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Sample preparation for integrated light and electron microscopy

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The structure function problem, and resolution

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Fluorescence microscopy

Dynamic molecular localisation and function

Reveals no underlying structure

Conventional resolution

Diffraction limited ~200 nm lateral, ~500 nm axial

Electron microscopy

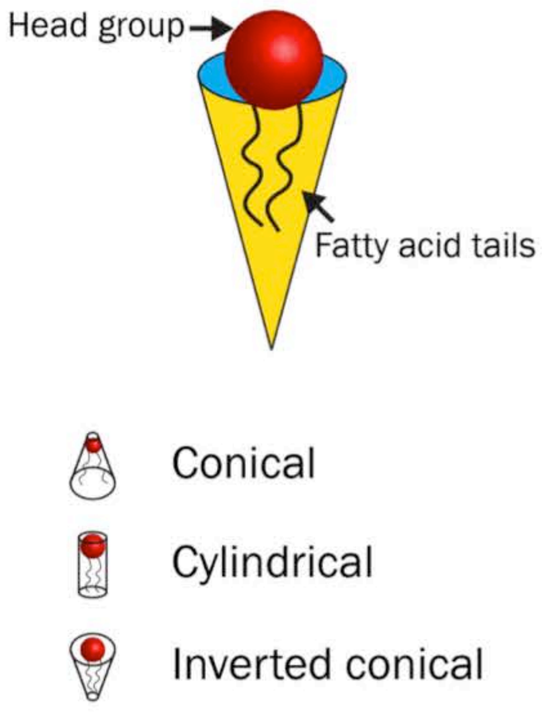
Contains no dynamic functional information

Highly detailed underlying structure

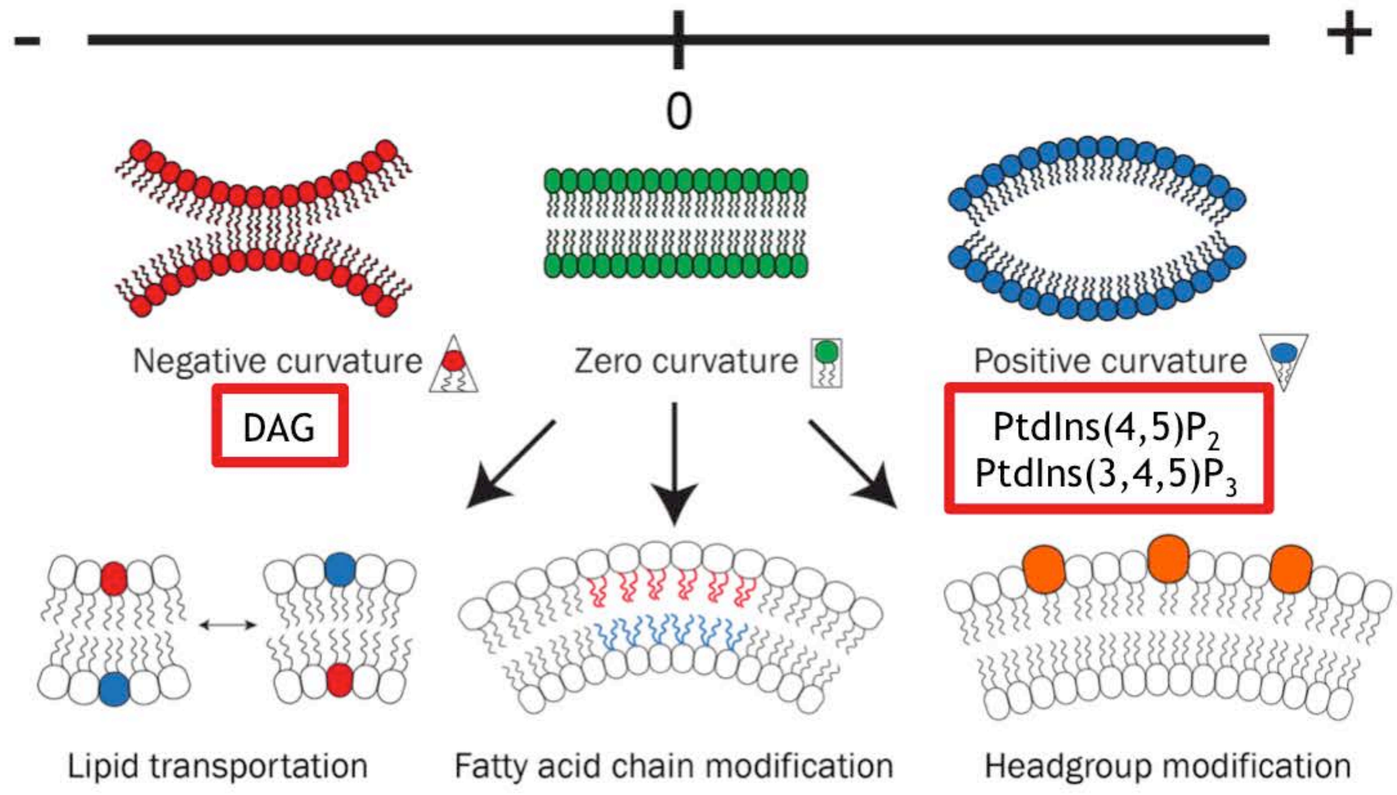
Conventional resolution (biological!)

Typically 2-6 nm lateral, and 50-70 nm axial

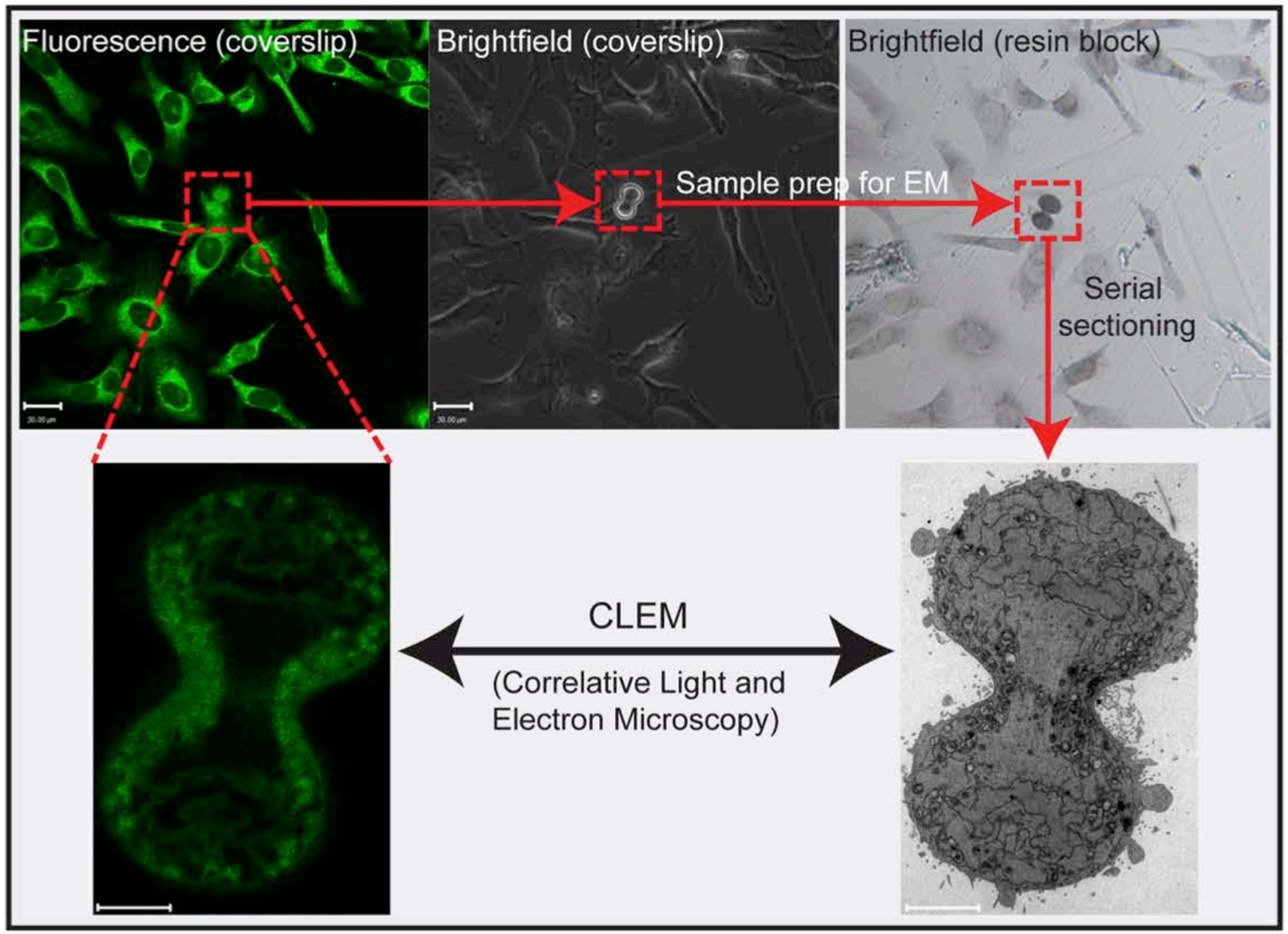
Lipids form structures according to their SC



Mechanisms to generate membrane curvature through lipids

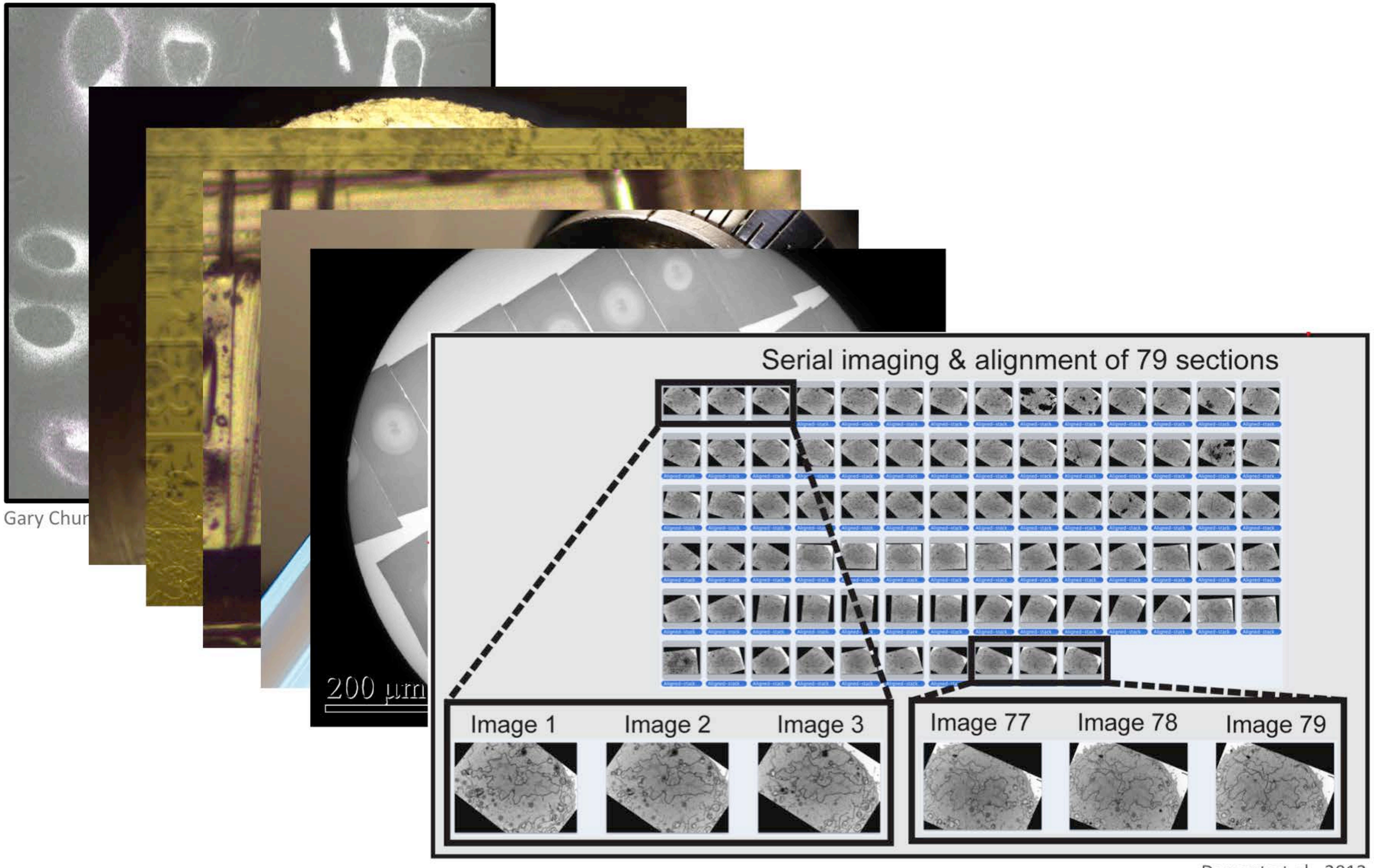


Correlative light and electron microscopy



Domart et al., 2012

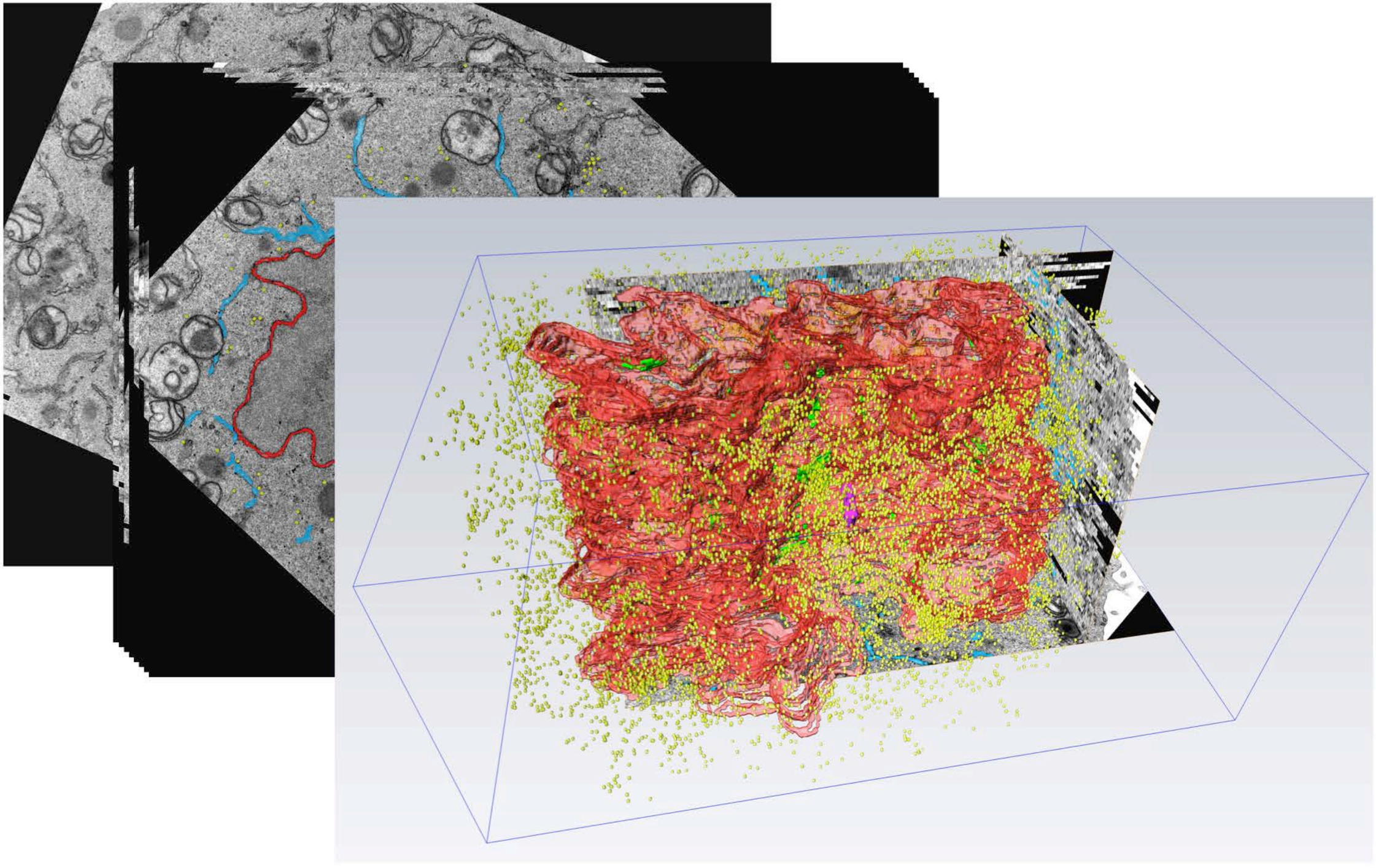
ROI relocation and serial section collection



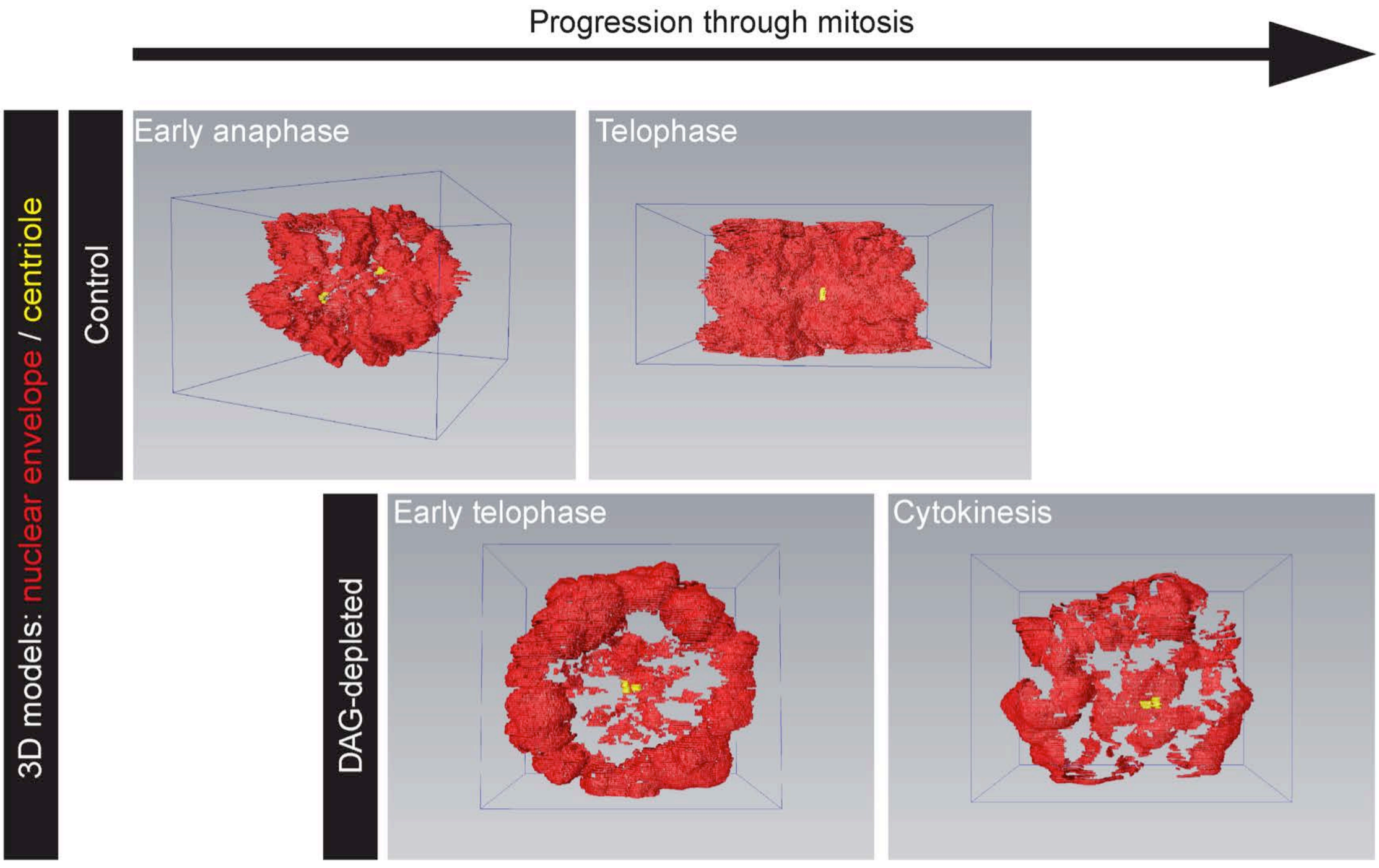
Gary Chur

Domart et al., 2012

Segmentation and reconstruction



Segmentation and reconstruction

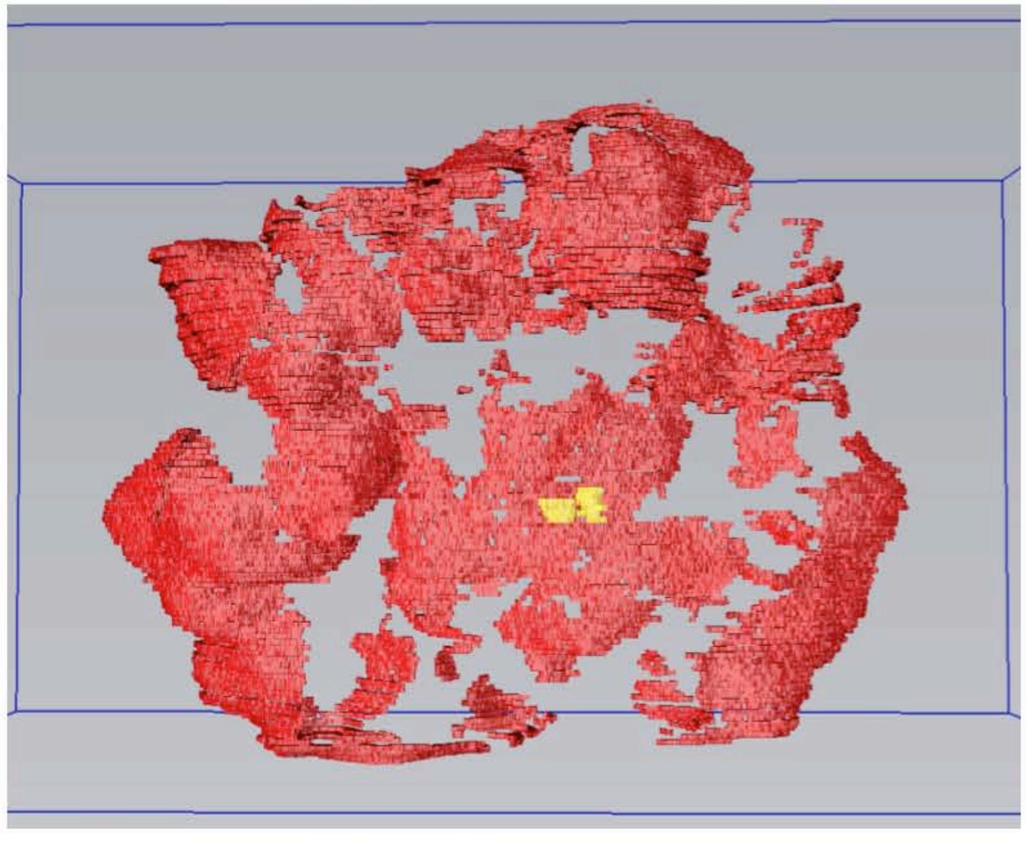


Domart et al., 2012, PLoS ONE

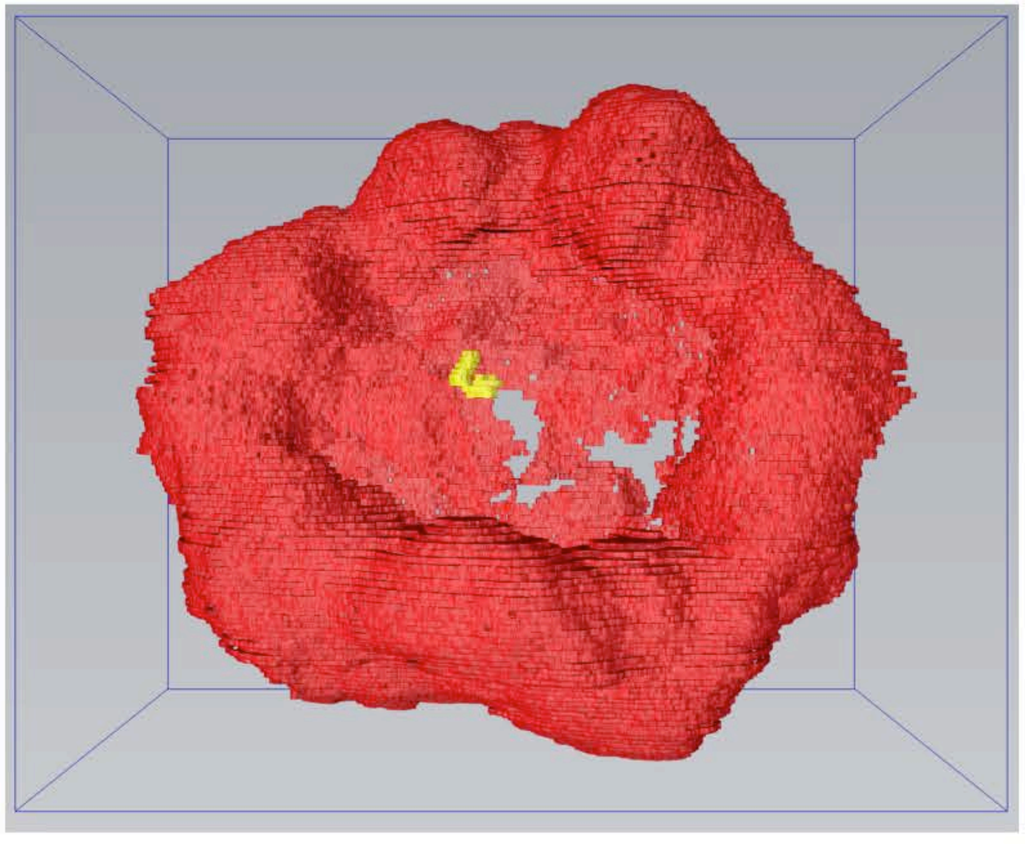
Segmentation and reconstruction

3D model / nuclear envelope / centrioles

DAG-depleted cell



DAG -rescued cell



→ Diacylglycerol is required for nuclear envelope formation

Domart et al., 2012, PLoS ONE

Aligned 3D LM + 3D EM over mm^3 with nm resolution

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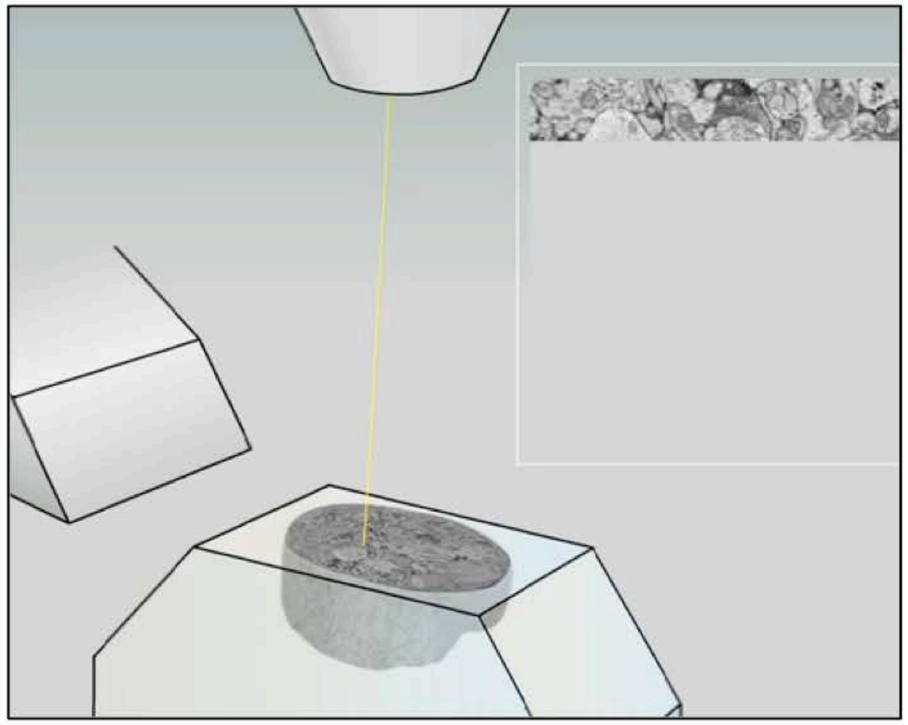
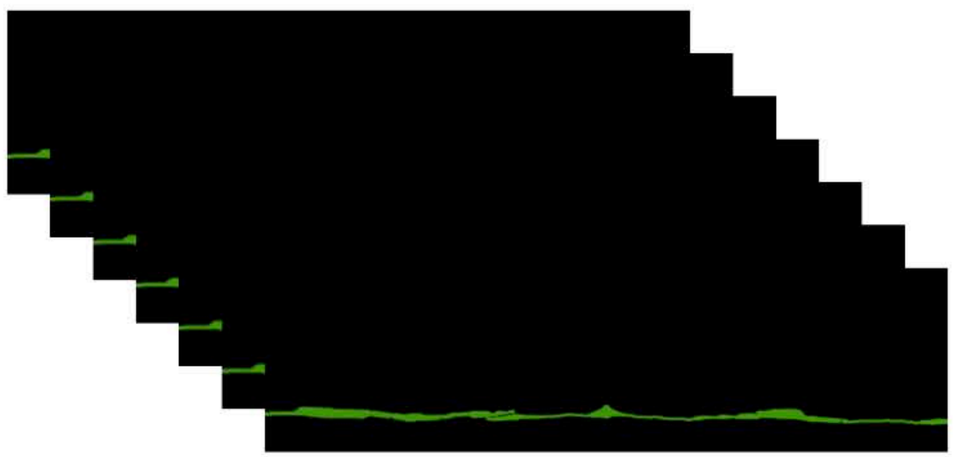
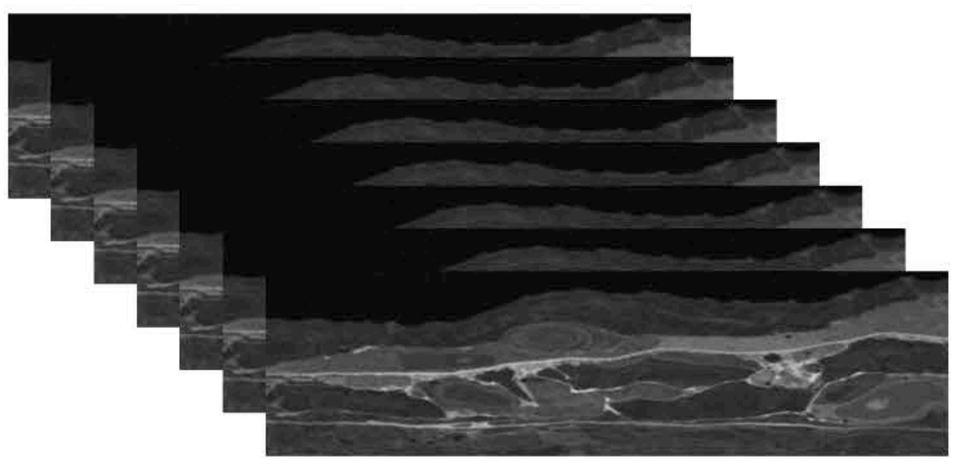
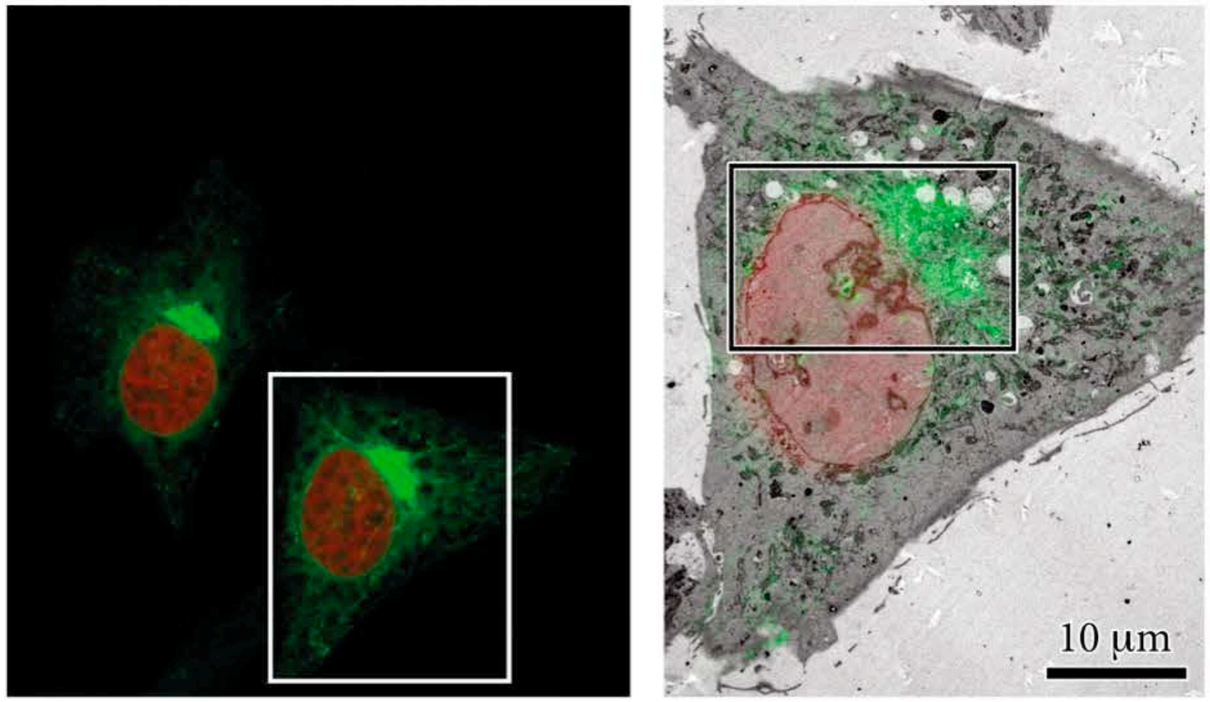
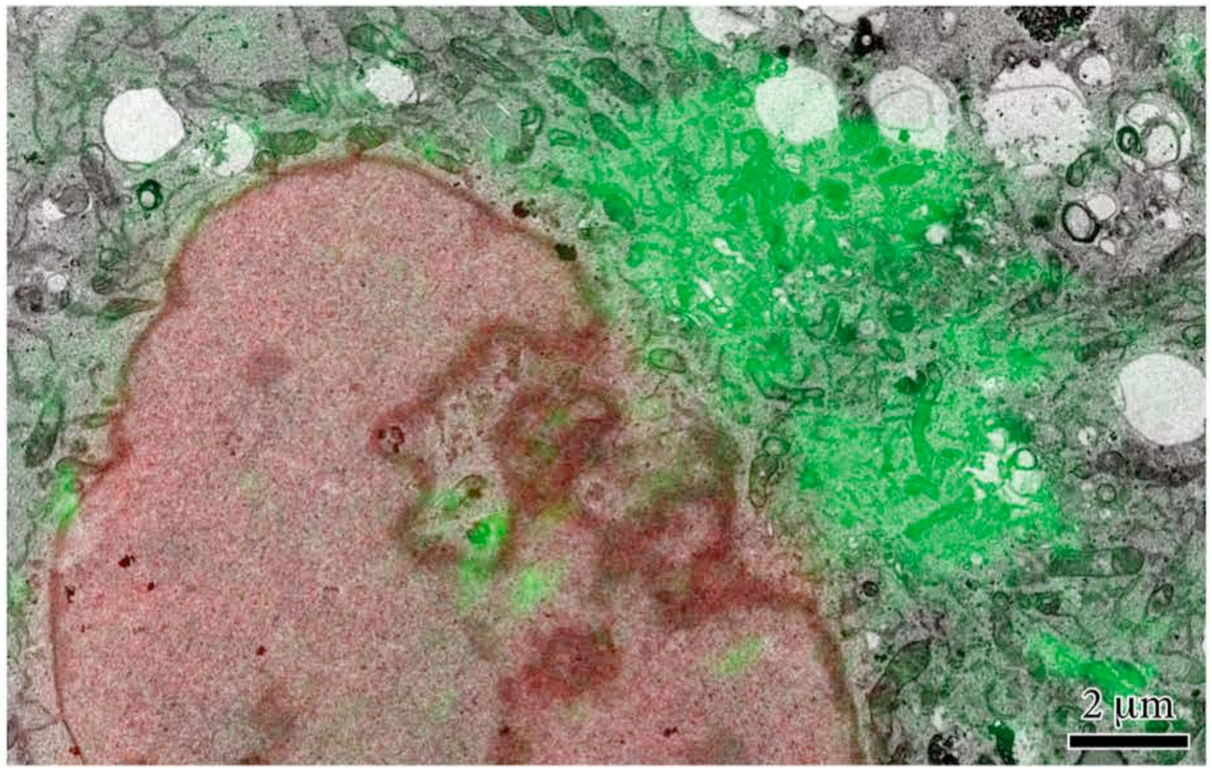




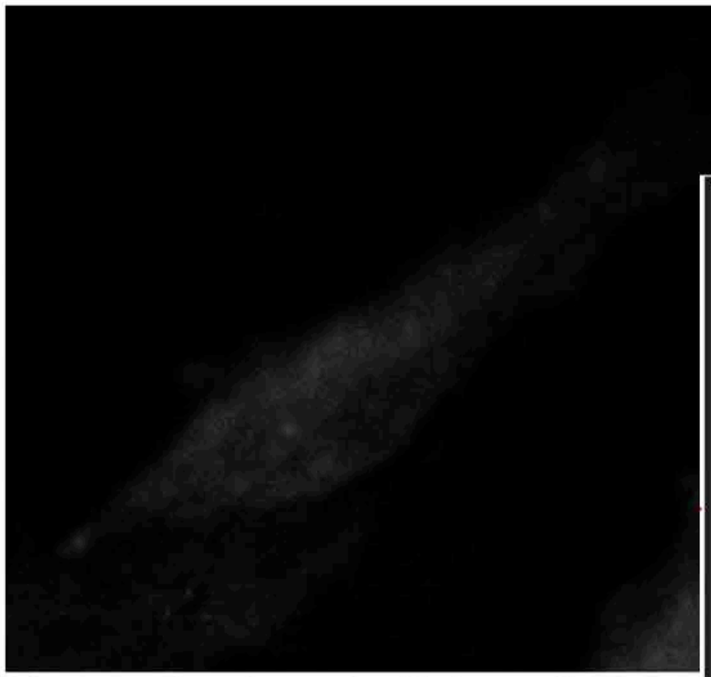


Resolution in XY axes (lateral)

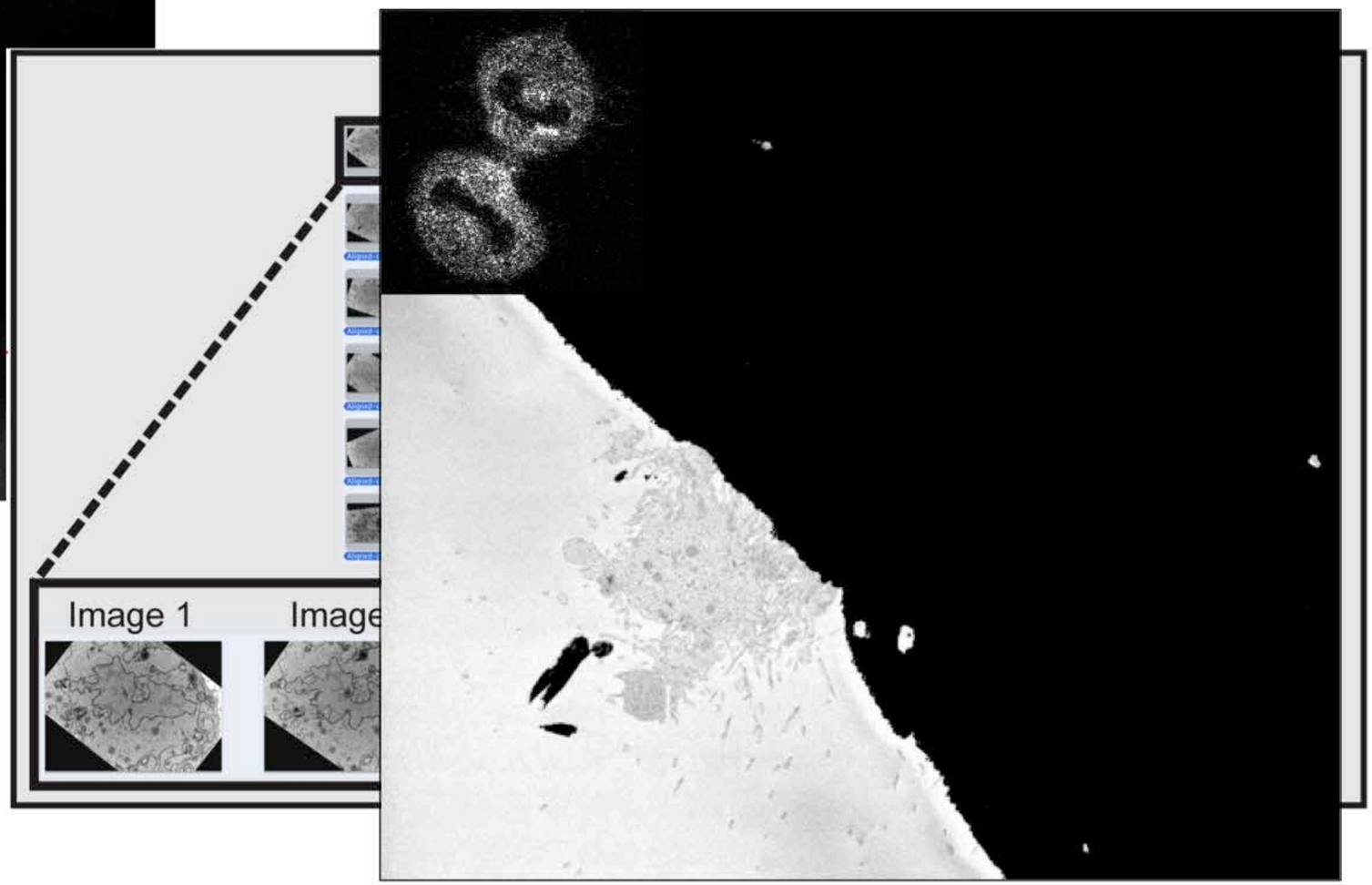




Resolution in Z axis (axial)



Live confocal stack: HeLa cell expressing EGFP-C1aC1b



But… what about putting ***both*** signals in the same specimen?

Integrated microscopy and fluorescence

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Why go for an integrated method?

No additional specimen movement or treatment
No deformation from sectioning
No additional staining for EM
No alignment for image overlays

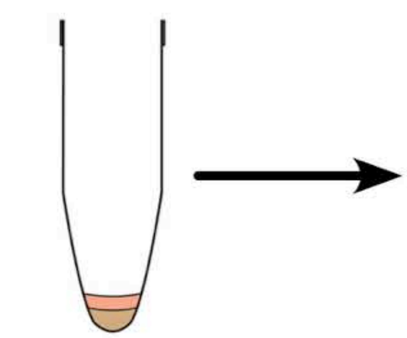
But, must maintain FL through EM processing and embedding…

Fixation
Dehydration
Resin embedding
Temperature

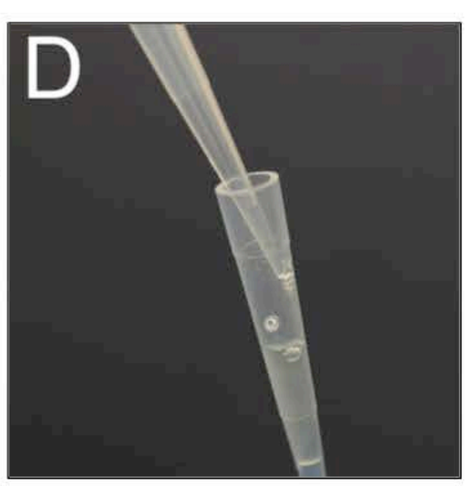
… and must do fluorescence imaging on ***dry*** sections

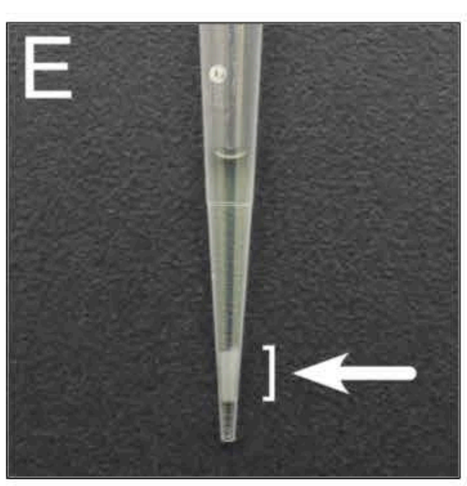
Ultimate goal High resolution correlative 3D analysis of near native state samples using integrated light and electron microscopy

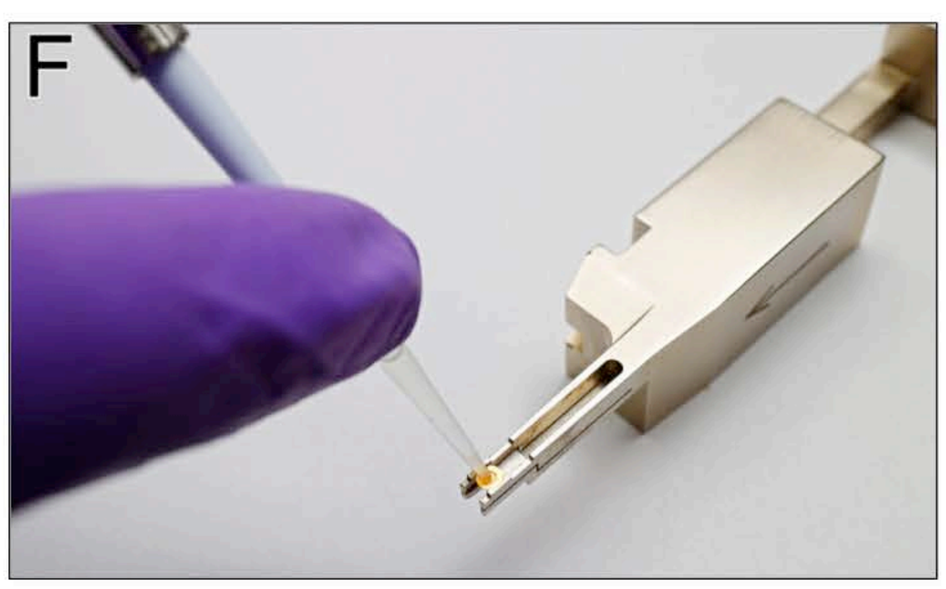
In-resin fluorescence (IRF)



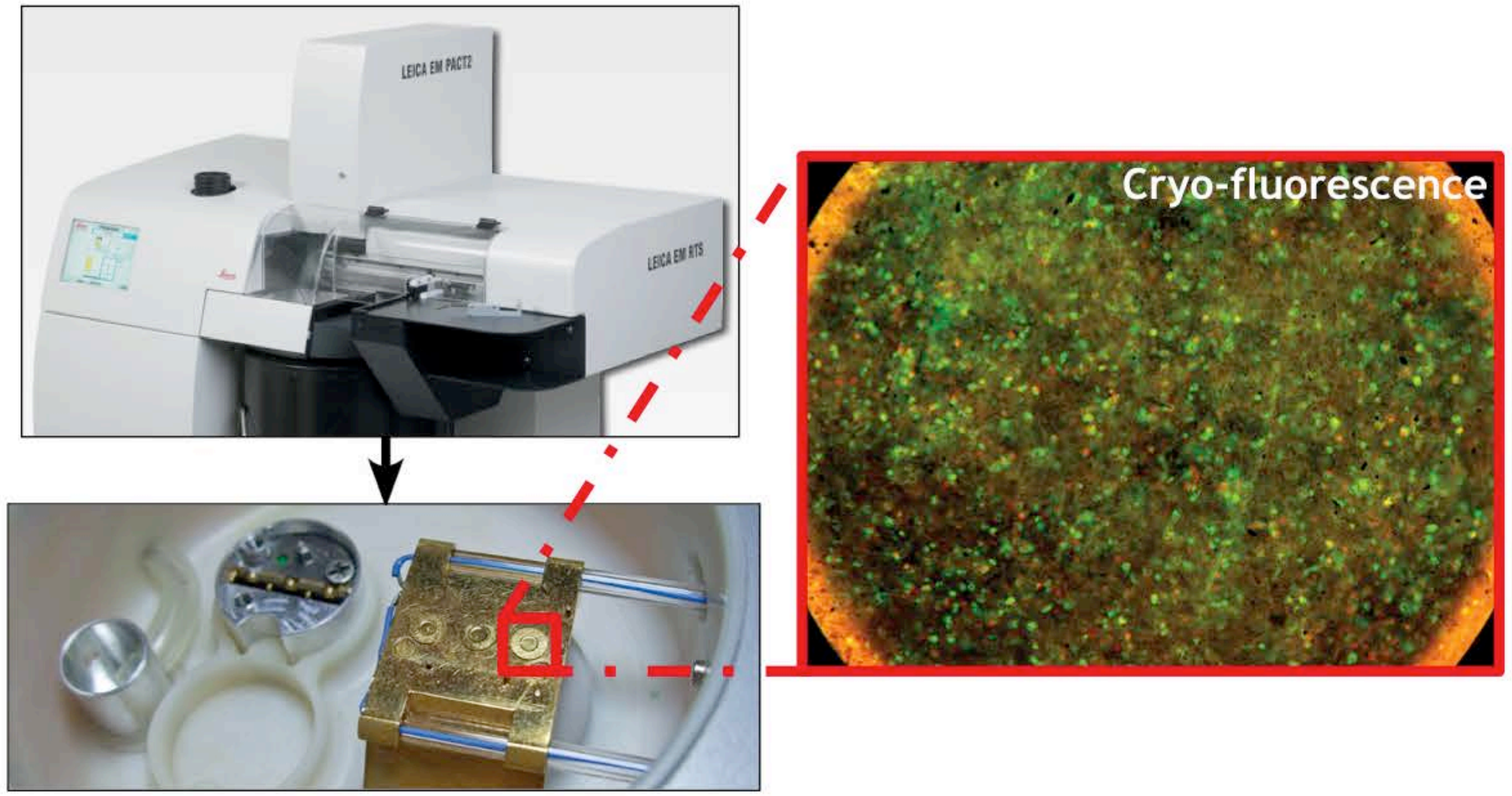
Spin and resuspend



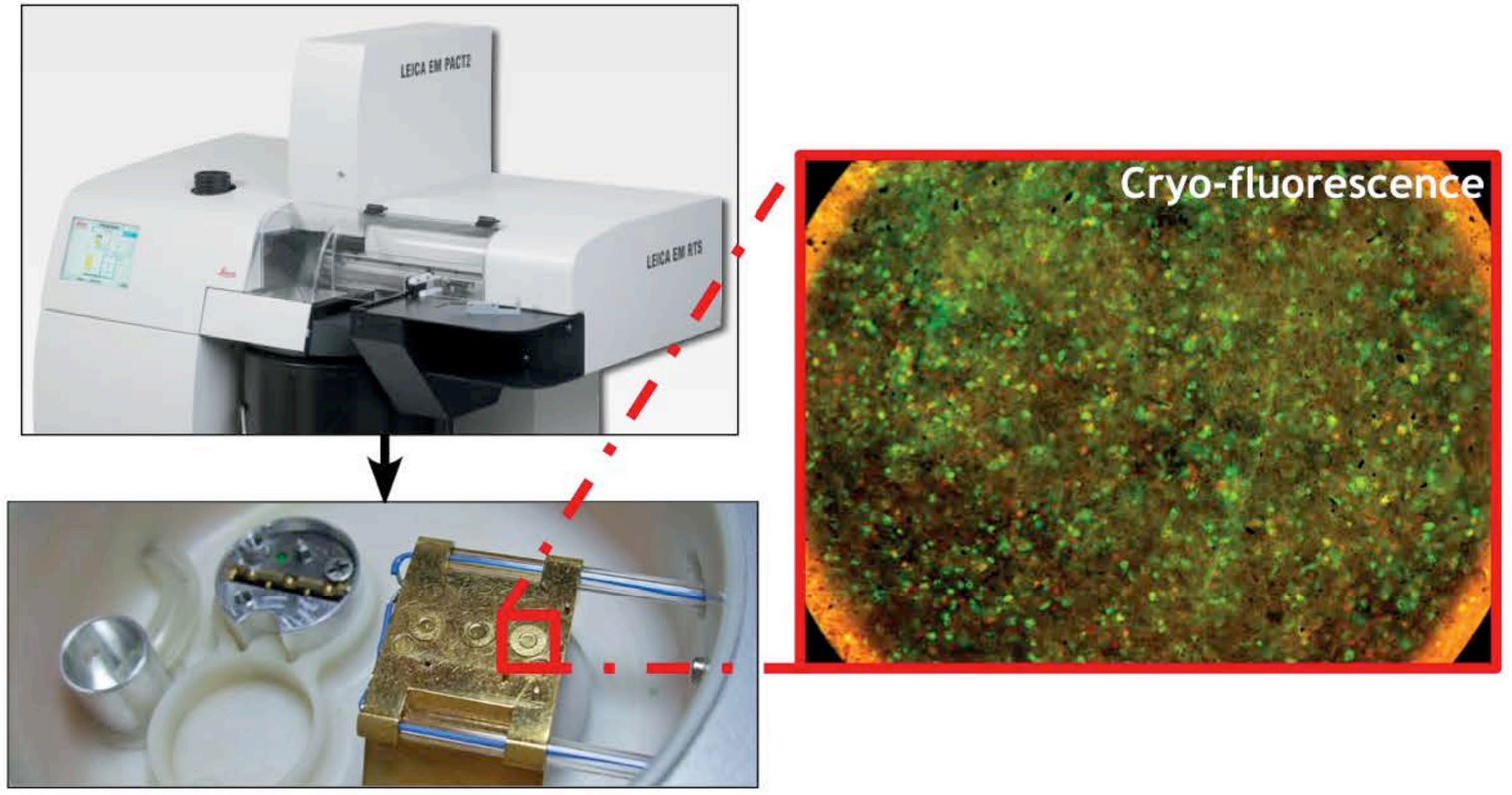




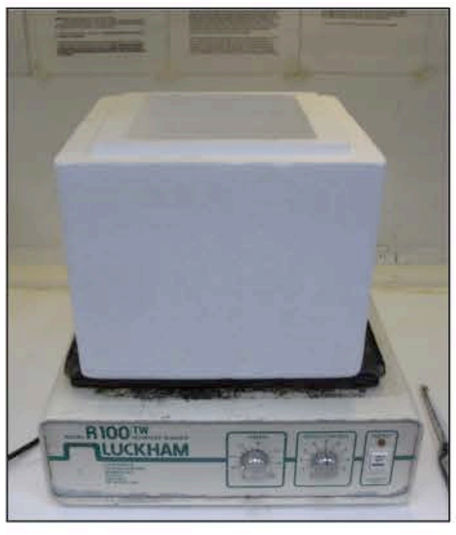
In-resin fluorescence (IRF)



In-resin fluorescence (IRF)



QFS modified from Nixon (2009)/McDonald and Webb (2011), embed in resin, and UV polymerise



Load into frozen FS media under LN_2

→ Acetone // H_2O // Uranyl acetate

Warm up phase

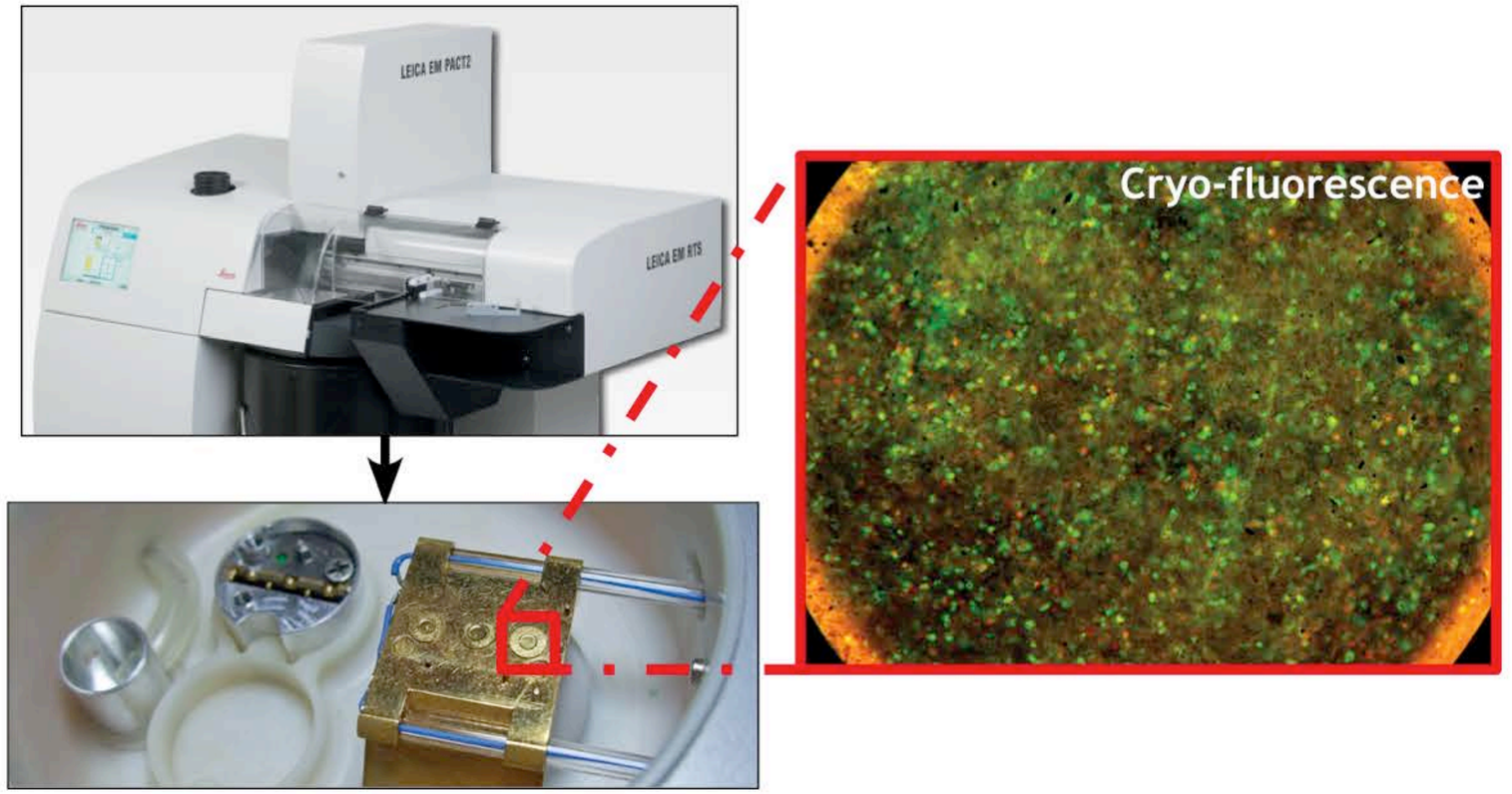
→ Remove LN_2, add dry ice, wait for -85°C

→ Remove dry ice, wait for -50°C

→ Transfer to AFS2 at -50°C

→ Acetone washes and infiltration with HM20

In-resin fluorescence (IRF)



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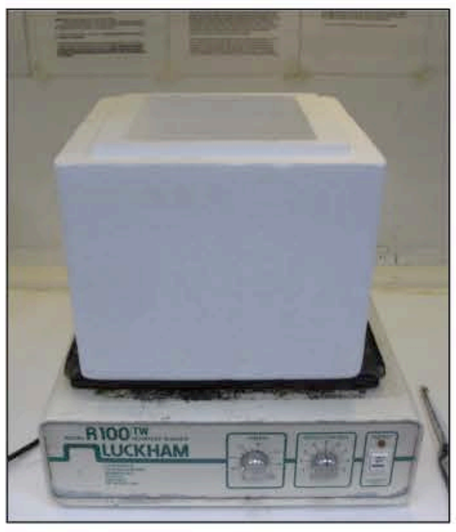
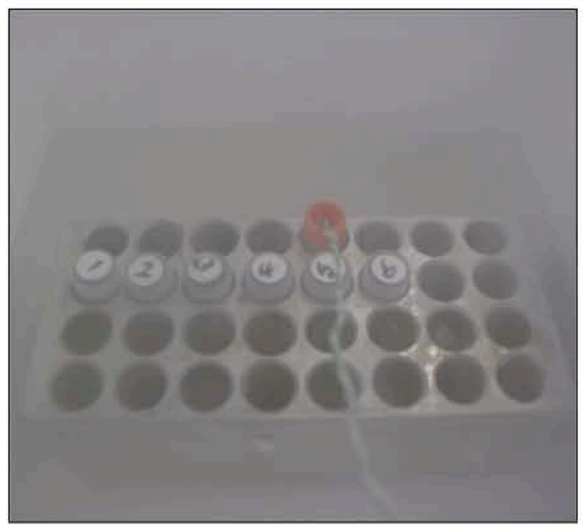
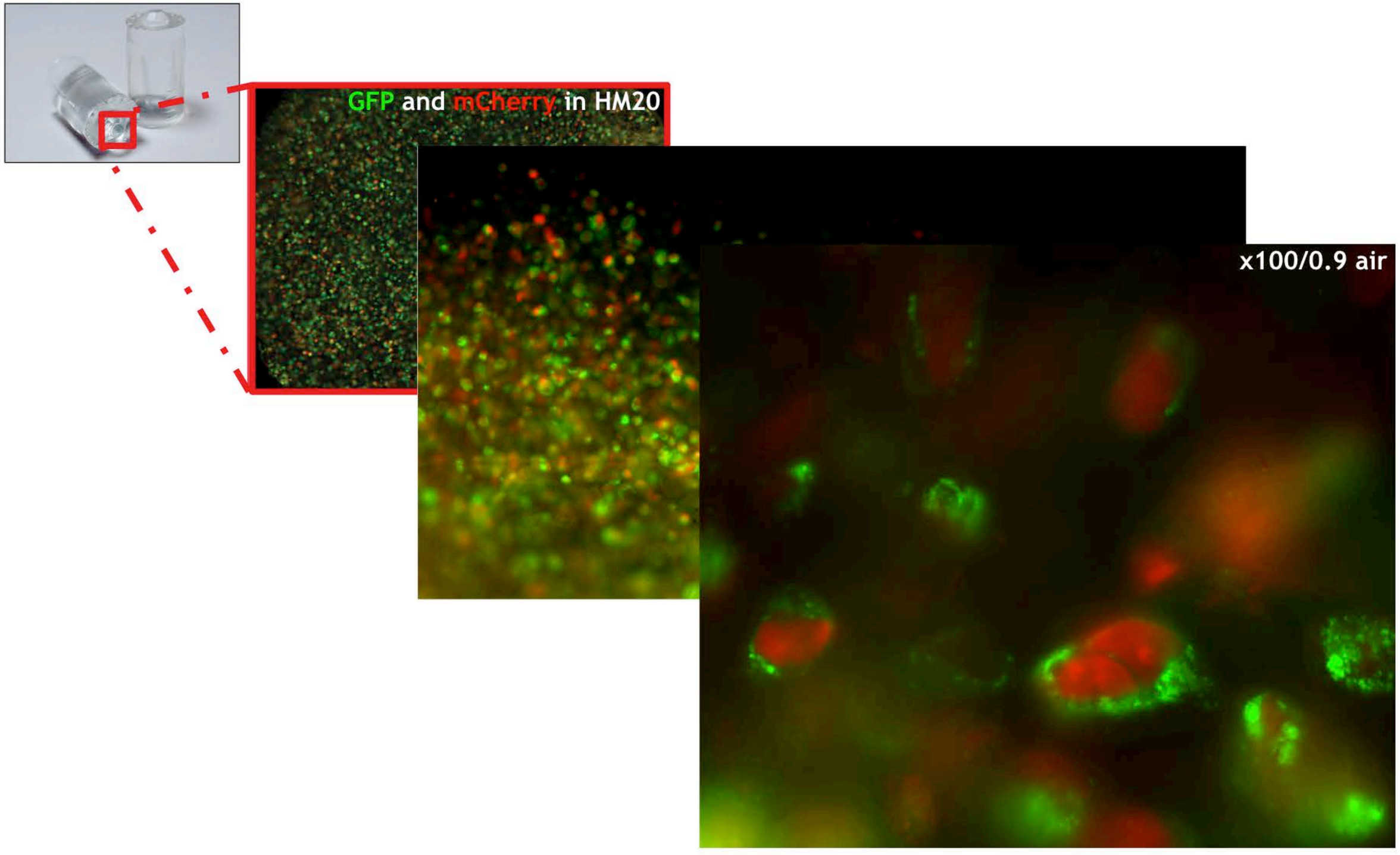


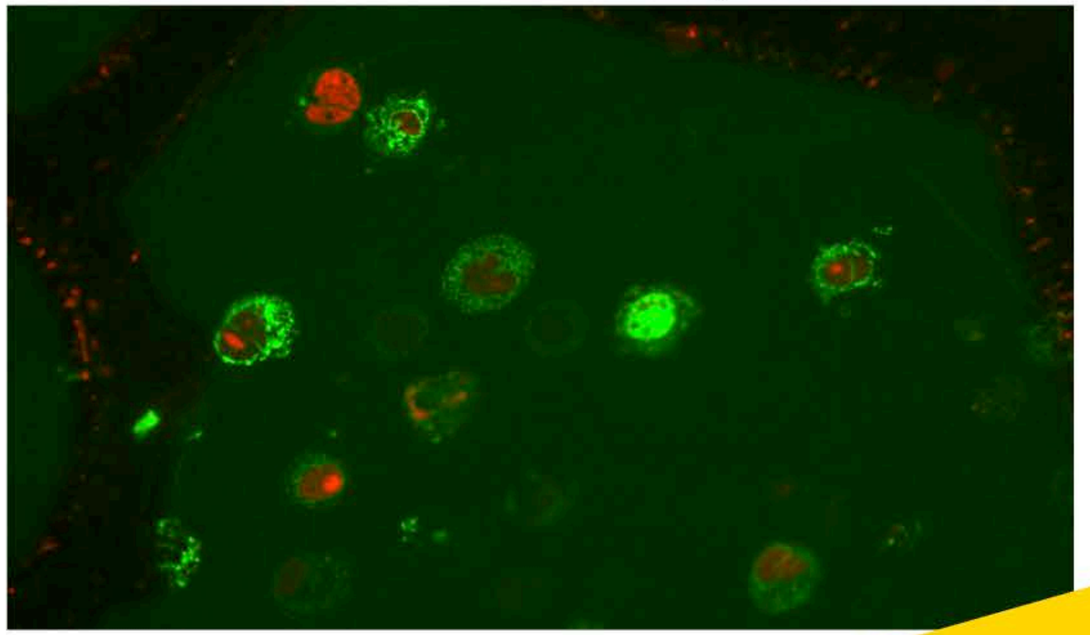


Imaging of IRF at the blockface

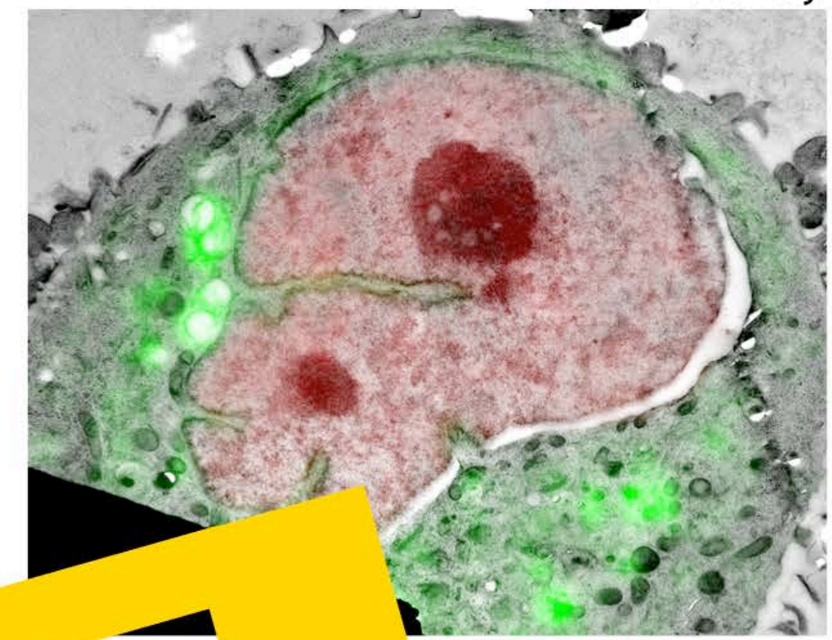


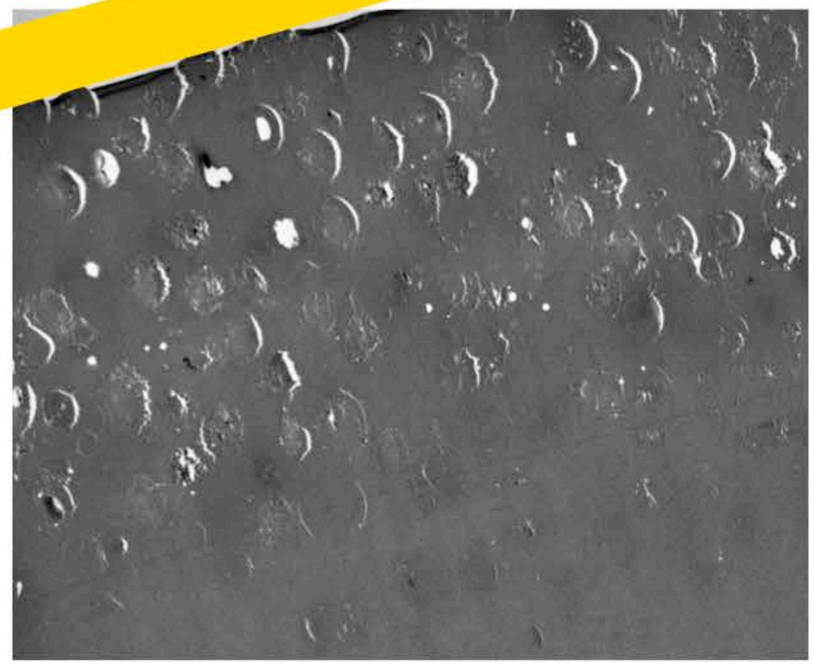
Preliminary results

HeLa; 200 nm HM20 LM



EM / LM overlay





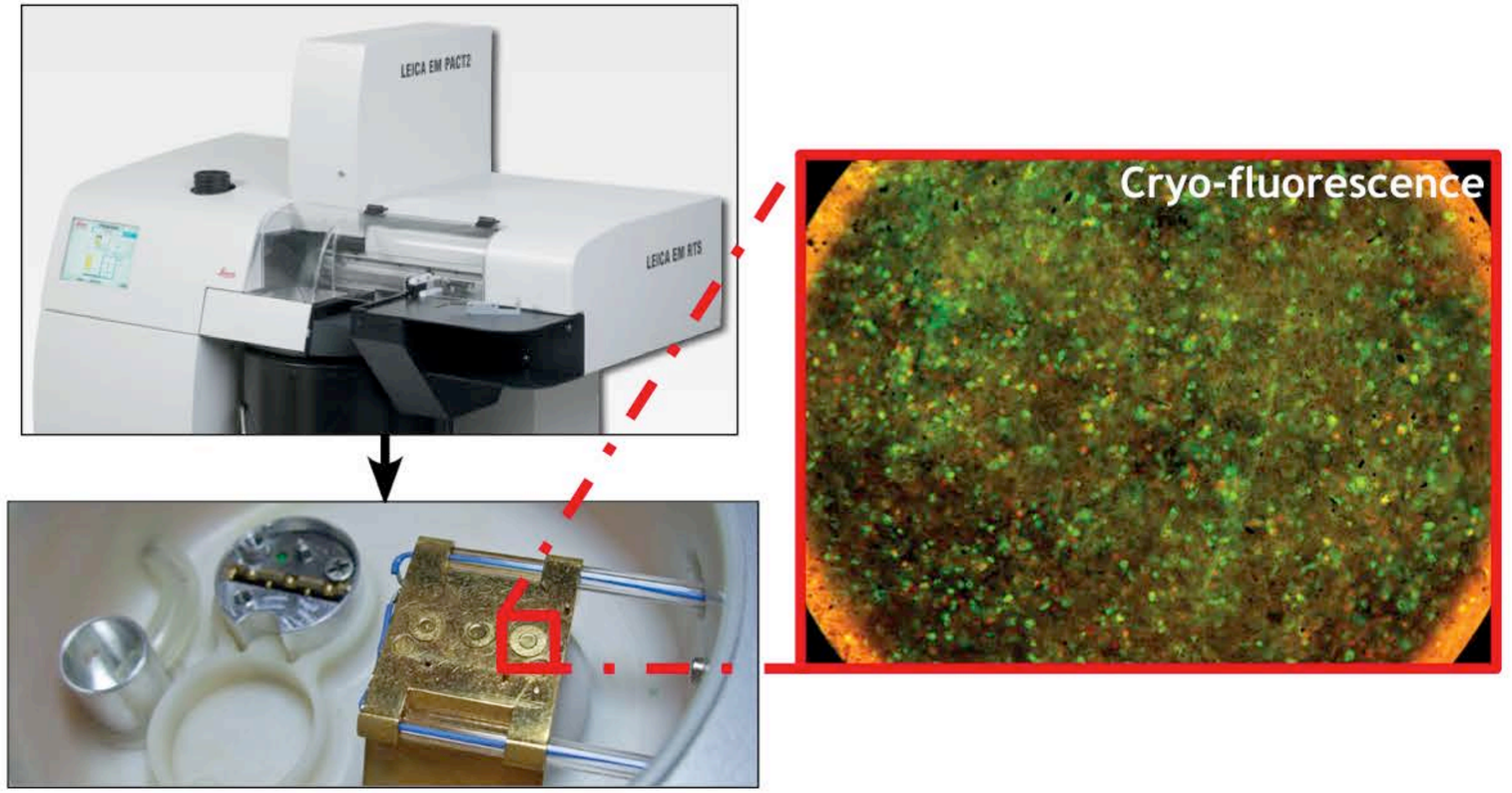
- ation
- n contrast

- Re uorescence

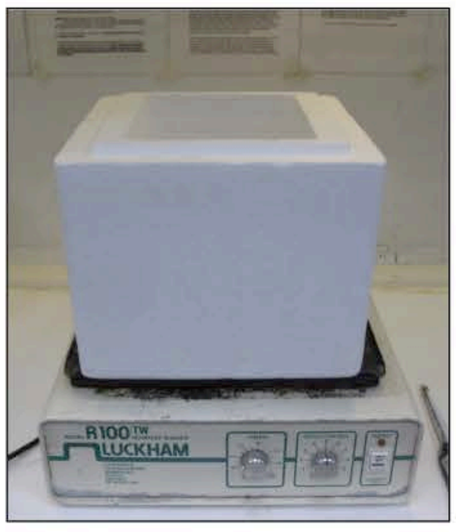
Evolution…

- → Routinely cut perpendicular to the cell layer
- → Reduce uranyl acetate concentration
- → (Change water concentration)
- → Extend overall substitution time
- → Widen resin infiltration dilutions and timings
- → Additional resins (K4M and LRWhite)

In-resin fluorescence (IRF)



QFS modified from Nixon (2009)/McDonald and Webb (2011), embed in resin, and UV polymerise



Load into frozen FS media under LN_2

→ 95% acetone // 5% H_2O, 0.1% uranyl acetate

Warm up phase

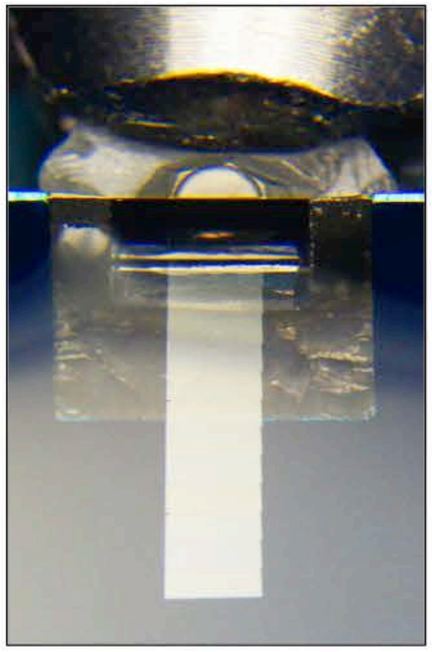
→ Remove LN_2, add dry ice, wait for -85°C

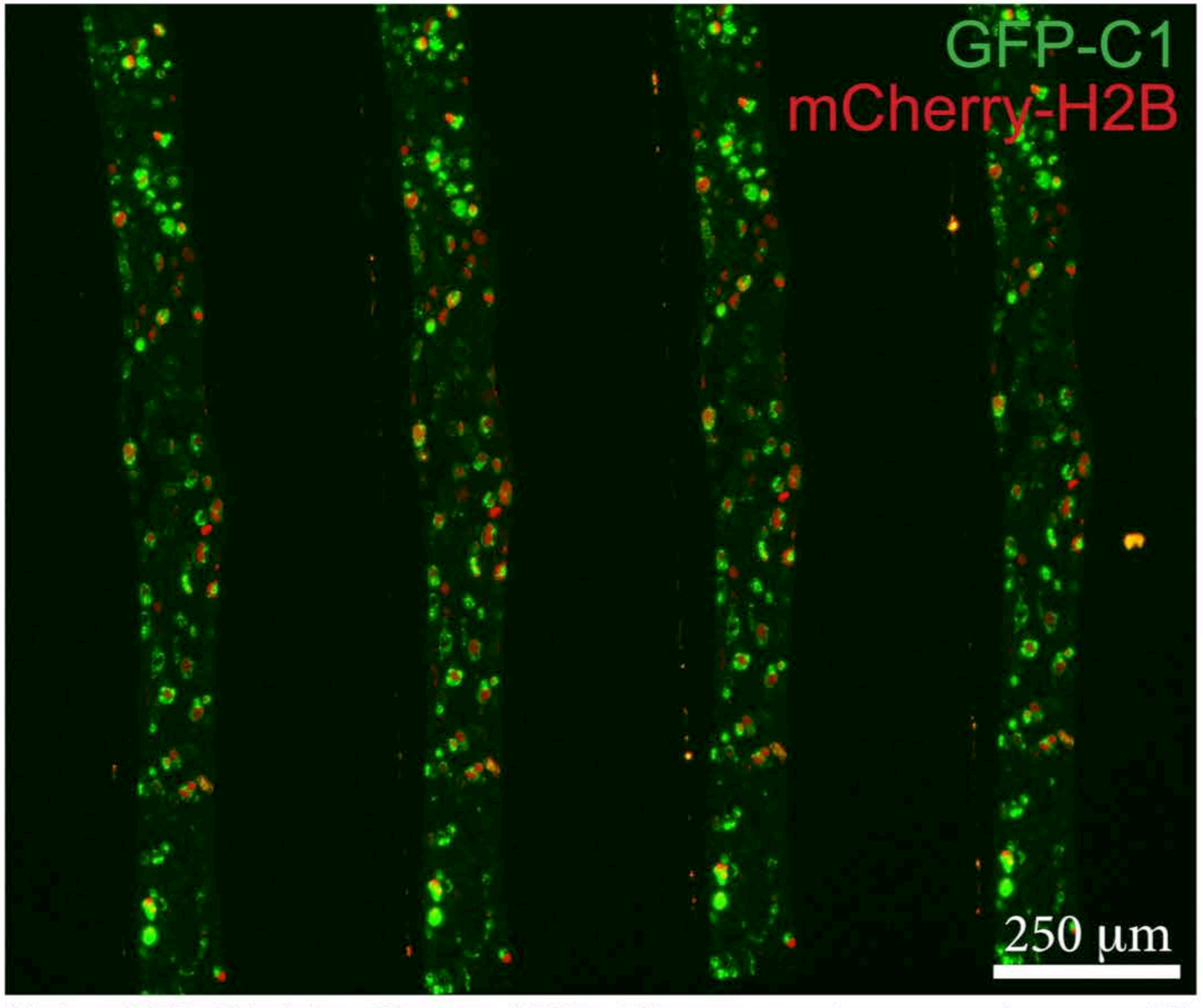
→ Remove dry ice, wait for -50°C

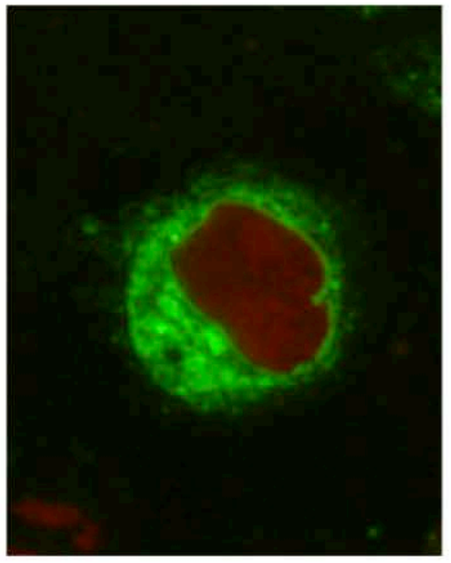
→ Transfer to AFS2 at -50°C, wait for FS time of 3 hours

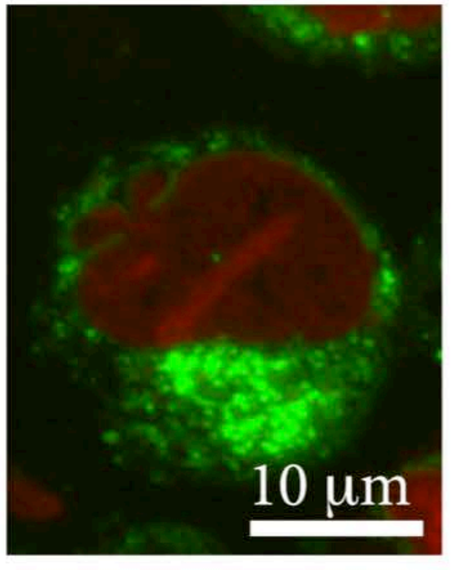
→ Acetone washes and infiltration with HM20

Ultrathin serial sectioning and light microscopy



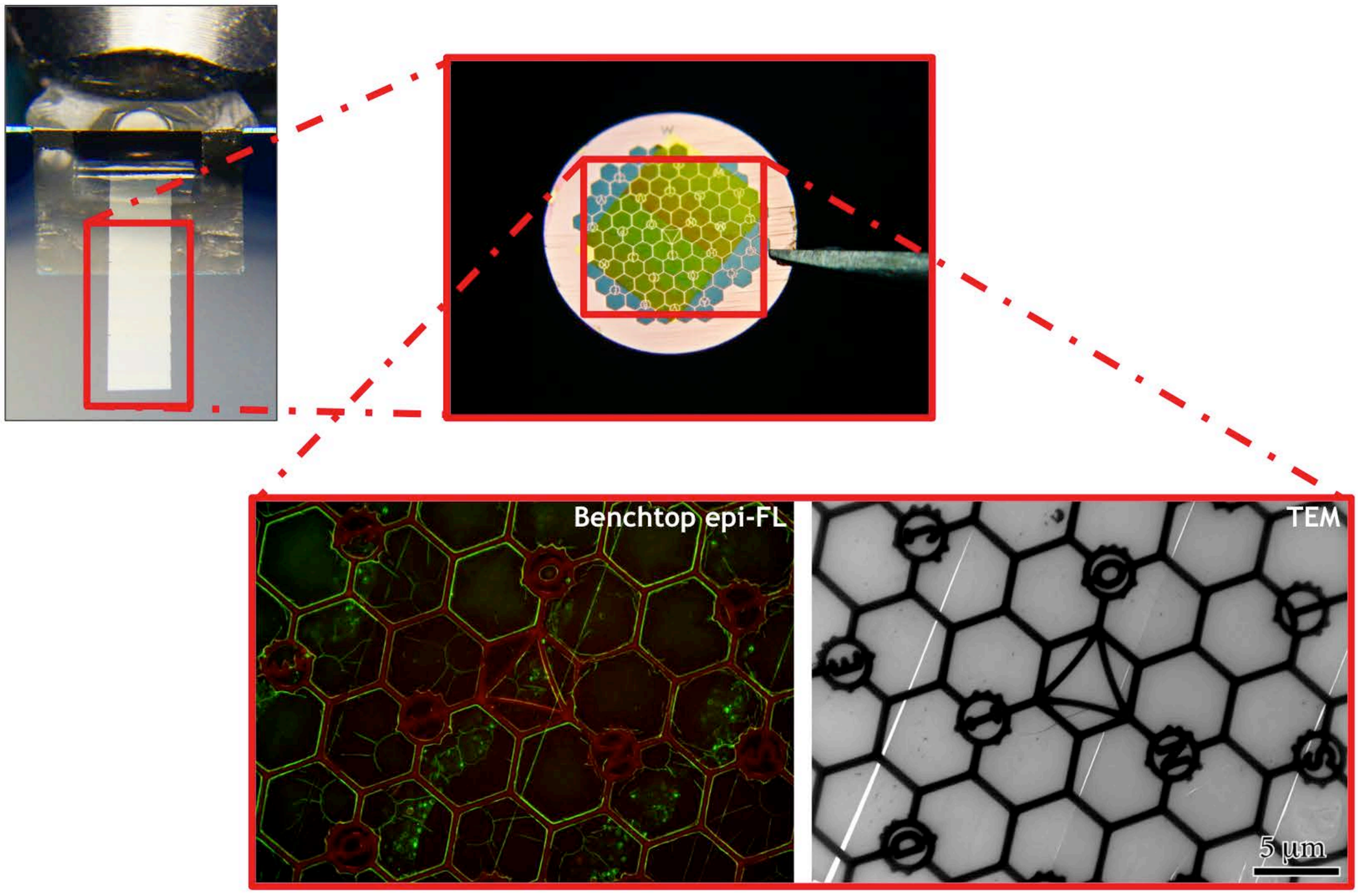






HeLa GFP-C1 // mCherry H2B, 70 nm sections on glass, epifluorescence

IRF analysis with separate instruments



IRF analysis: HeLa cells expressing GFP-C1

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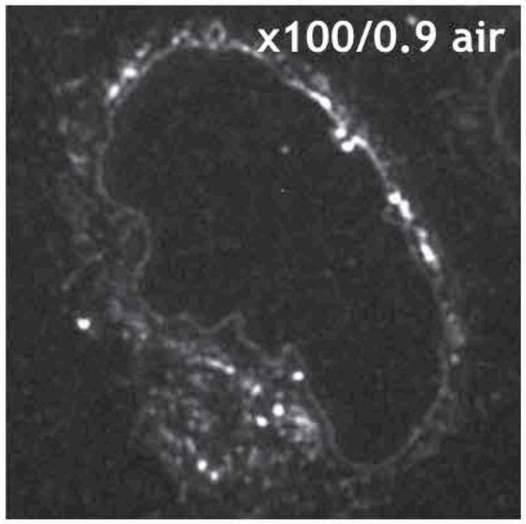
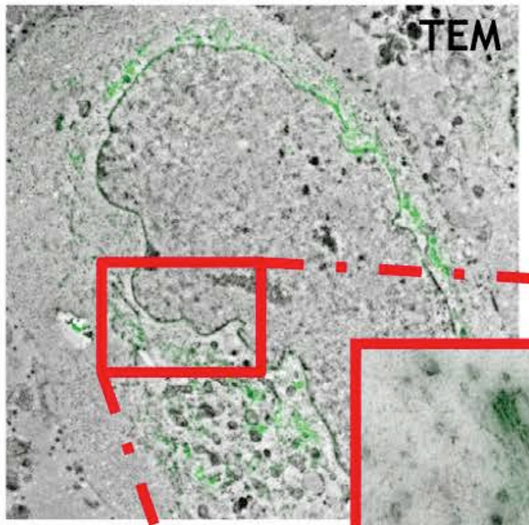
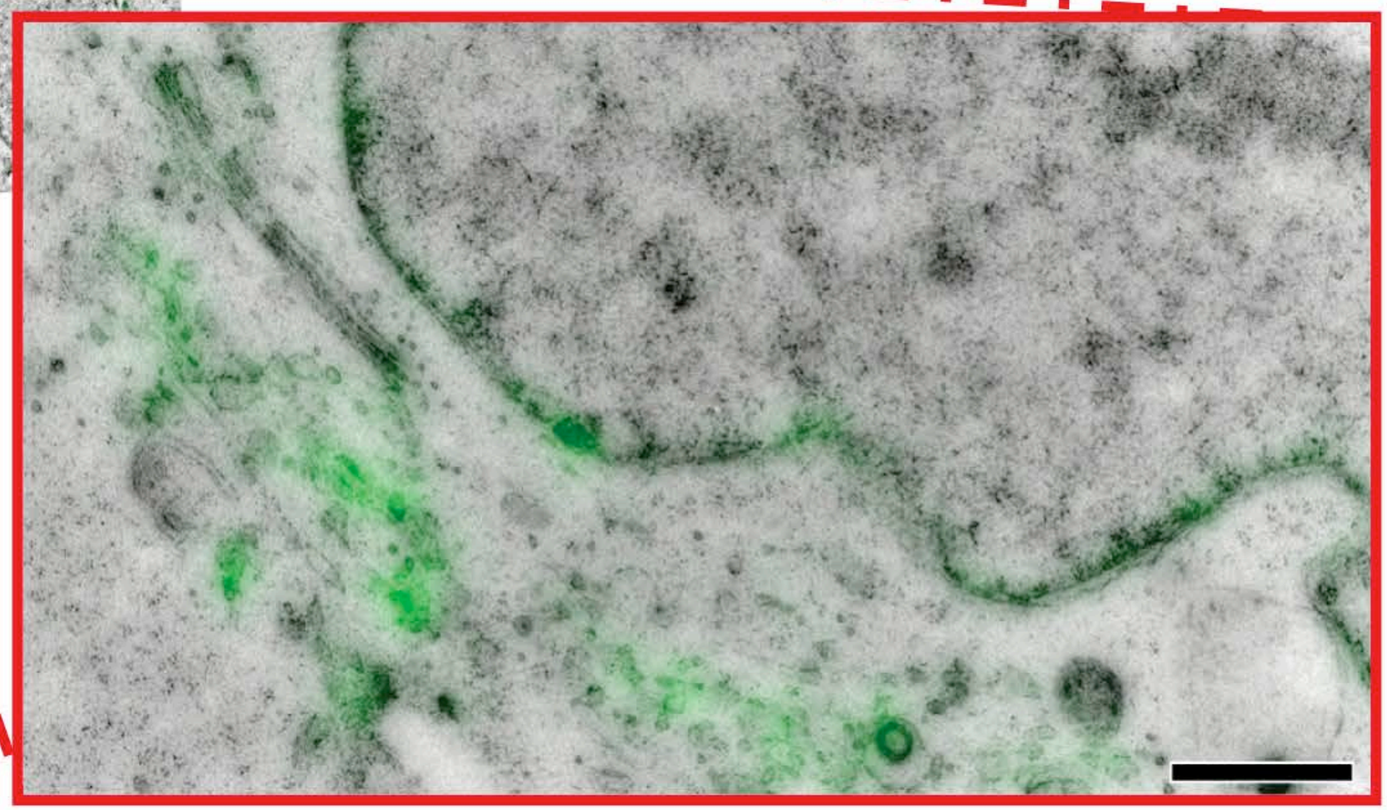
HM20

70 nm sections







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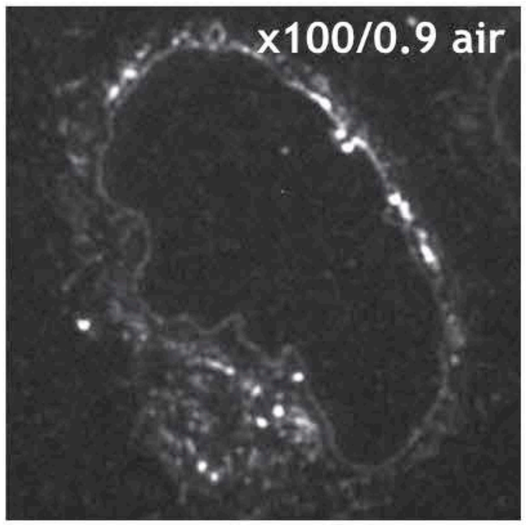
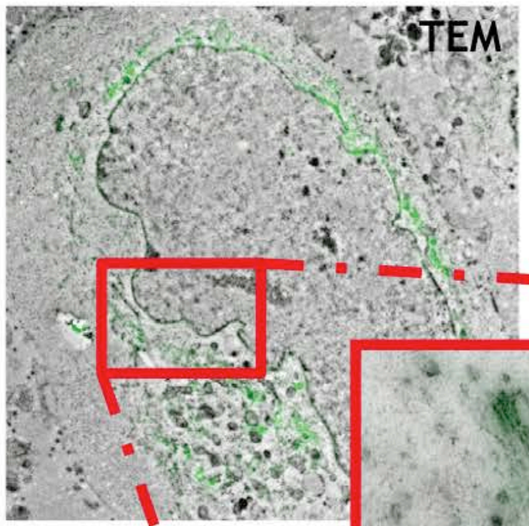
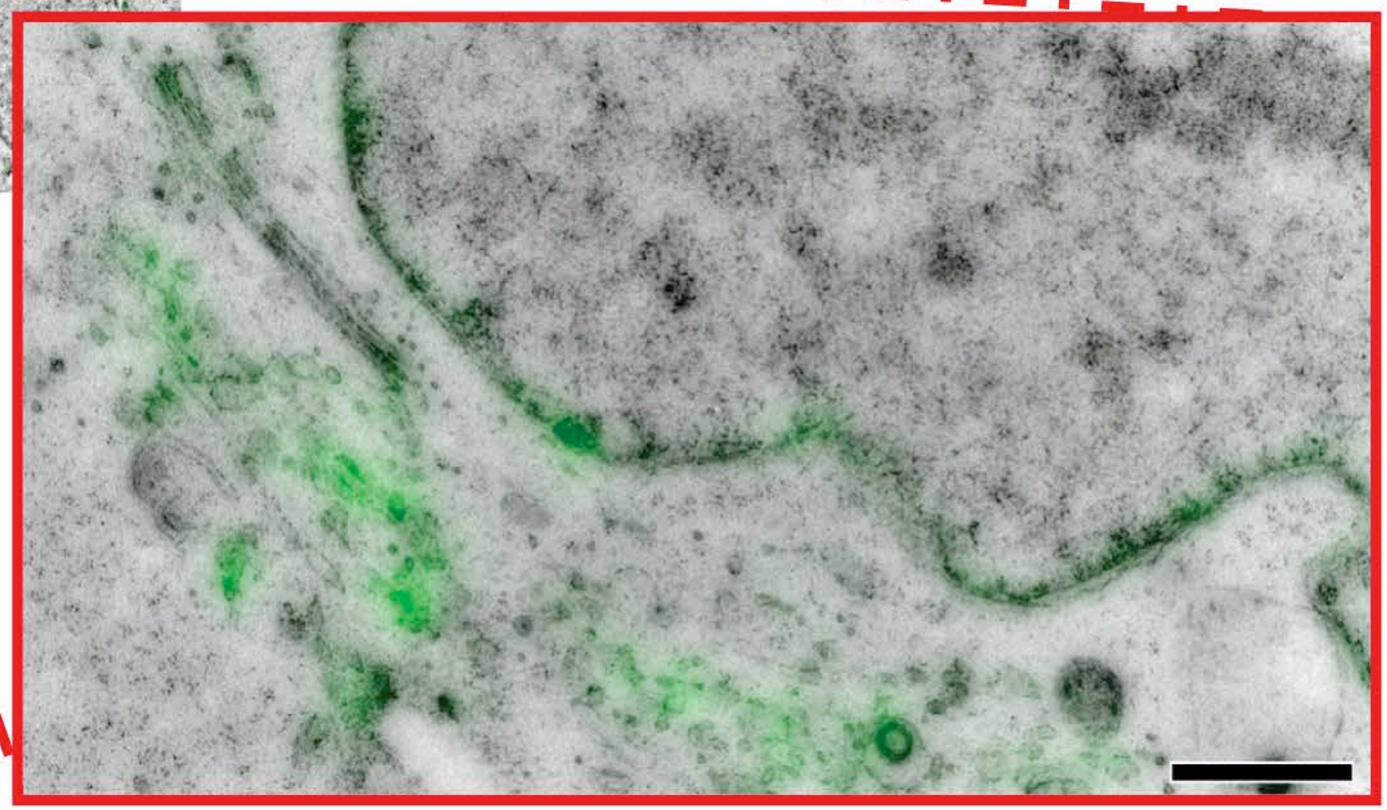
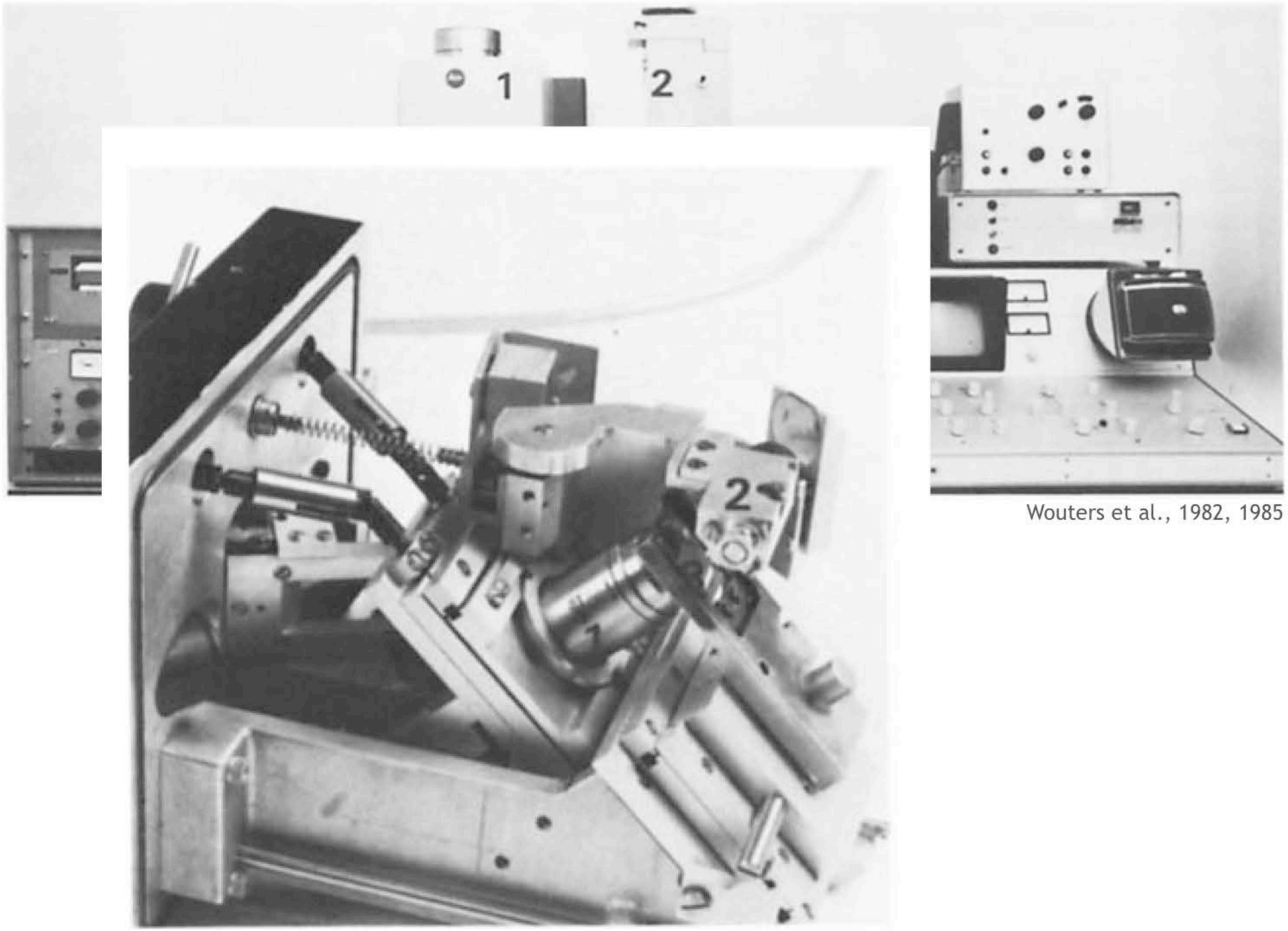






So… how about imaging ***both*** signals using an integrated microscope, under vacuum?

Integration of microscopes



Wouters et al., 1982, 1985

ILSEM → SECOM with IRF sections

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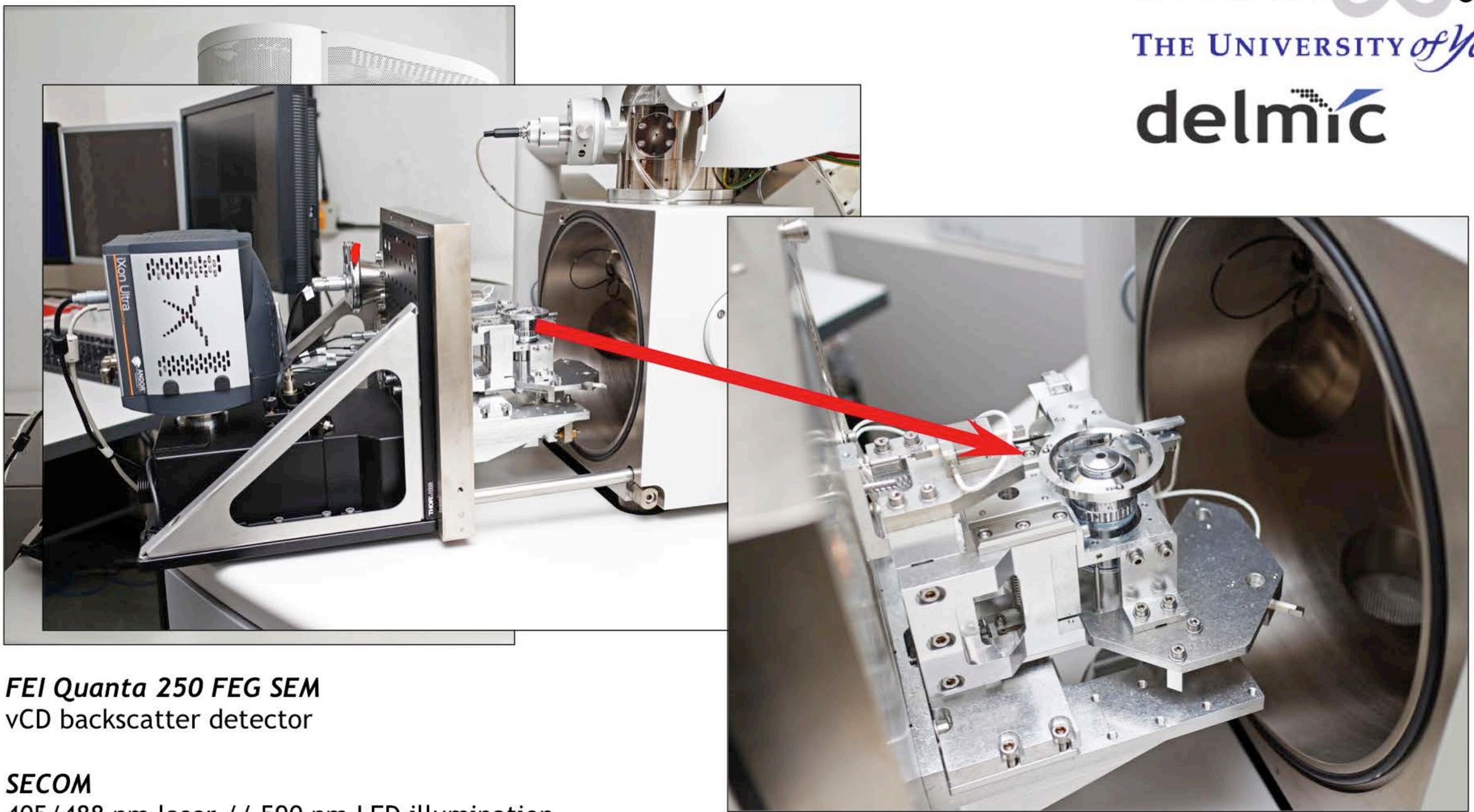
Bio-Continuum Imaging

THE UNIVERSITY of York

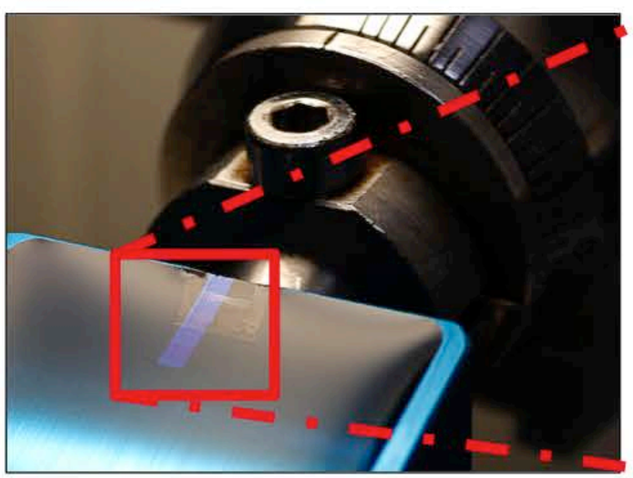
delmic

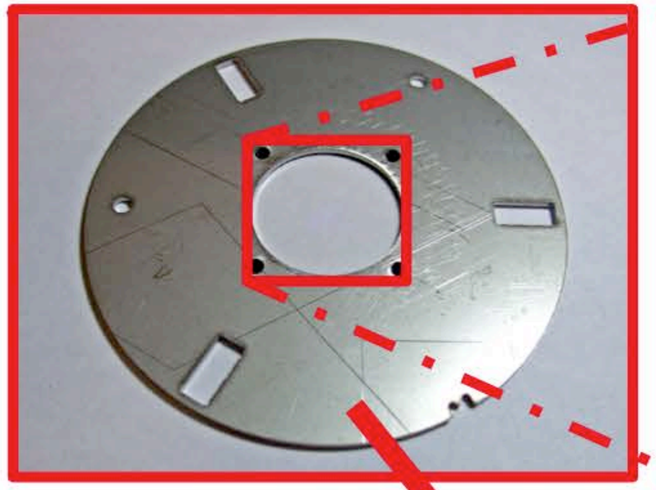


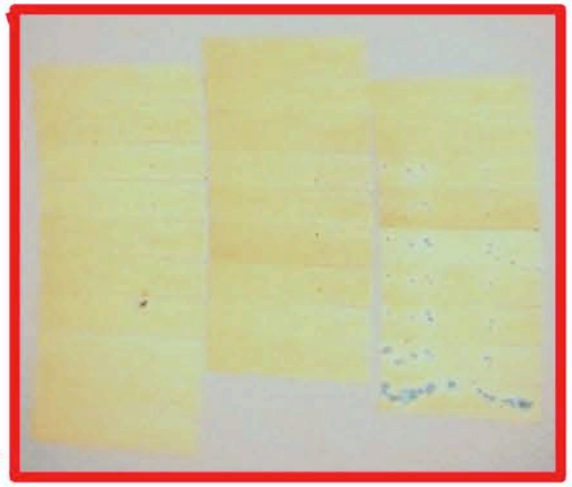
FEI Quanta 250 FEG SEM
vCD backscatter detector

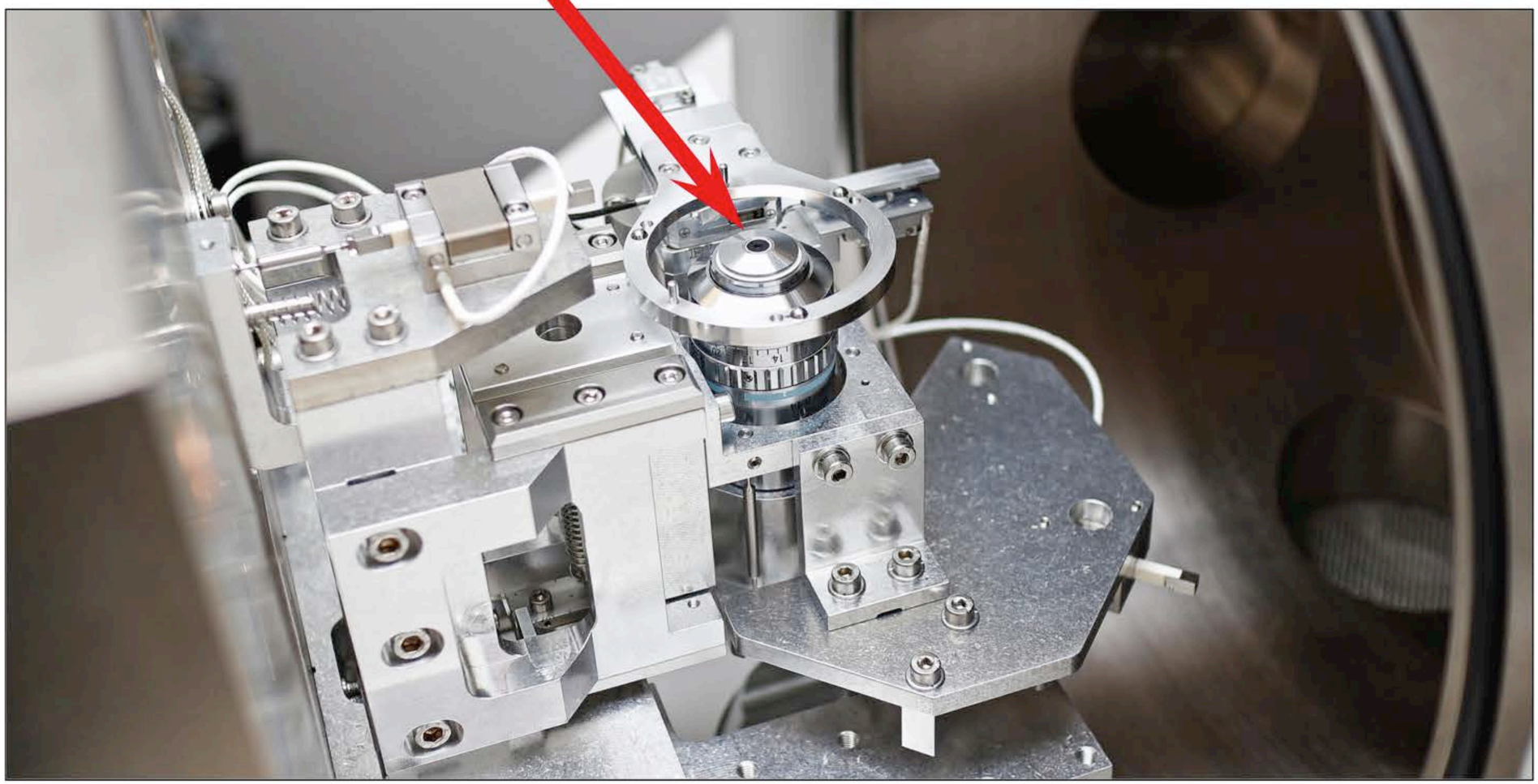
SECOM
405/488 nm laser // 590 nm LED illumination
x40/0.9 (air) and x100/1.4 (oil) objectives
Andor EMCCD and sCMOS cameras

SECOM sample preparation









ILSEM with SECOM: current status

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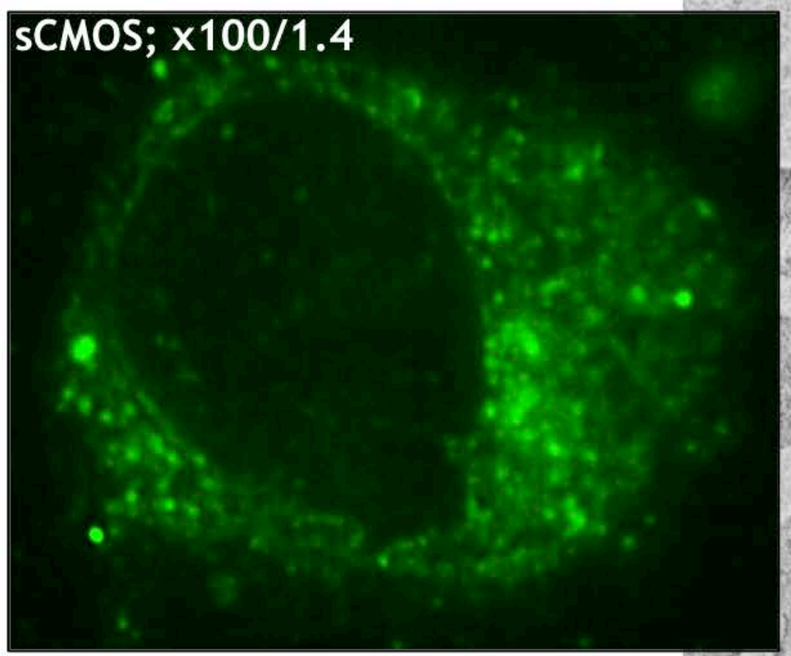
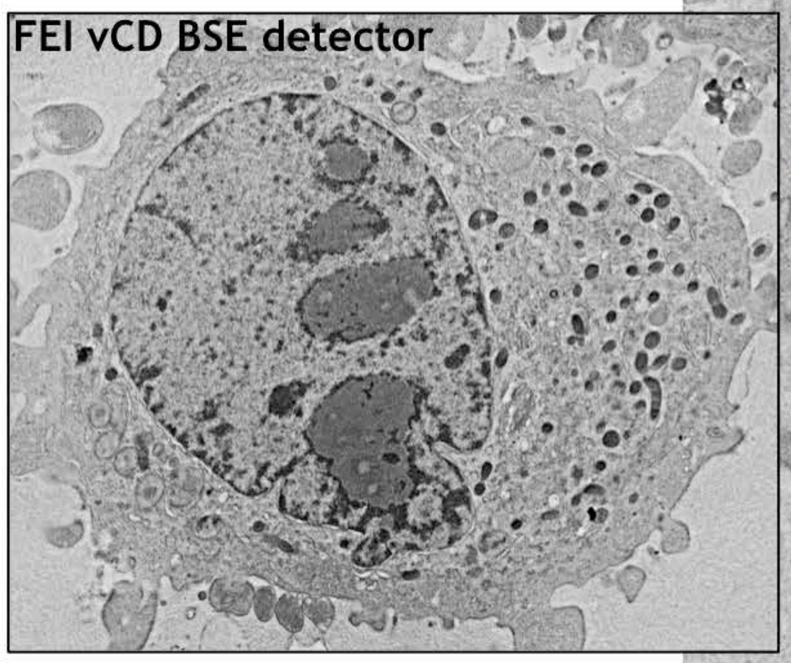
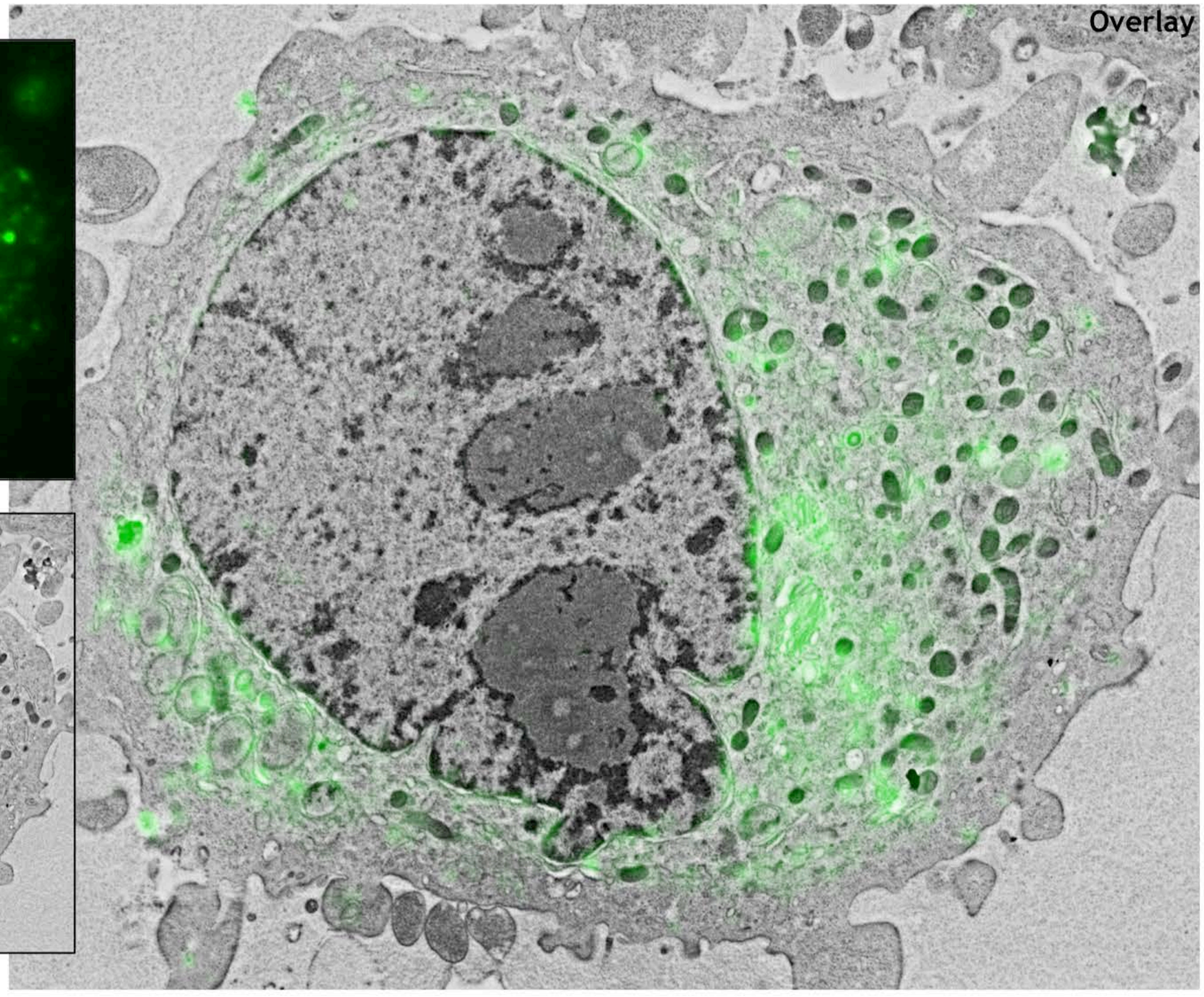


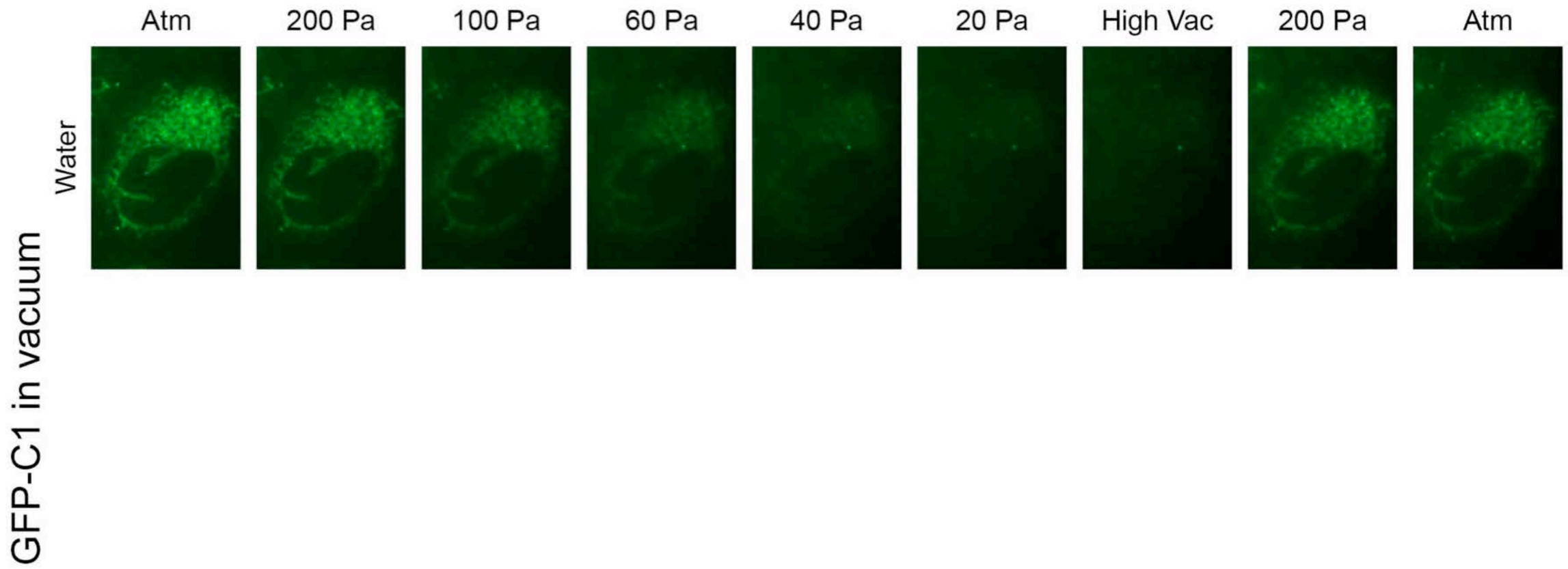




HeLa GFP-C1, 200nm section, sequential fluorescence and electron imaging in vacuo

ILSEM with SECOM: GFP vacuum behaviour

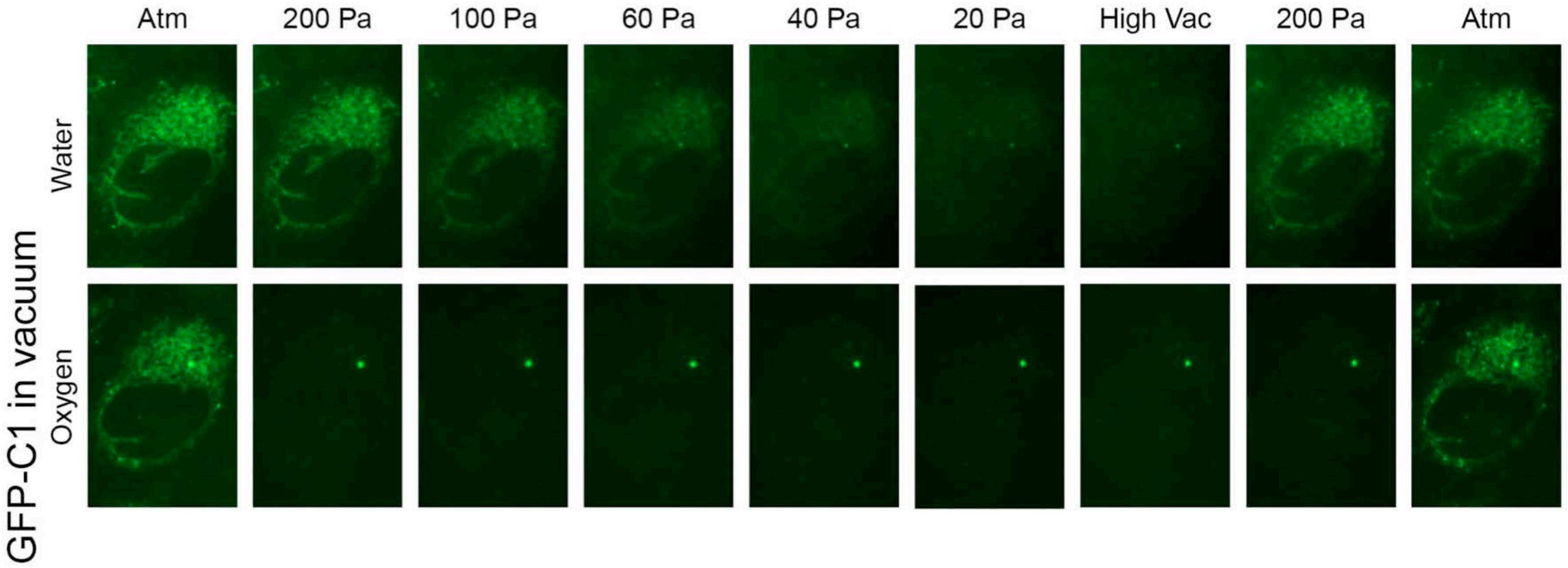
GFP fluorescence intensity reduces as vacuum improves, but does not photobleach significantly



Brama et al., 2015

ILSEM with SECOM: GFP vacuum behaviour

Water is essential for GFP fluorescence in partial pressure



Brama et al., 2015

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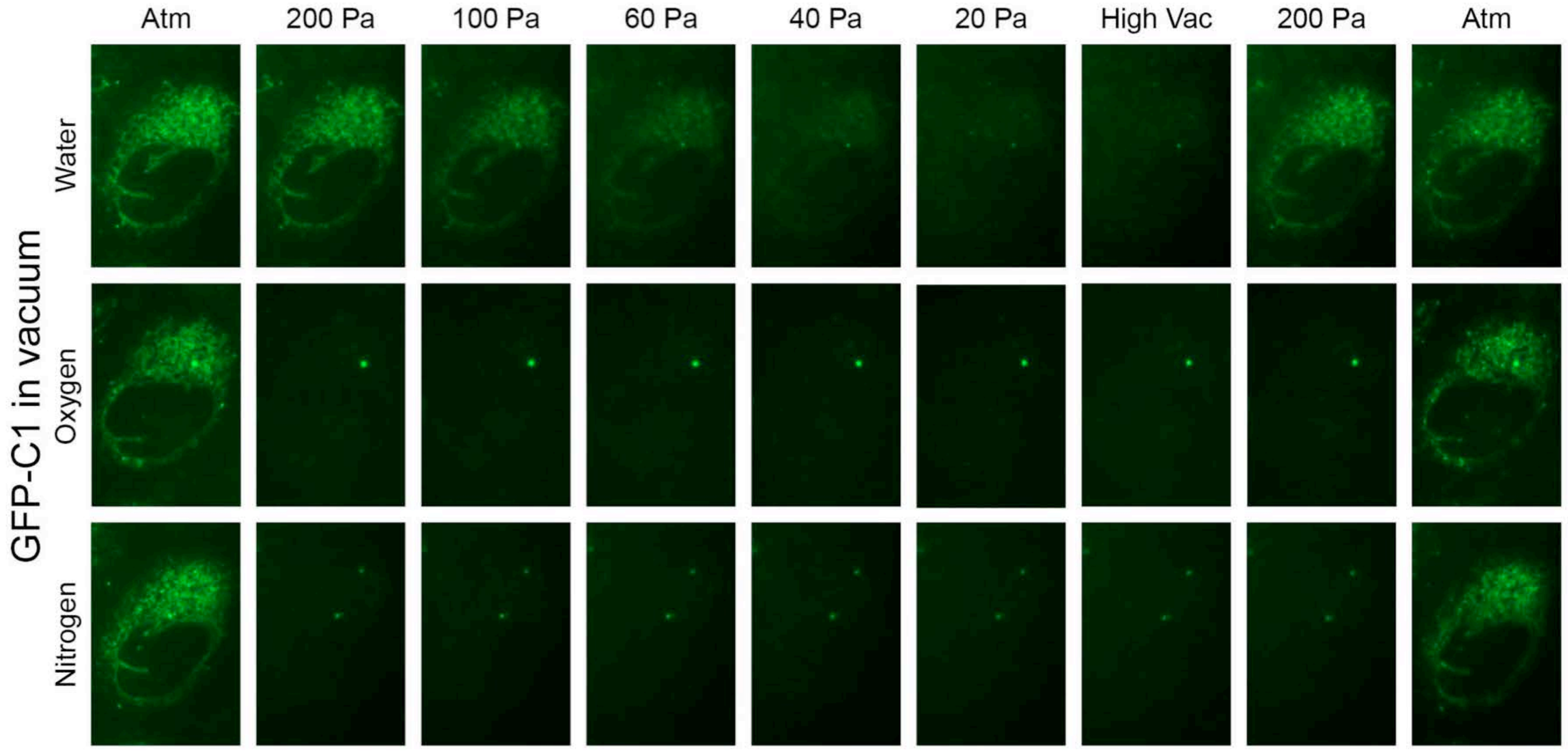
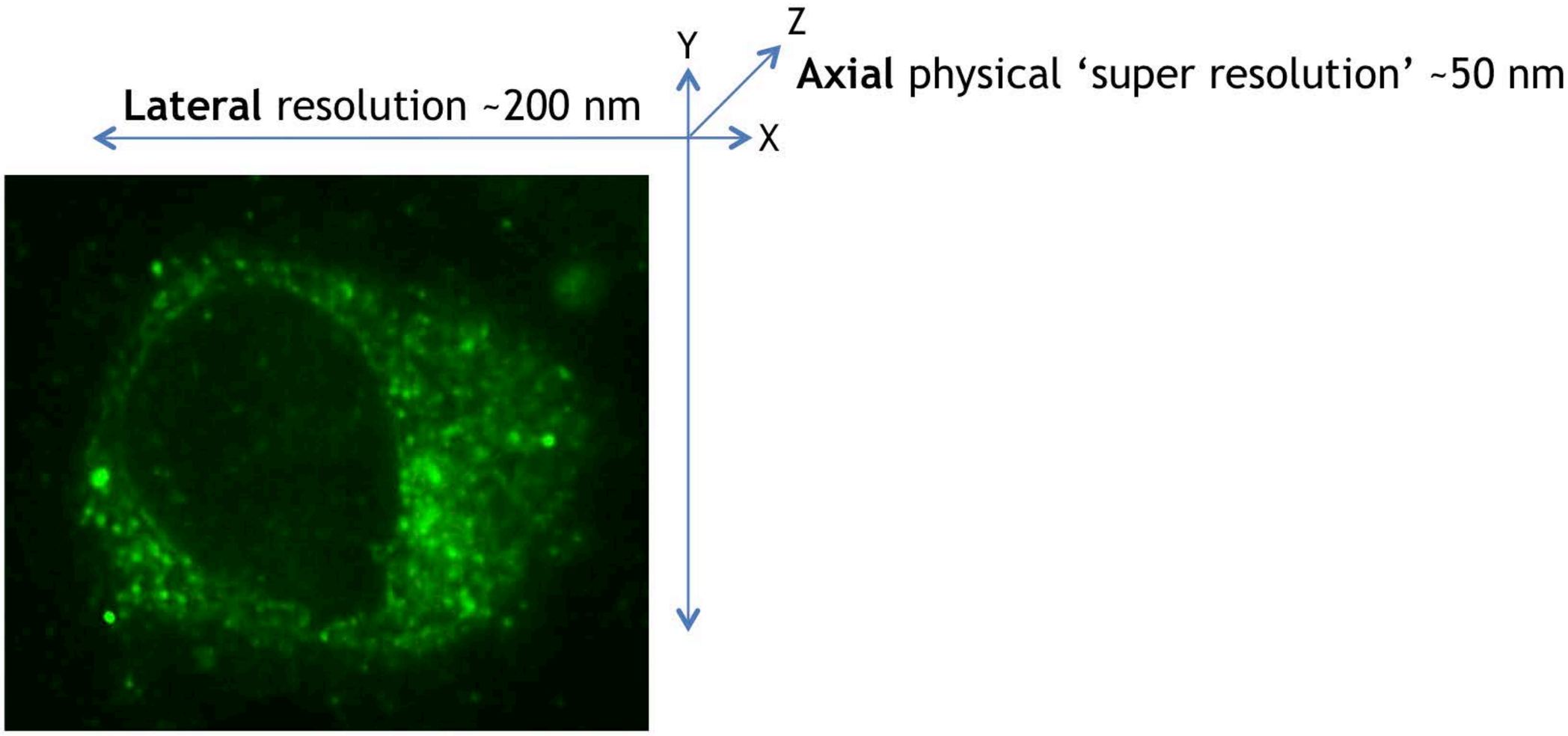
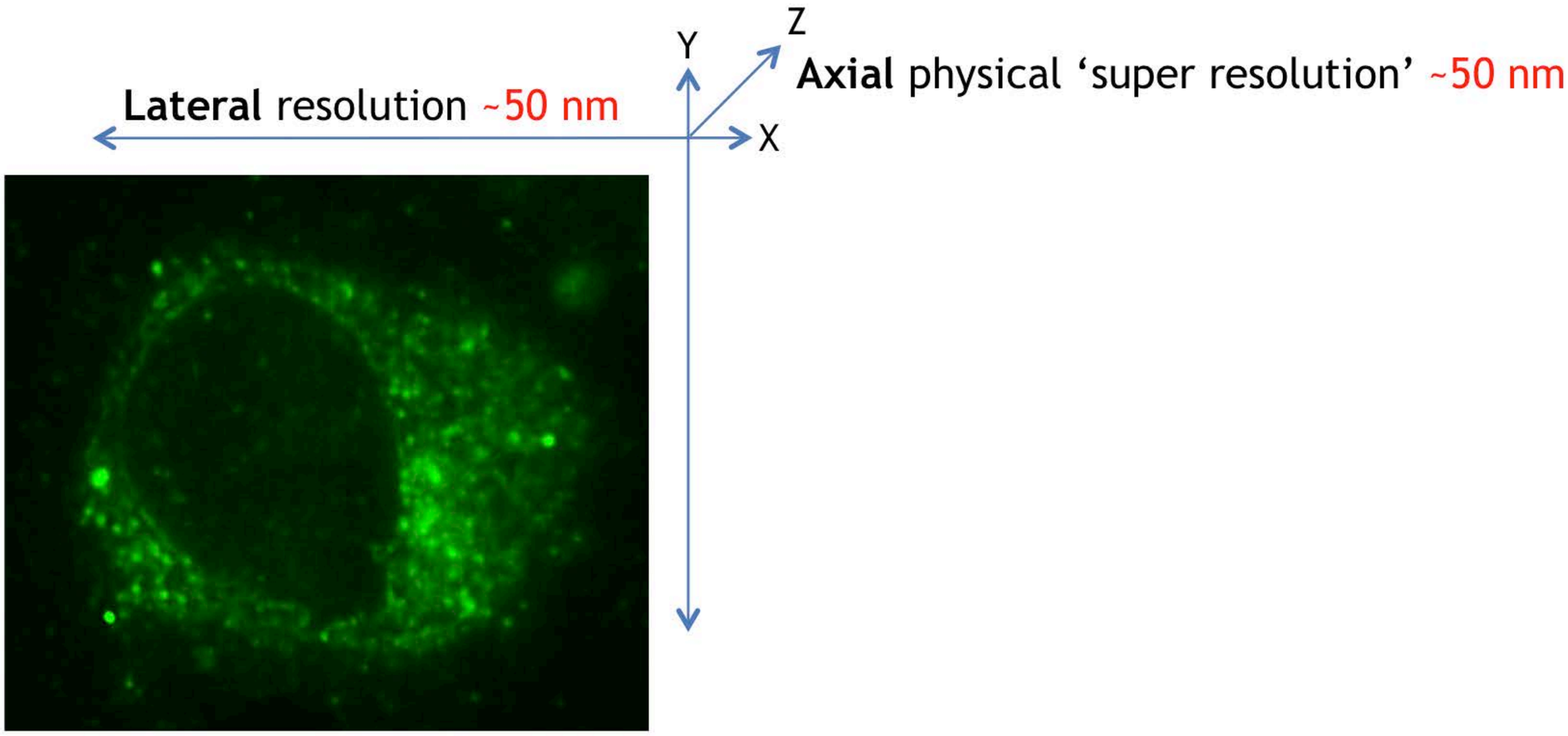


Brama et al., 2015

ILSEM developments: improving lateral resolution



ILSEM developments: improving lateral resolution



Localisation microscopy with blinking proteins

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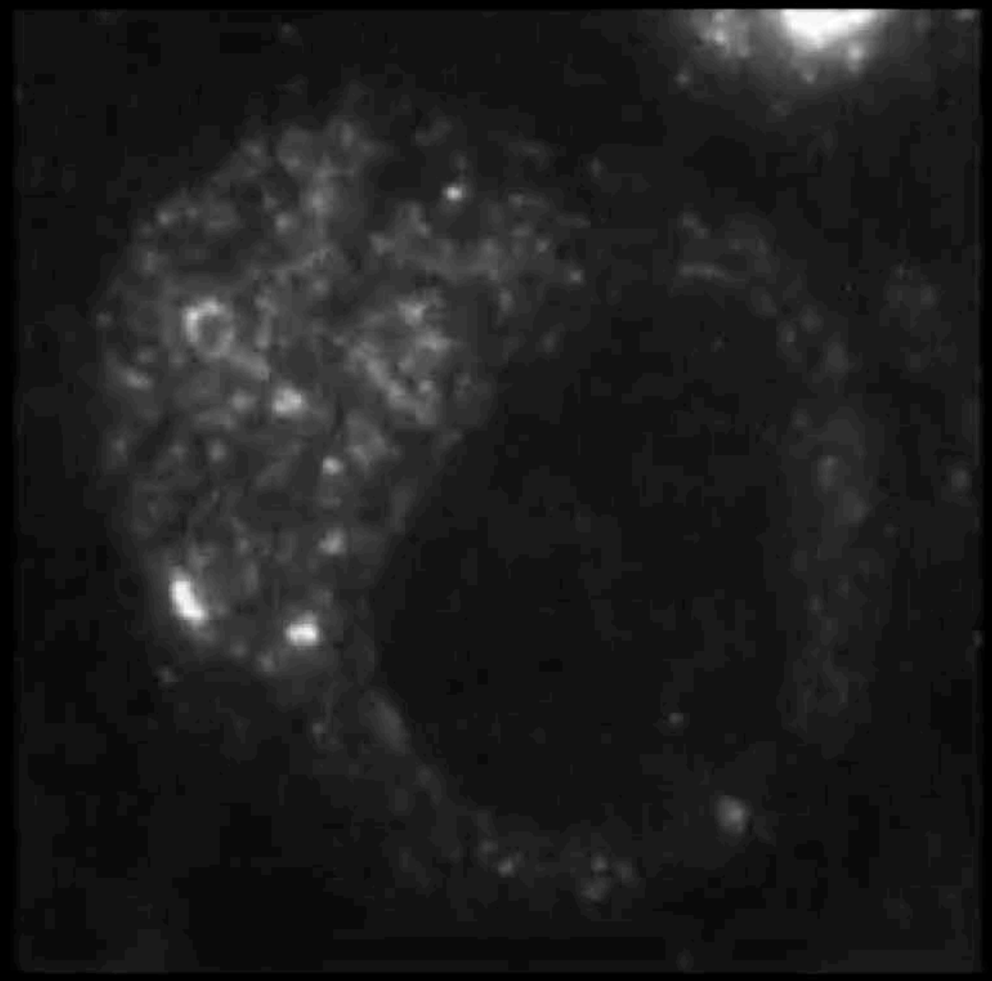
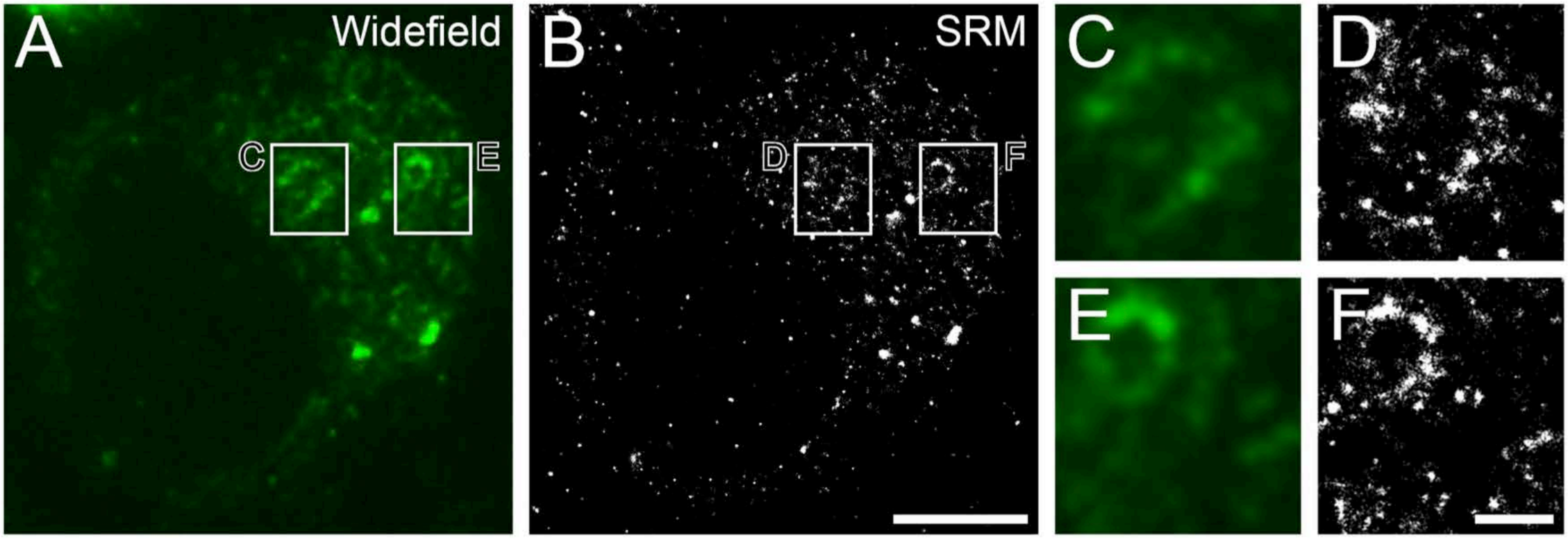
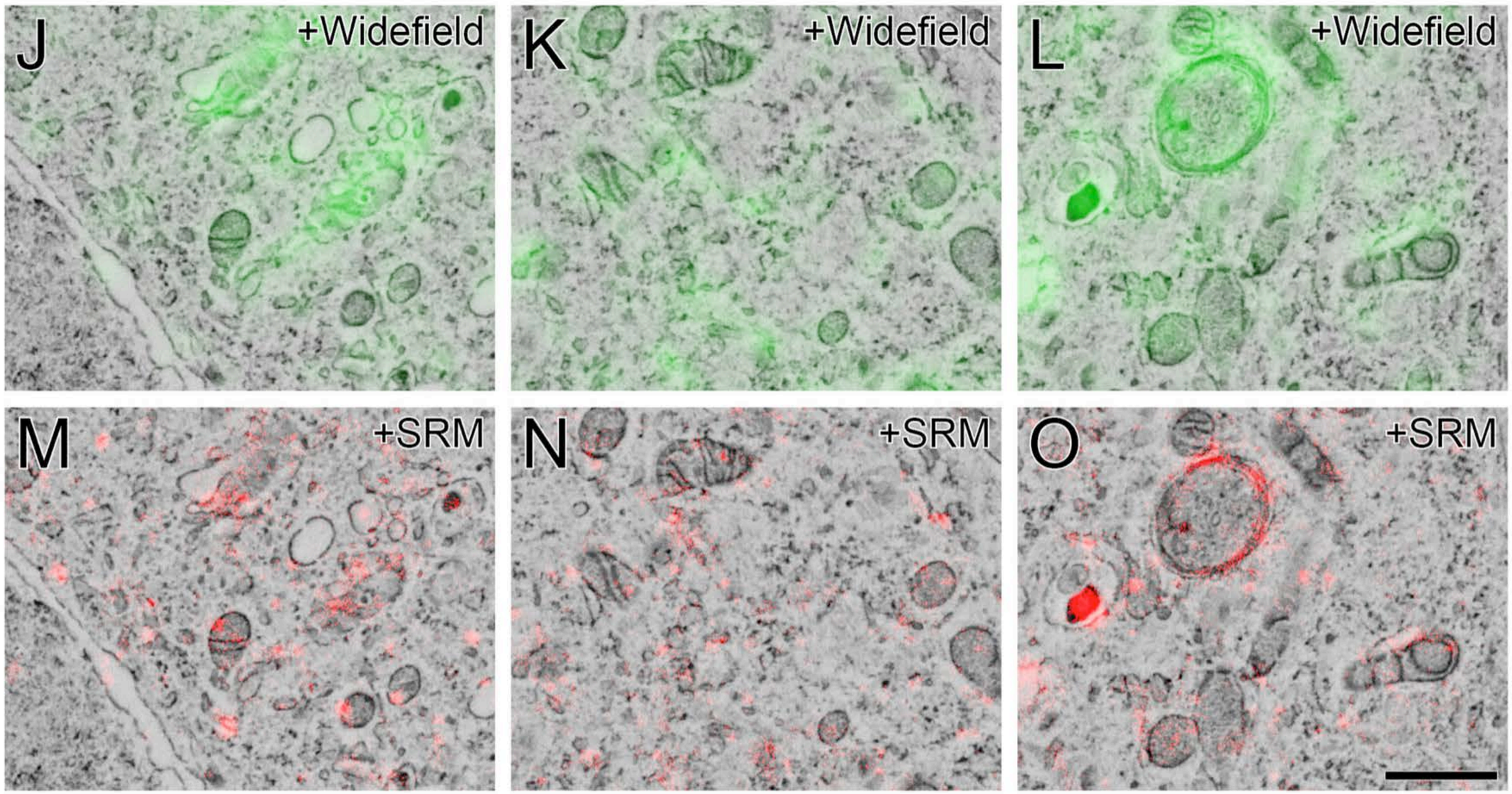
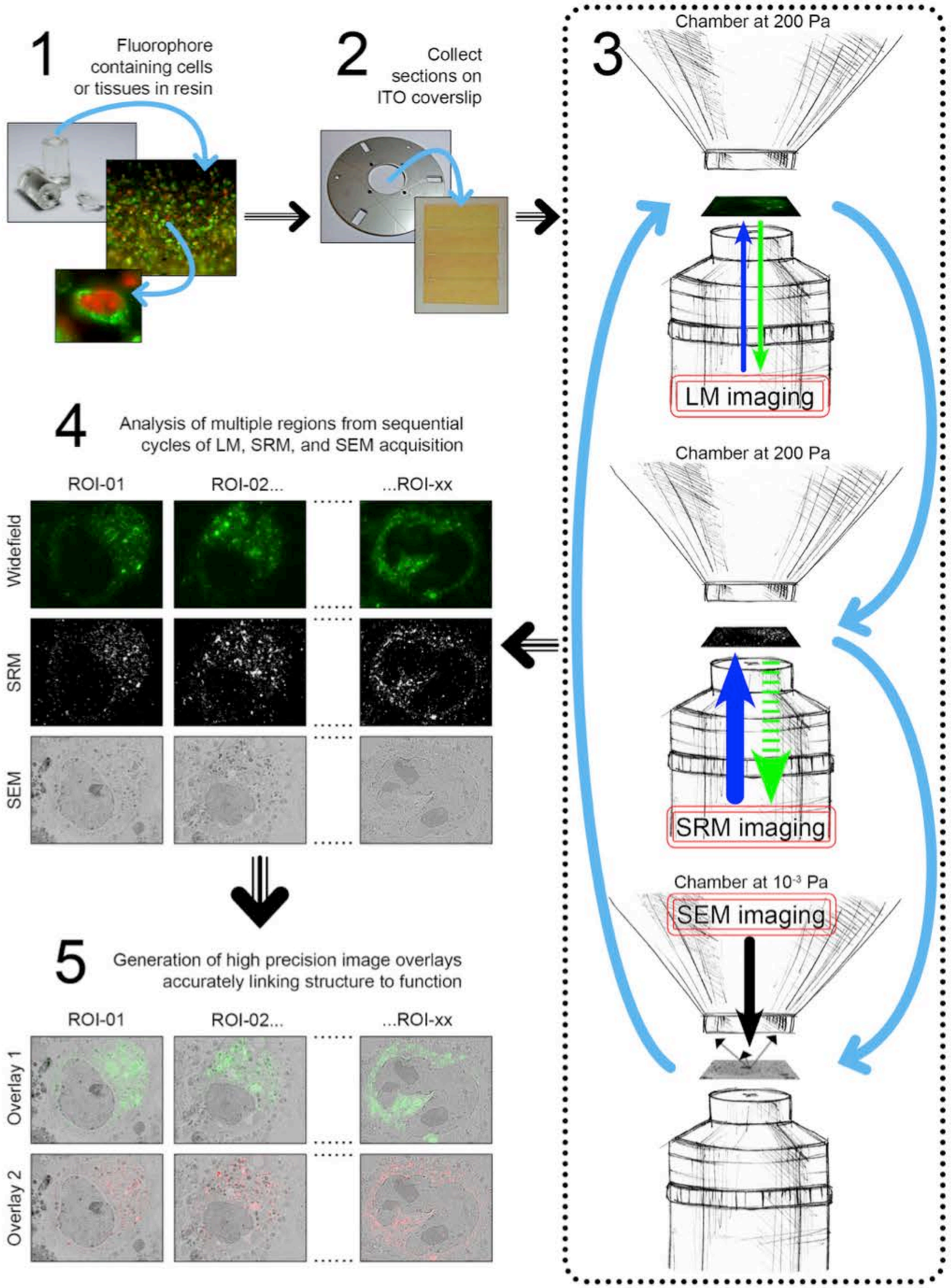


x100/1.4 lens with vacuum oil, 40 ms exposures for 31,000 frames, laser at 100% (~70 mW at sample)


A
Widefield
C
E
B
SRM
D
F
C
D
E
F



Workflow for integrated SR light and SEM



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MRC Medical Research Council

bbsrc biotechnology and biological sciences research council

EPSRC Engineering and Physical Sciences Research Council

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