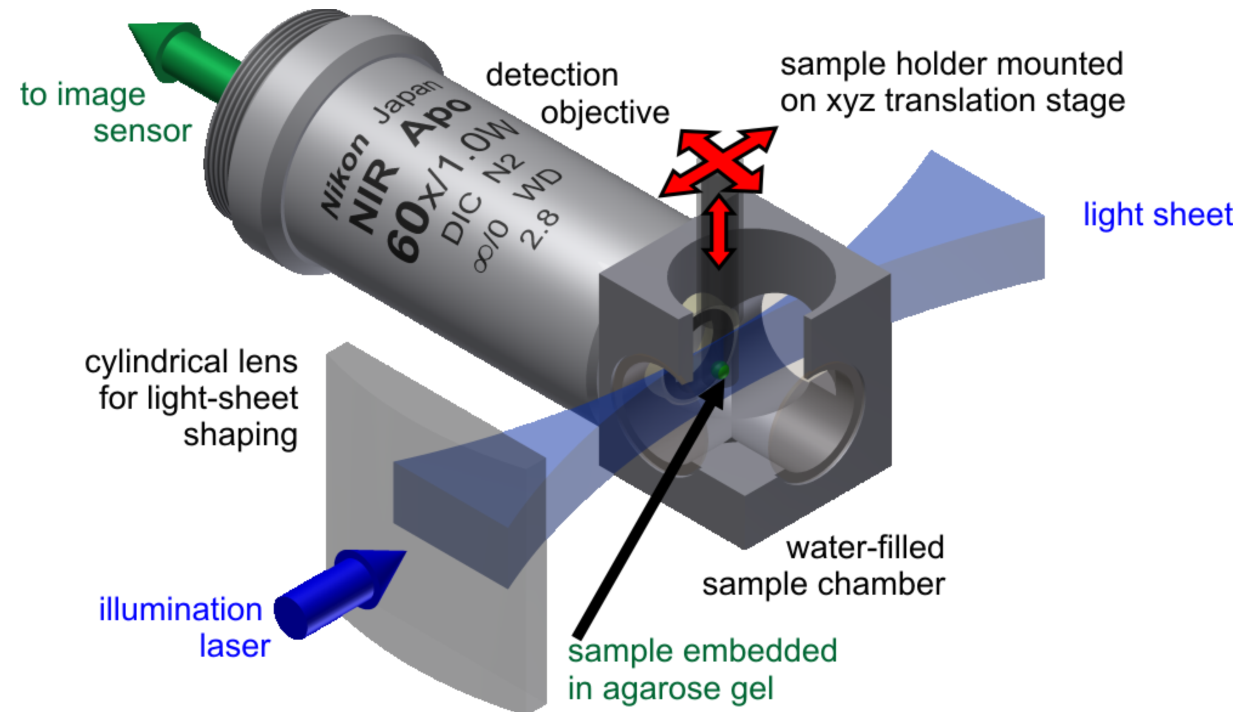




Typical setup

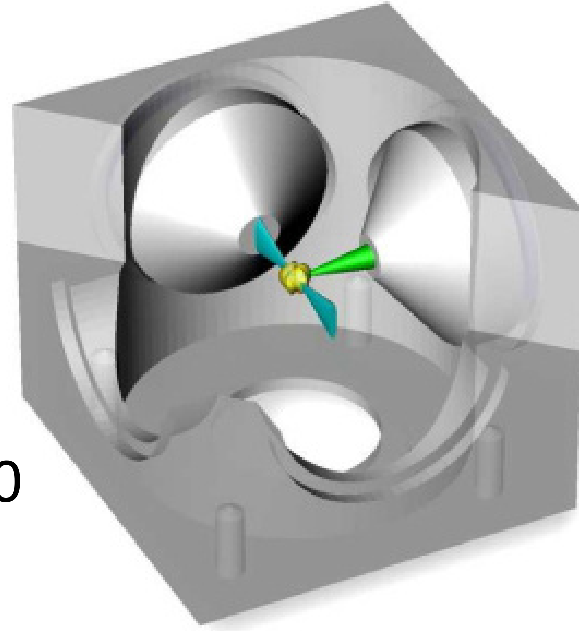
- Samples need to be optically transparent in all 3 dimensions, typically encased in agarose
- Elliptical lens brings laser to a focus on back focal plane of objective
- Light sheet is created on sample
- Detector objective typically doesn't move, sample is on a translation stage

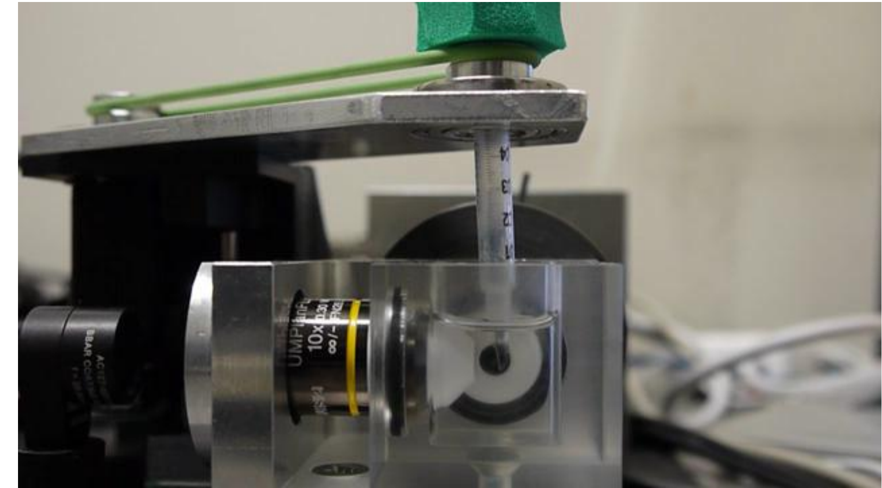


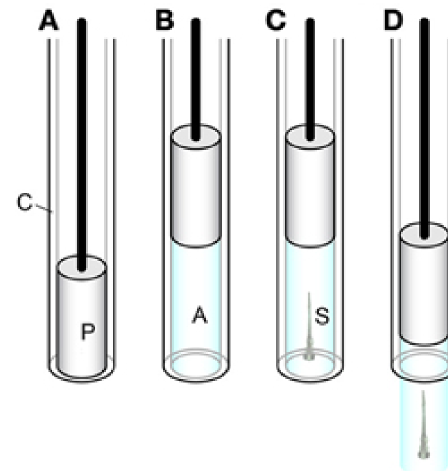


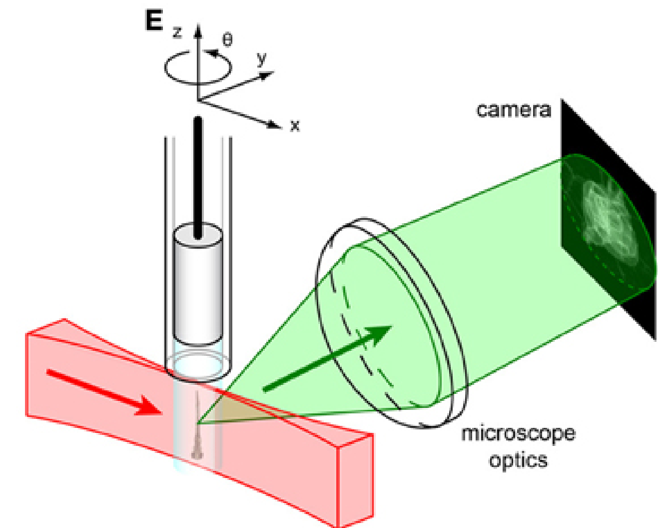
Sample mounting

- Unconventional geometry makes sample mounting tough
- Detection objective has to be 90 degrees to excitation beam
- Sample has to be immobilized, but has to have the same index of refraction as water
- Plane is formed to illuminate large cross section of sample
- Often use agarose tubes with plungers
- Objectives are water immersion, and can not move relative to the sample



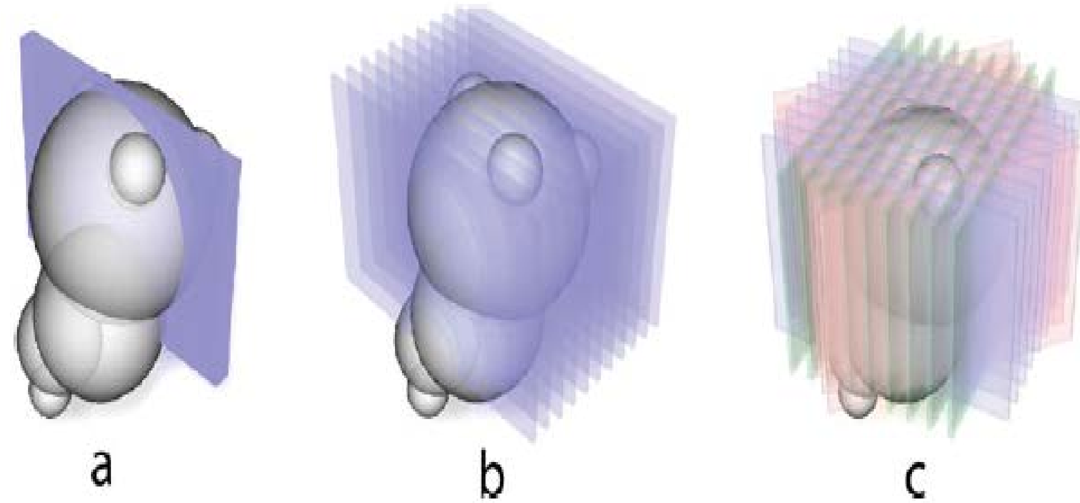


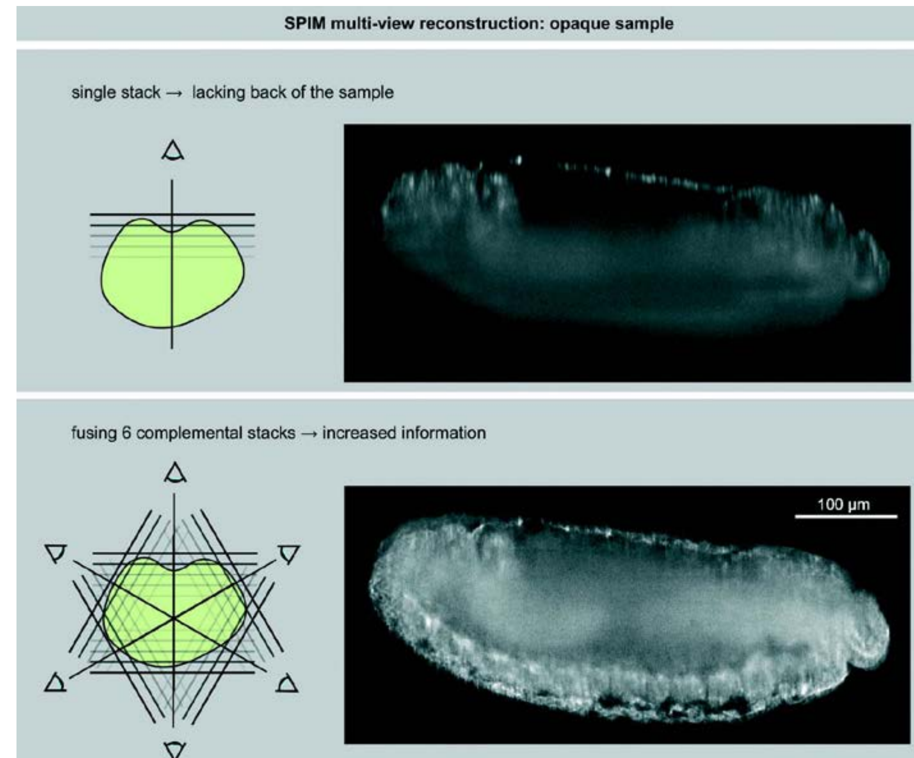




Sample imaging

- Easy to imagine a single plane, and you can watch with very high time resolution
- Translating sample through the light sheet will build up 3D image
- With rotations and translations, can build up full 3D tomographic map with isotropic resolution across all 3 dimensions

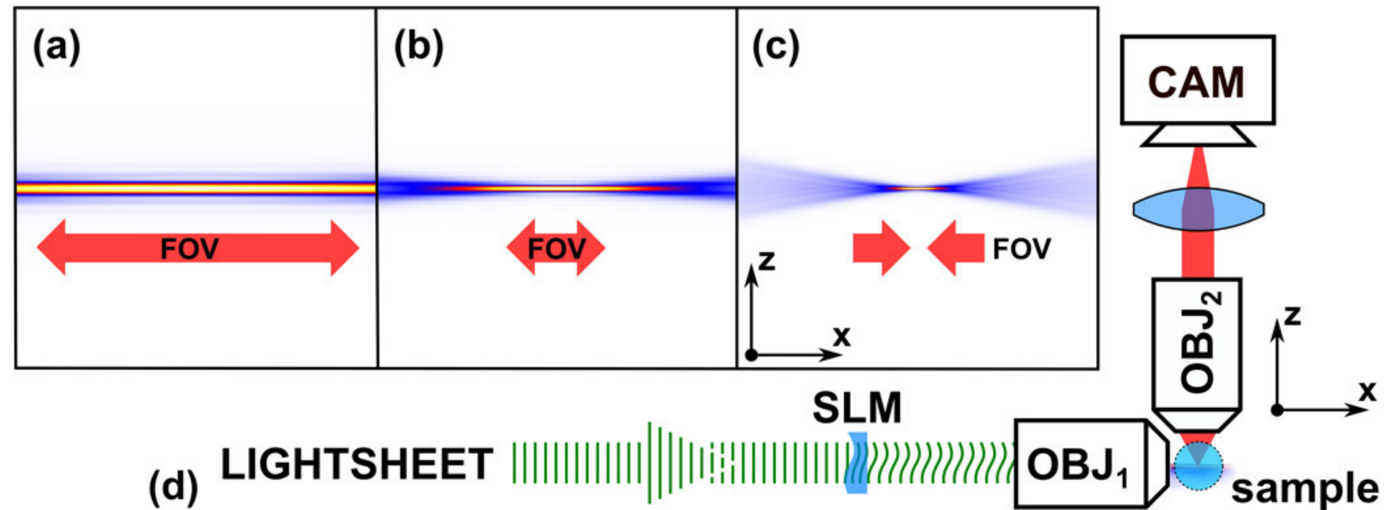




SPIM resolution

- Lateral resolution is given by the NA of the detector objective as per usual
- Axial resolution is set by the NA of the SPIM objective and the detection objective
- The higher the NA, the better the axial resolution, the smaller the size of the sheet
- Tradeoff between sheet thickness, and sheet size

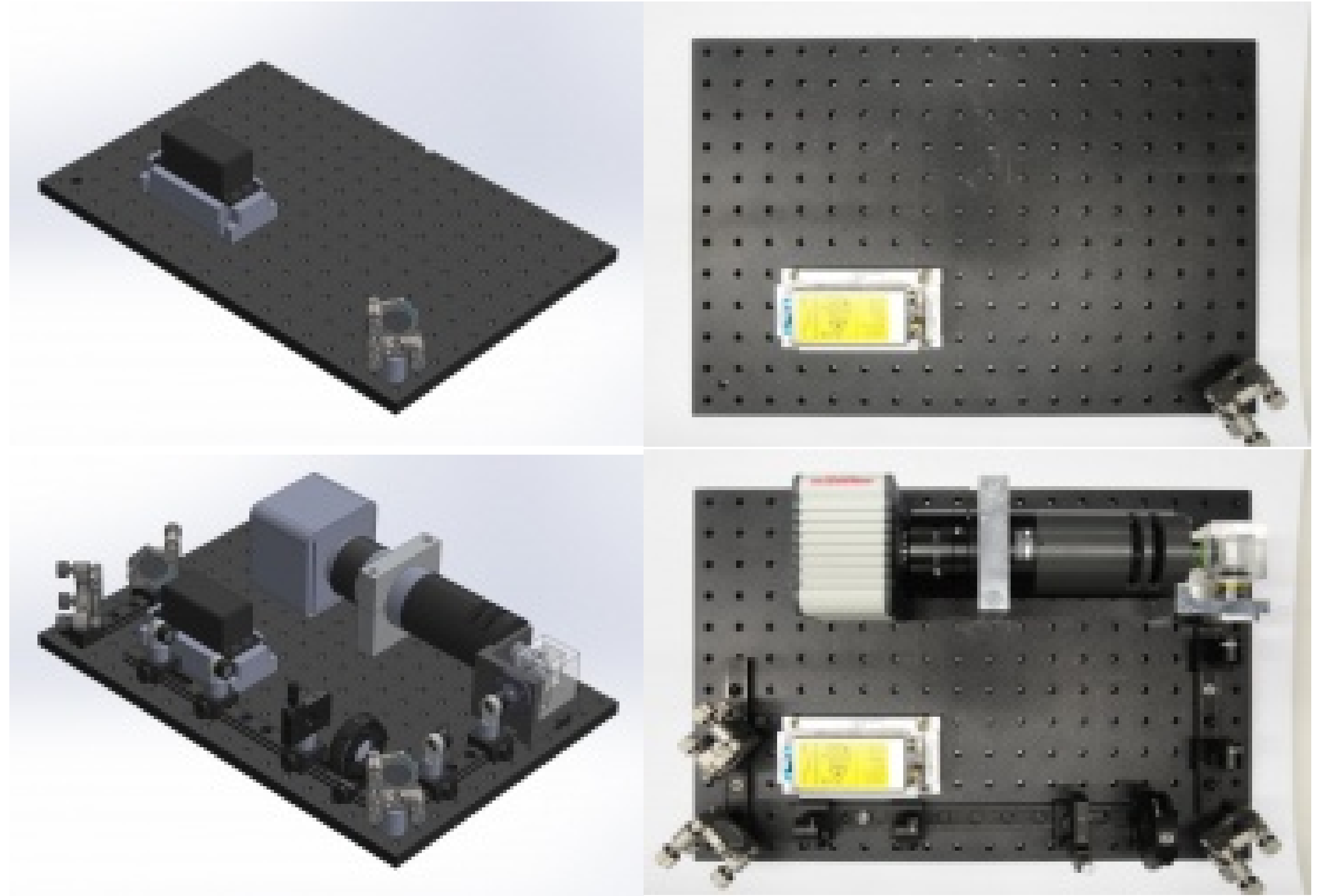
$$d_{x,y} = \frac{\lambda_{em}}{2 * NA} \qquad d_z = \left(\frac{2 * NA}{\lambda_{ex}} + \frac{n(1 - cos\theta)}{\lambda_{em}}\right)^{-1}$$



2 μm thickness sheet will cover 30 μm distance

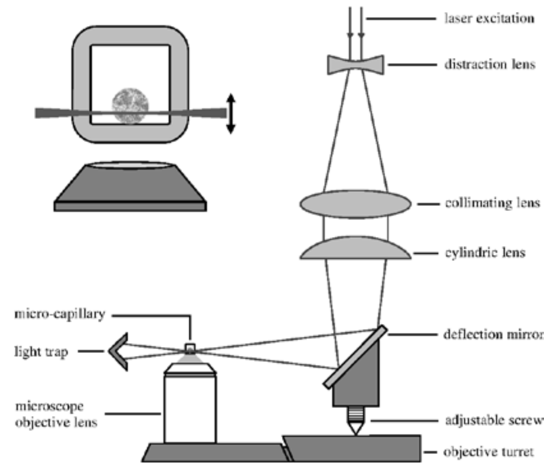
OpenSPIM

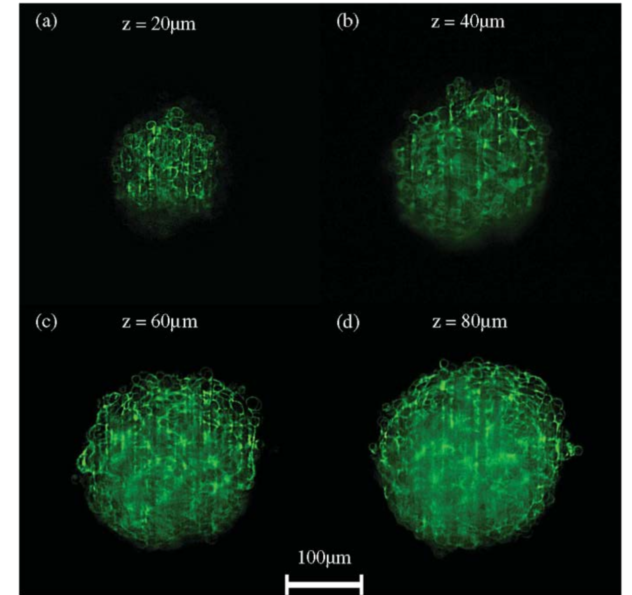
- www.openspim.org
- Complete list of components and blueprints
- Gives software to both drive instrument and analyze data
- Far cheaper than spinning disk
- Major expenses are objective, laser, camera

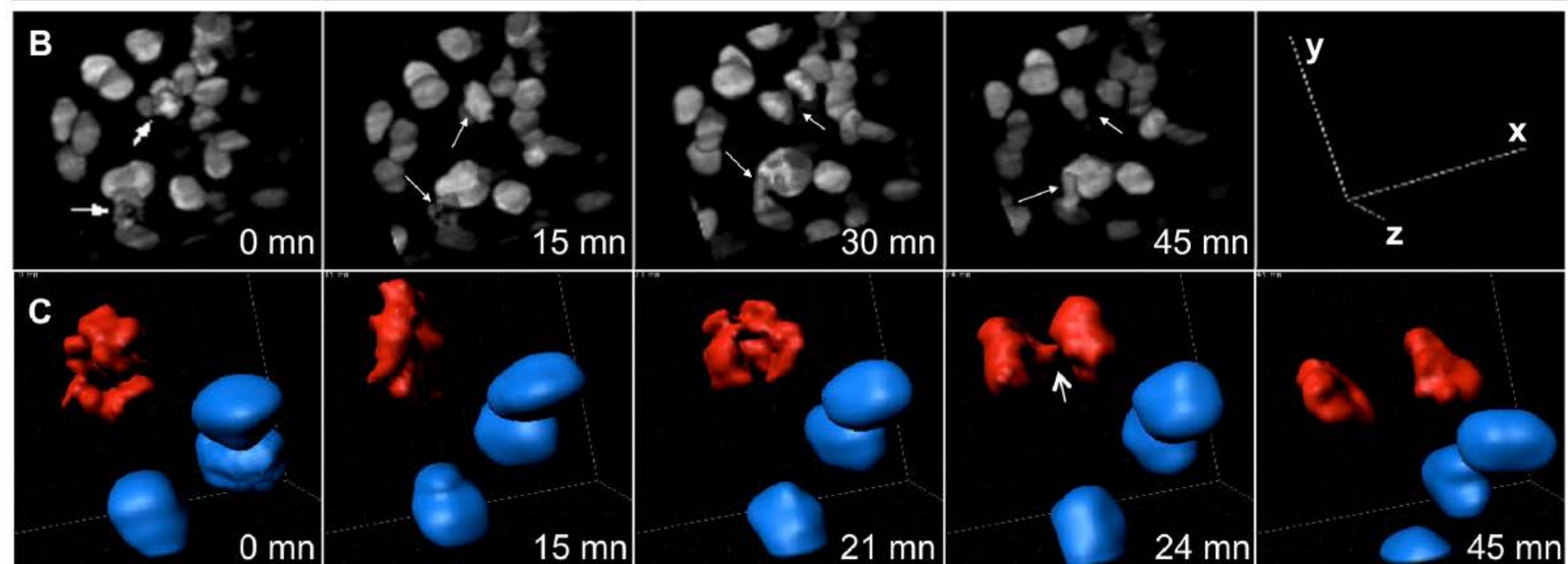


SPIM applications – 3D cell culture

- Need to get clever with sectioning set up
- Allows long term imaging of 3D culture cells
- Hard to maintain physiological conditions over the time course

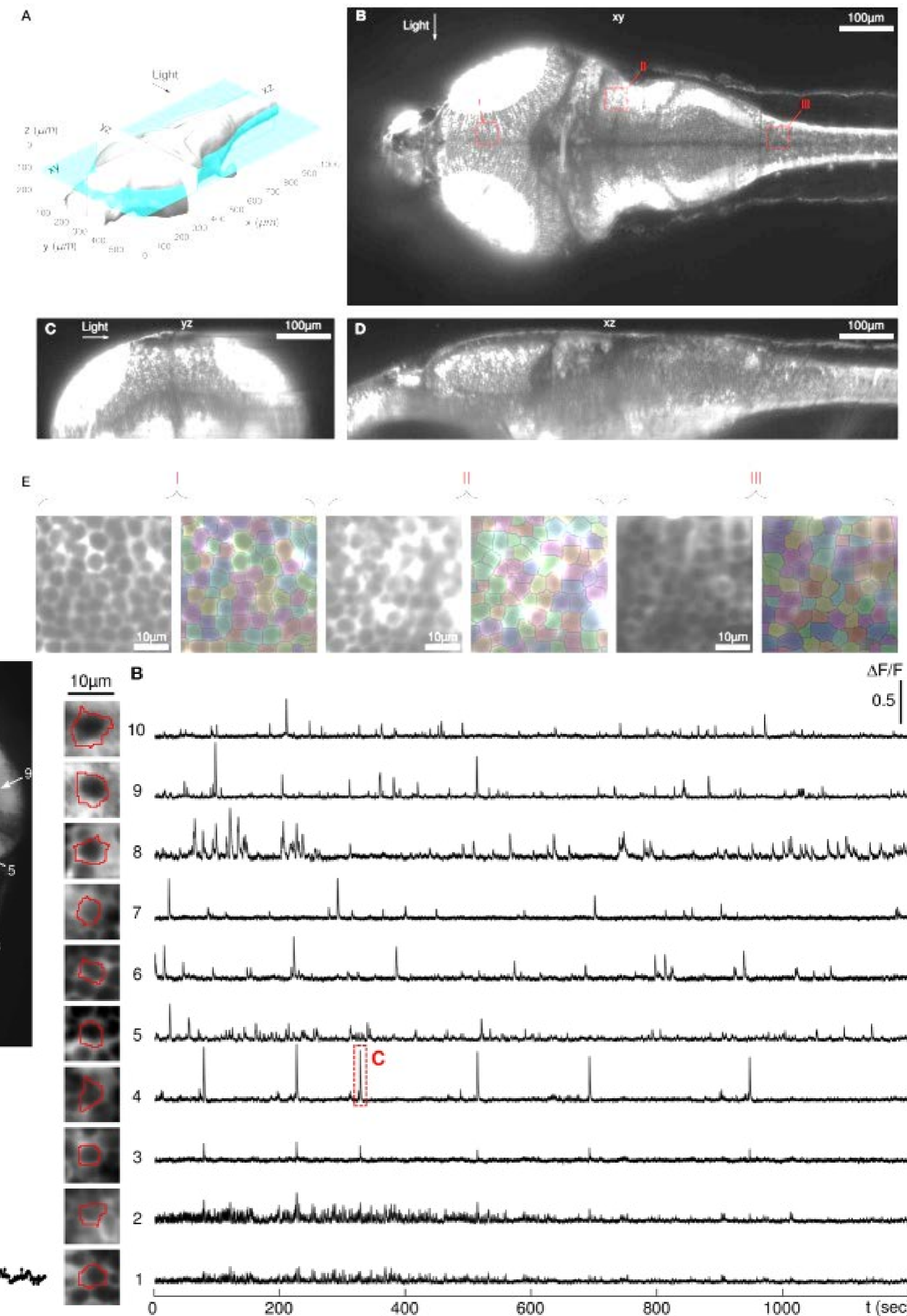






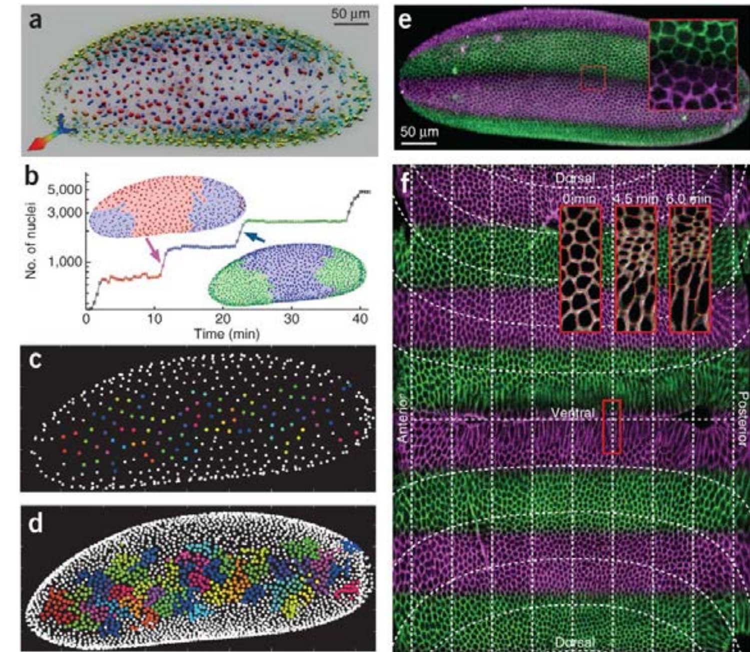
Applications of SPIM – zebrafish

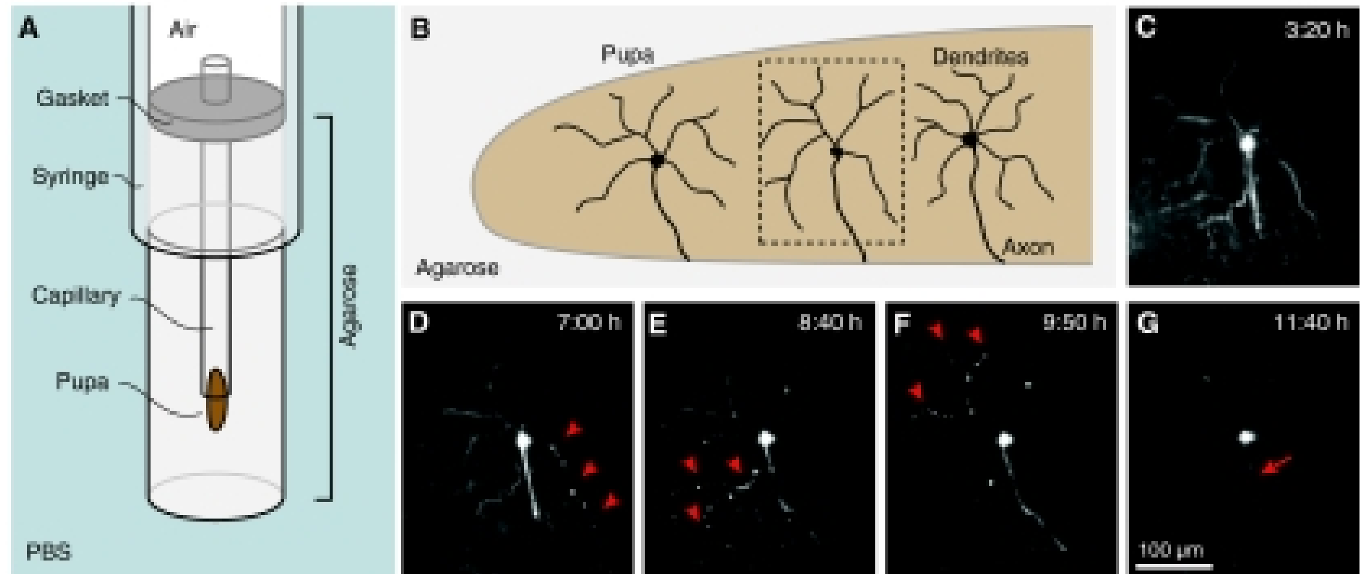
- Useful when you have a large, transparent, non-scattering object you want to image quickly
- Less photodamage than spinning disk
- SPIM is useful for extremely long term imaging



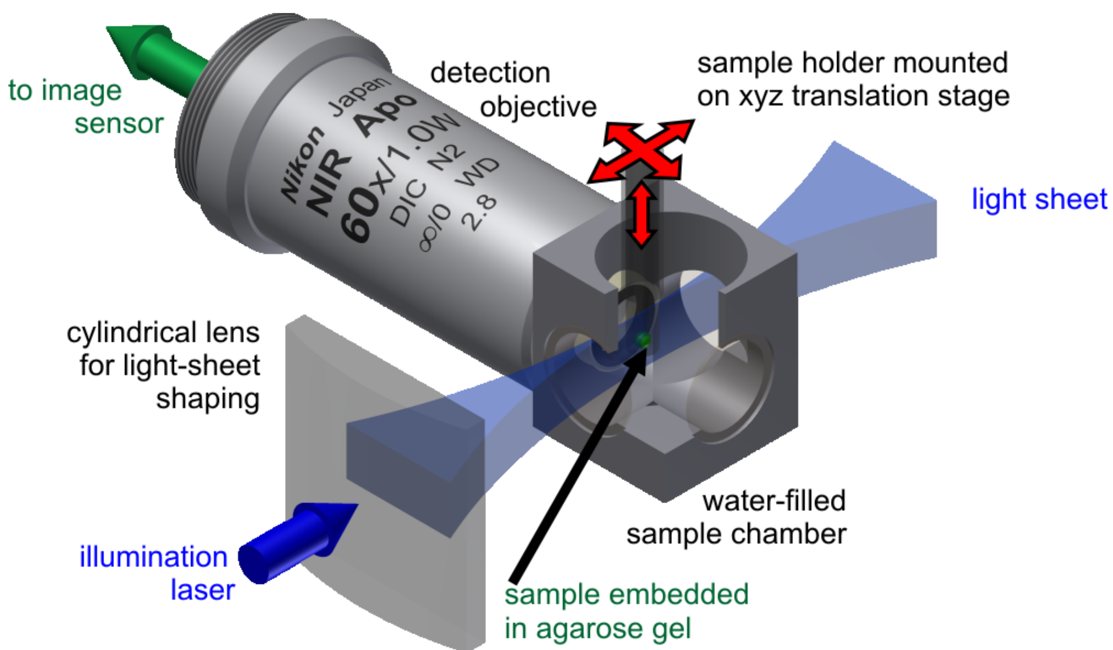
SPIM applications - Drosophila

- Drosophila larvae are transparent, and great models of development
- SPIM allows continuous monitoring over days
- Lots of good cell labels to mark different types of cells, and there 3D location
- Can watch neuro-development at sub-cellular resolution



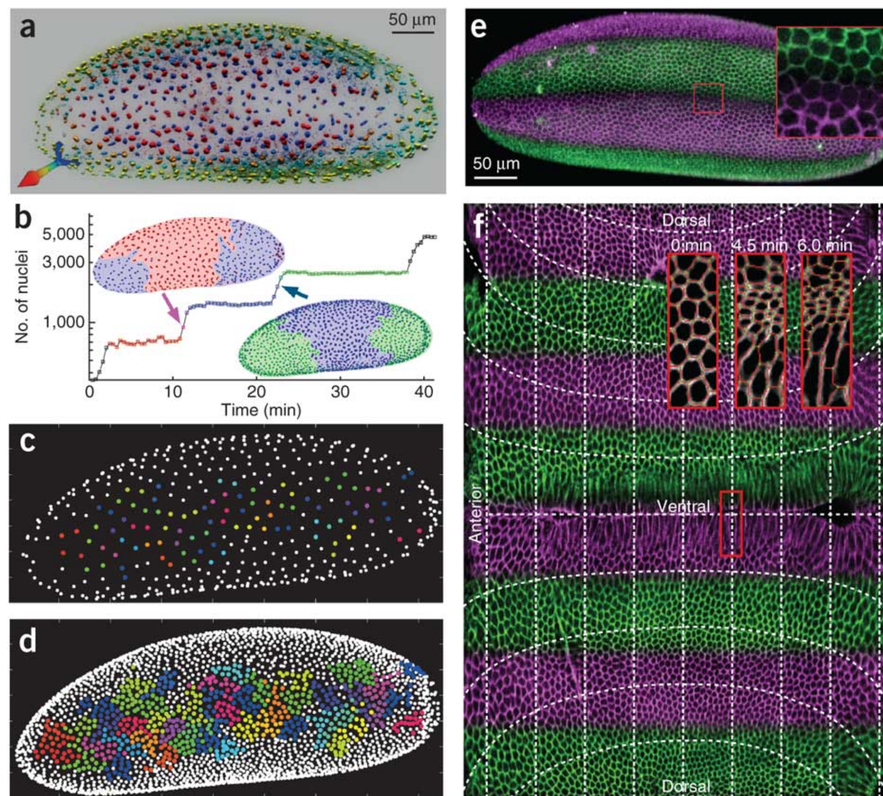


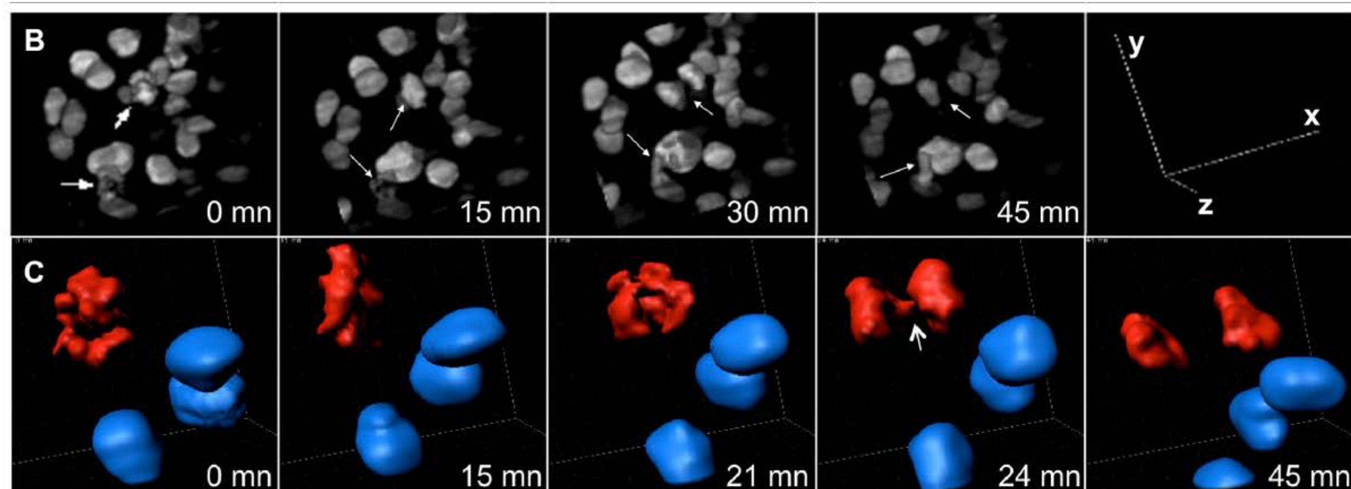
SPIM



$$d_{x,y} = \frac{\lambda_{em}}{2 * NA} \qquad s_{length} = \frac{\lambda_{em}}{n(1 - cos\theta)}$$

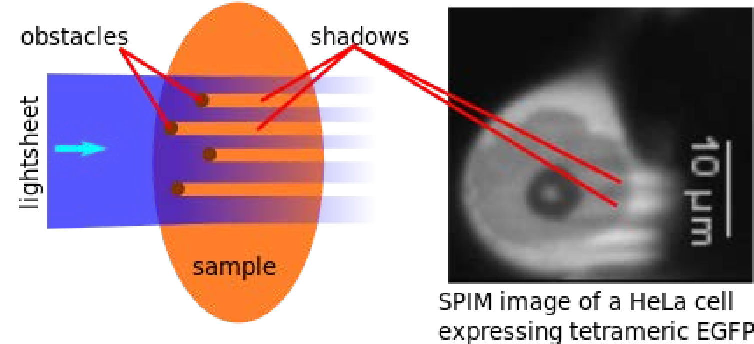
$$d_z = \left(\frac{2 * NA}{\lambda_{ex}} + \frac{n(1 - cos\theta)}{\lambda_{em}}\right)^{-1}$$



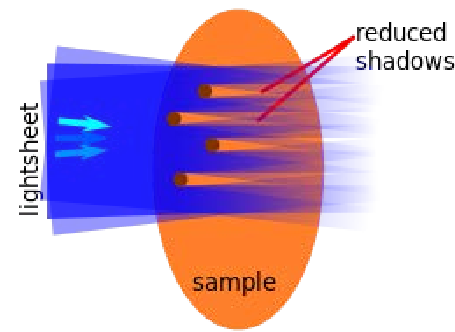


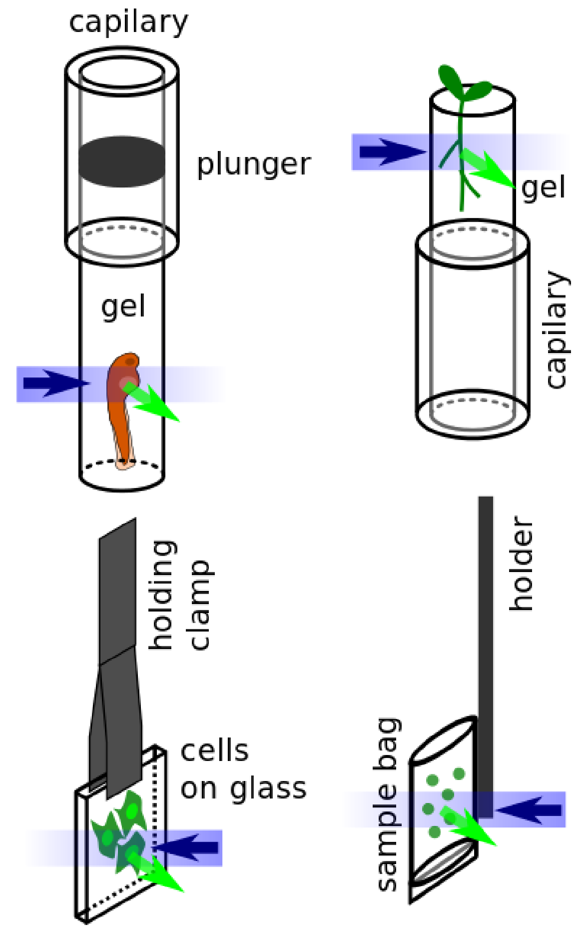
SPIM limitations

- Striping – if there are objects that scatter or absorb a lot of light, you can get stripes in your illumination
- Can get around this by rotating the angle of illumination slightly to cover the whole object
- Resolution is not as good as STORM, STED, SIM
- Very annoying sample geometries
- If your embryo/sample needs to move, agarose won't be an option



SPIM image of a HeLa cell expressing tetrameric EGFP

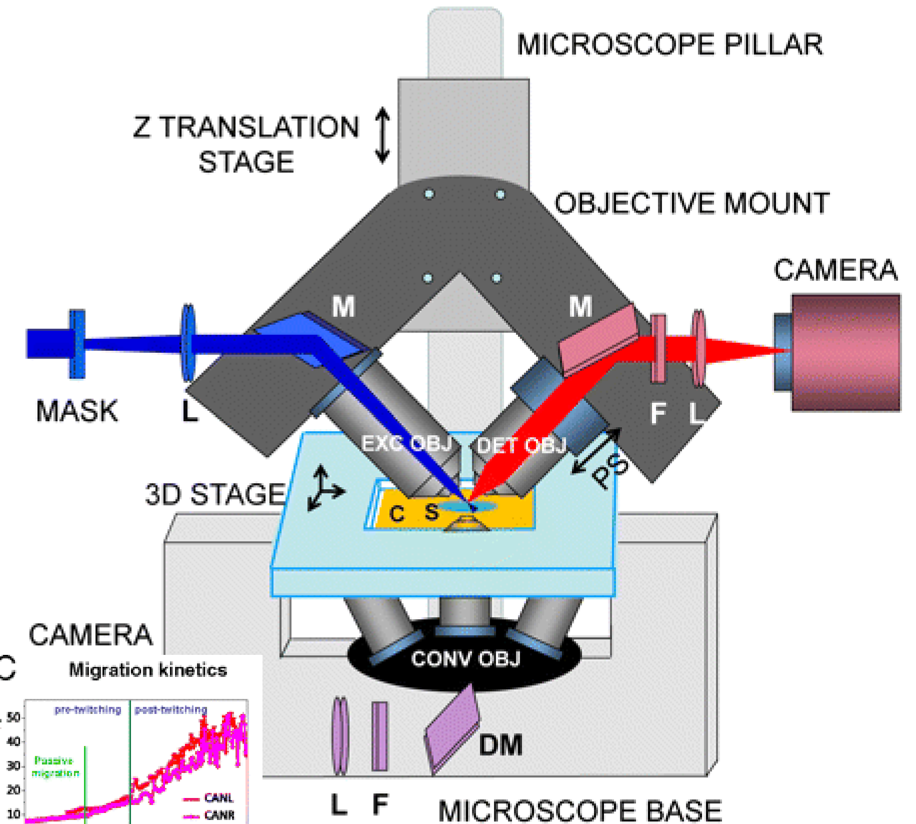


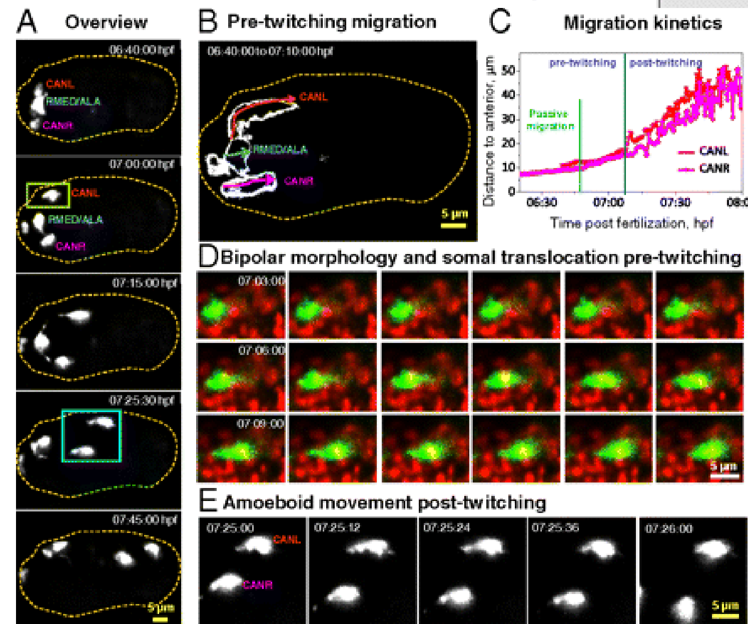


Interesting geometries

iSPIM – Shroff in 2011 – Fast enough to allow samples to move

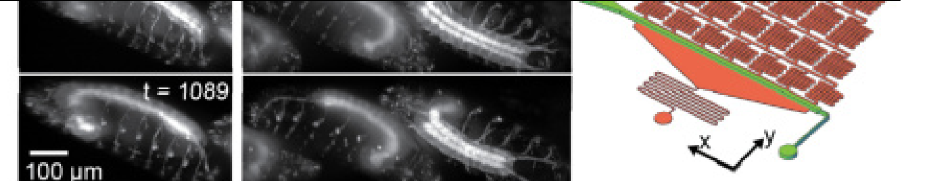
- Annoyance at the agarose popsicle has led to additional geometries
- Often much more difficult to set up the optics, but easier sample prep
- Ideally want it to be like an inverted scope – you never have to touch the alignment, and it's very easy to get samples on the dish
- Have to maintain 90 degree angle between objectives, and deal with gravity





Interesting geometries

- Open top SPIM
- Capable of imaging microfluidic chambers or multiwell plates
- Imaging at 45° through coverglass causes huge astigmatism
- Solution is to put 45 degree prism underneath glass
- Put in extra lens to compensate astigmatism
- Objectives must have long working distance



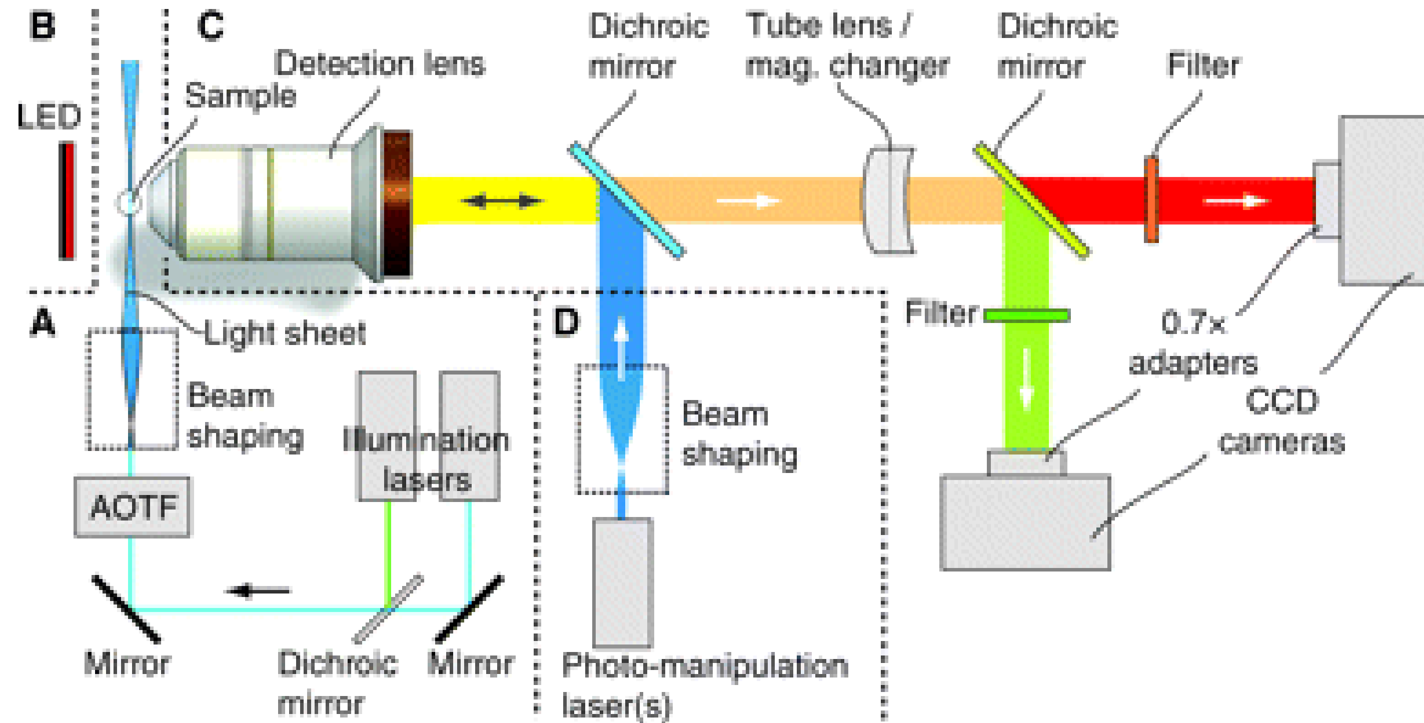
Fancy SPIM

- Possible to include more than one camera for detection
- SPIM can (and has) been combined with the other superresolution techniques we’ve seen
- Further shaping of the beam leads to Bessel illumination
- Always trending towards higher intensity, but briefer exposures are better for sample health

Solar constant at equator	$1{,}366\ W/m^2 = 1{,}366\ J/s \cdot m^2$
Solar constant in central Europe	$\approx 1\ kW/m^2$
Microscopy relevant units	$\approx 1\ nW/\mu m^2 = 100\ mW/cm^2$
Energy density within 600 s	$\approx 0.6\ \mu J/\mu m^2$
Cell diameter of 100 μm × (0.3 s or 10 min)	$\approx$ 2.4 μJ or 4.8 mJ
Embryo diameter of 900 μm × (0.3 s or 10 min)	$\approx$ 190 μJ or 380 mJ

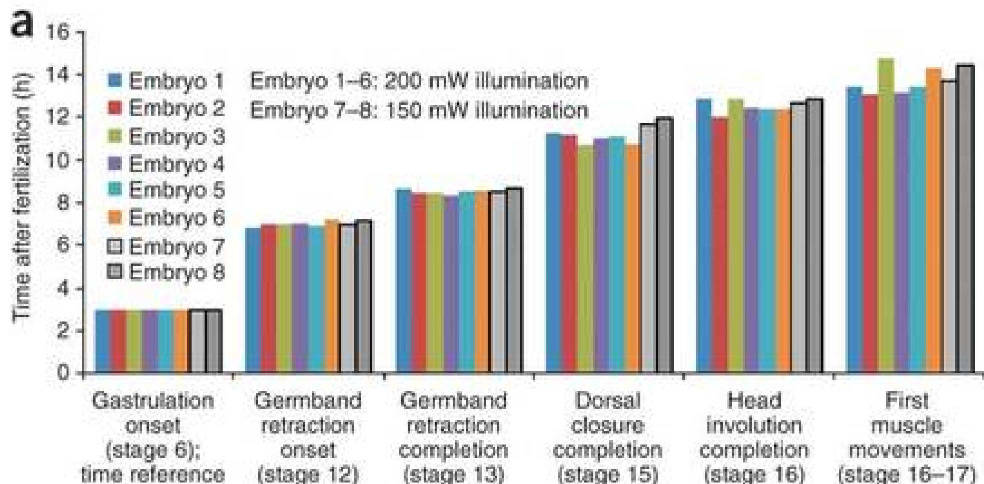
Adding functionality to SPIM

- It’s very easy to pair other techniques with SPIM to get benefits of sectioning and low phototoxicity
- 2 Colors are very easy to add – just need to combine them with a dichroic mirror
- Patterned illumination with a DMD or holographic plate can be added on to detection pathway
- Dual view emission is no problem
- Two photon SPIM is easy by just hooking up ultrafast laser



2 Photon SPIM

- All the advantages of 2 photon – can go deeper into tissue
- 10x faster than point scanning 2 photon
- Less phototoxic
- Dispersion through sample will decrease intensity – loss of fluorescent signal



Time after onset of gastrulation (min)

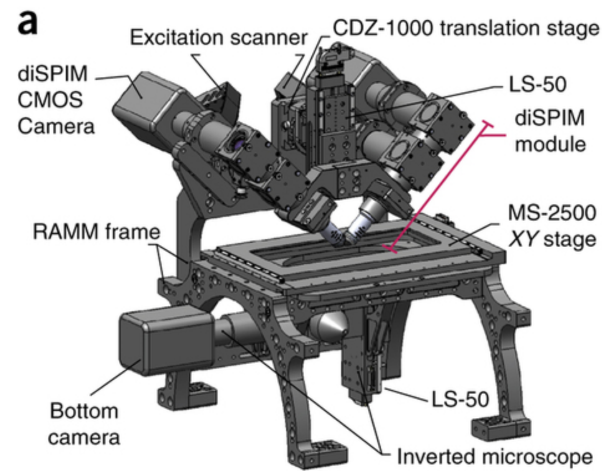
Middle of embryo

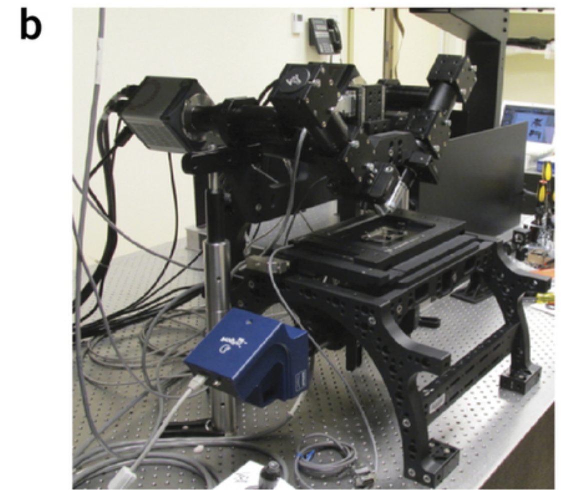
x depth from embryo surface (μm)

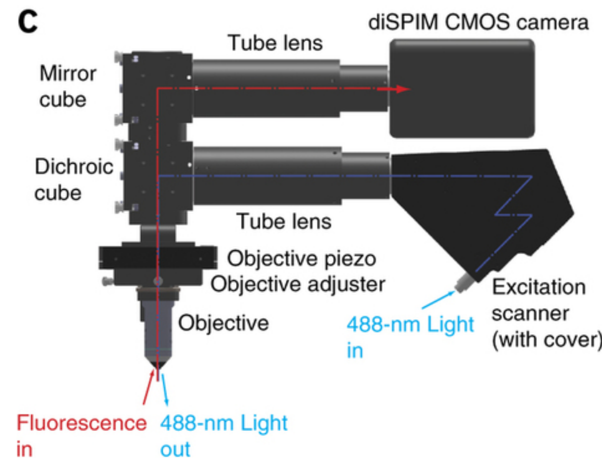
x stack of y-z slices

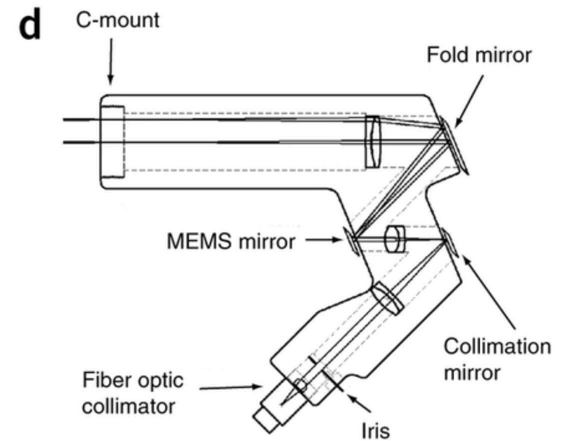
Getting crazy with SPIM

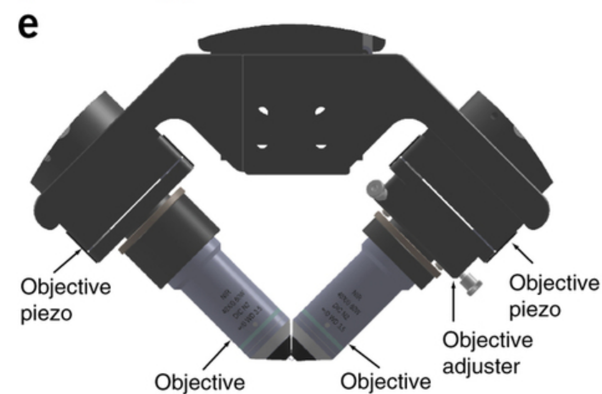
- Two cameras can section 3D object twice as fast
- Photons from opposite beam will contribute very little out of focus blur
- Image registration becomes critical between alternating cameras

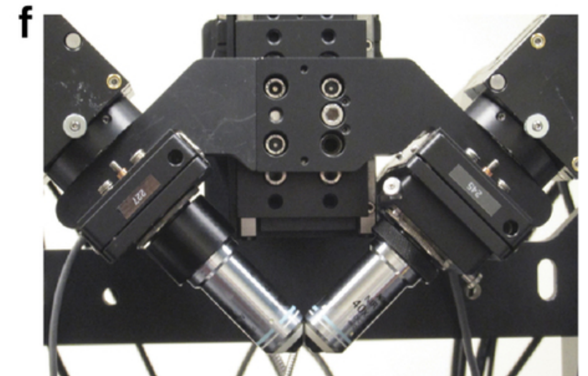












diSPIM – 2x the camera, 4x the alignment

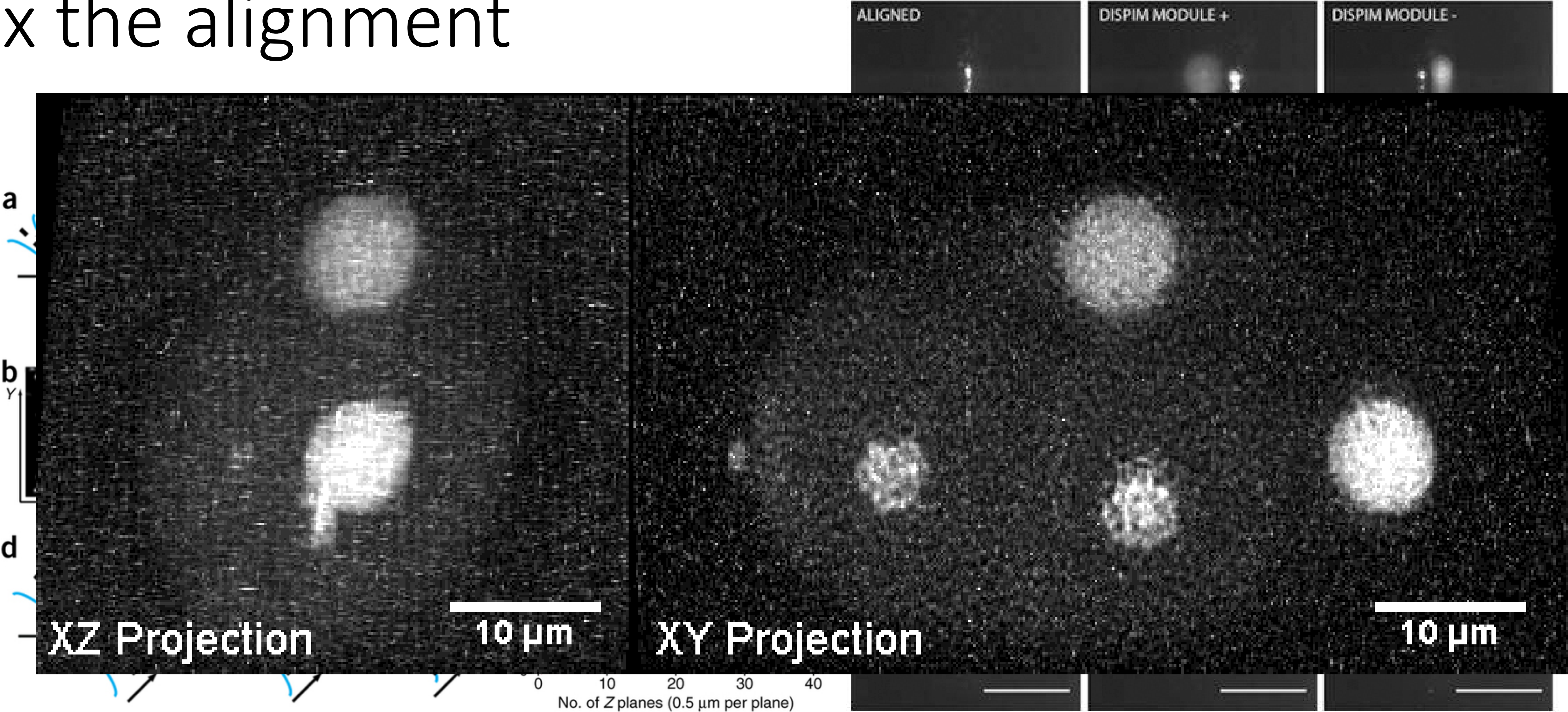
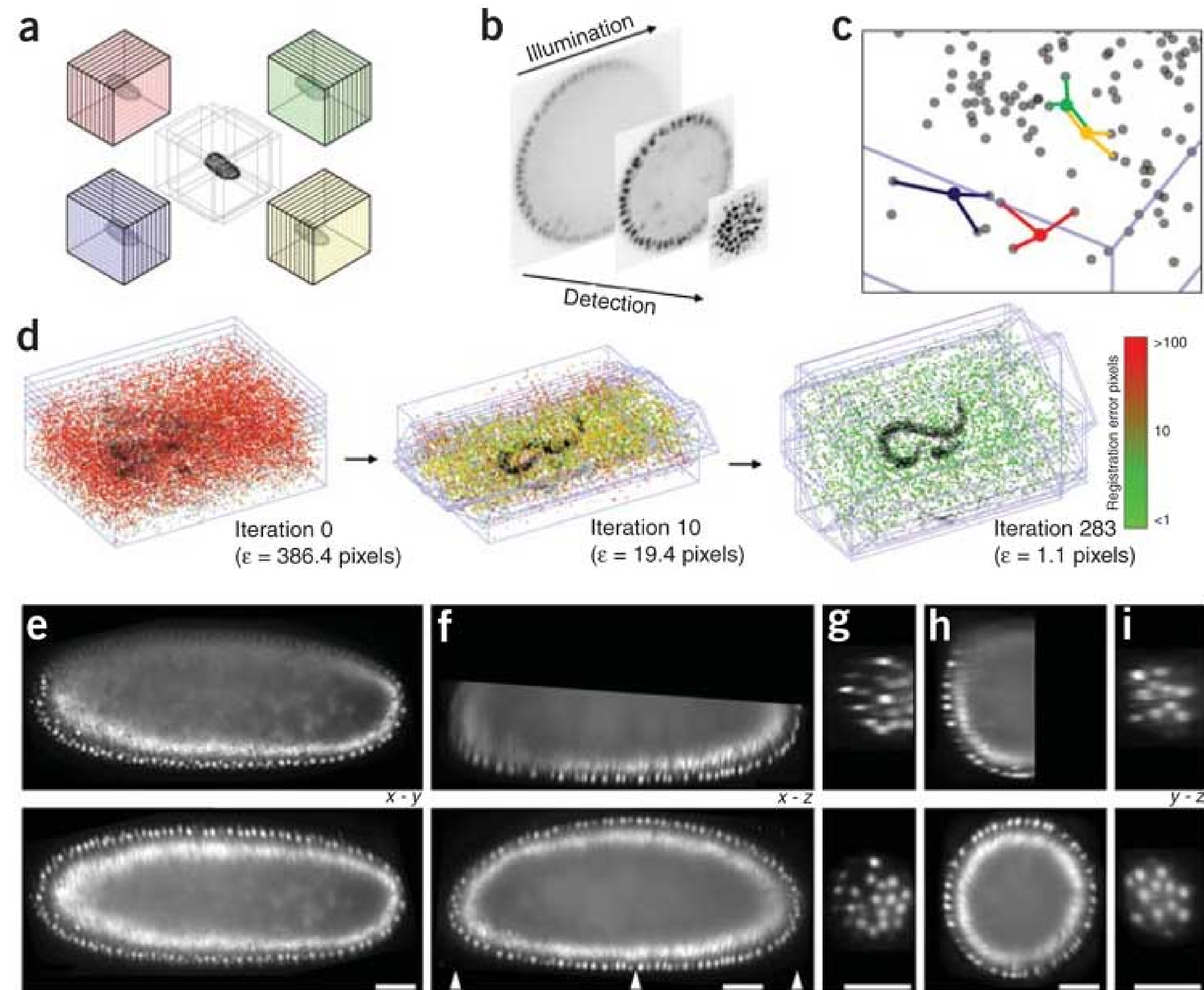


Image processing for SPIM

- Difficulties reconstructing image since the signal is degraded along the SPIM axis
- There may be limited overlap between views
- Real time algorithm developed by Tomancak
- For each point, find the relationship between 4 beads which can be represented by a single vector
- This vector is invariant depending on imaging orientation
- This package uses thousands of beads around sample



On to Matlab…