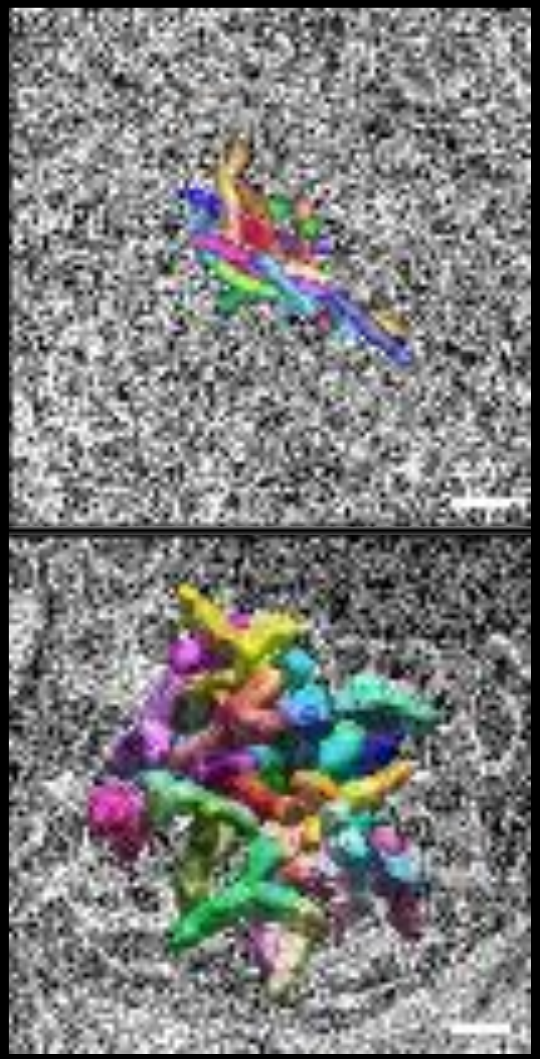
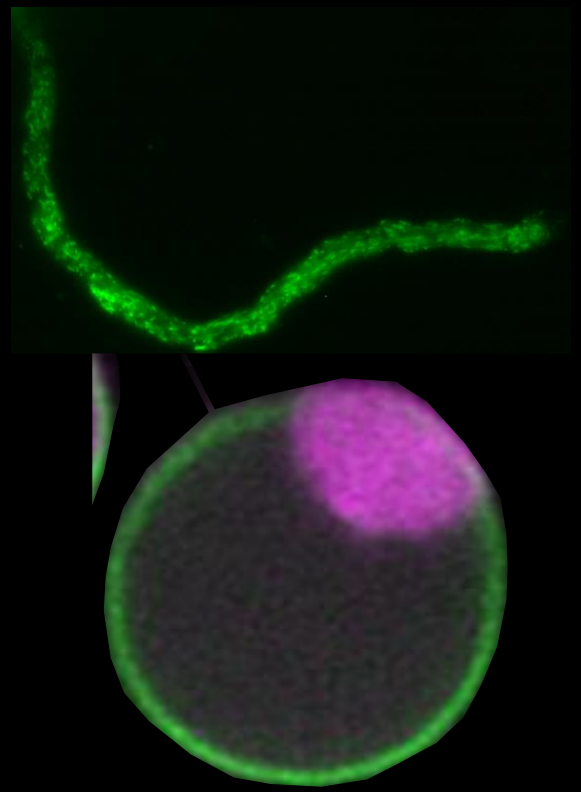
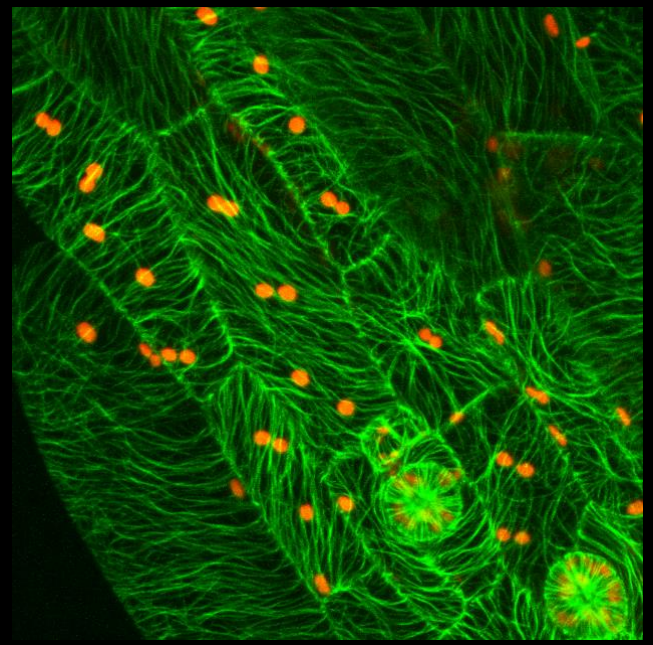
# Challenges and solutions for optimal plant sample preparation

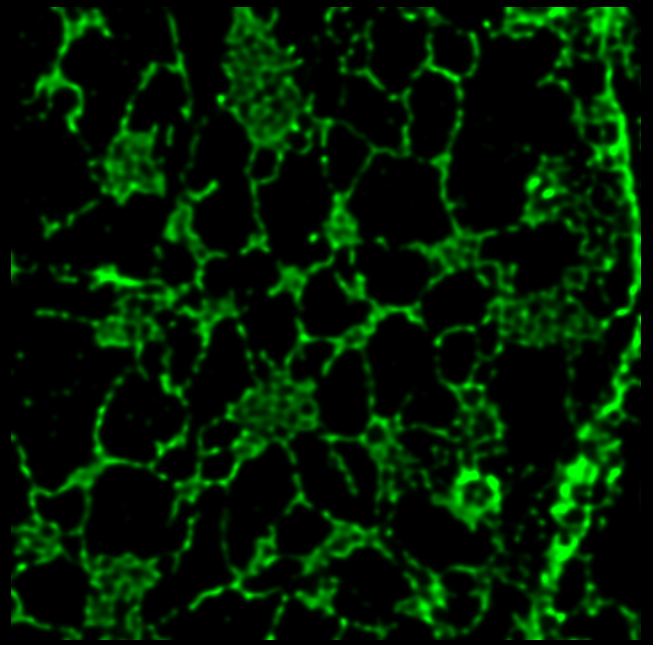
AGILE

Funded by the European Union









@joemckenna.bsky.social

## AGILE Training Event #2: Fundamentals of Microscopy Imaging for plant sciences - IPK

19th November 2026

Joe McKenna, University of Warwick

What do you need to understand to generate reliable data?

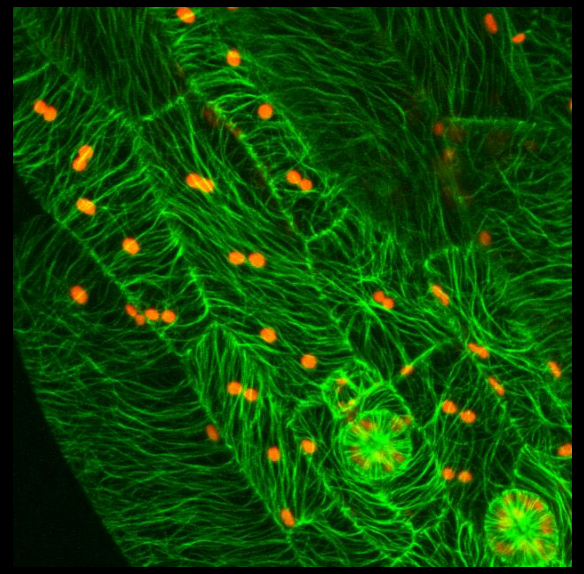


Microscope

+

?

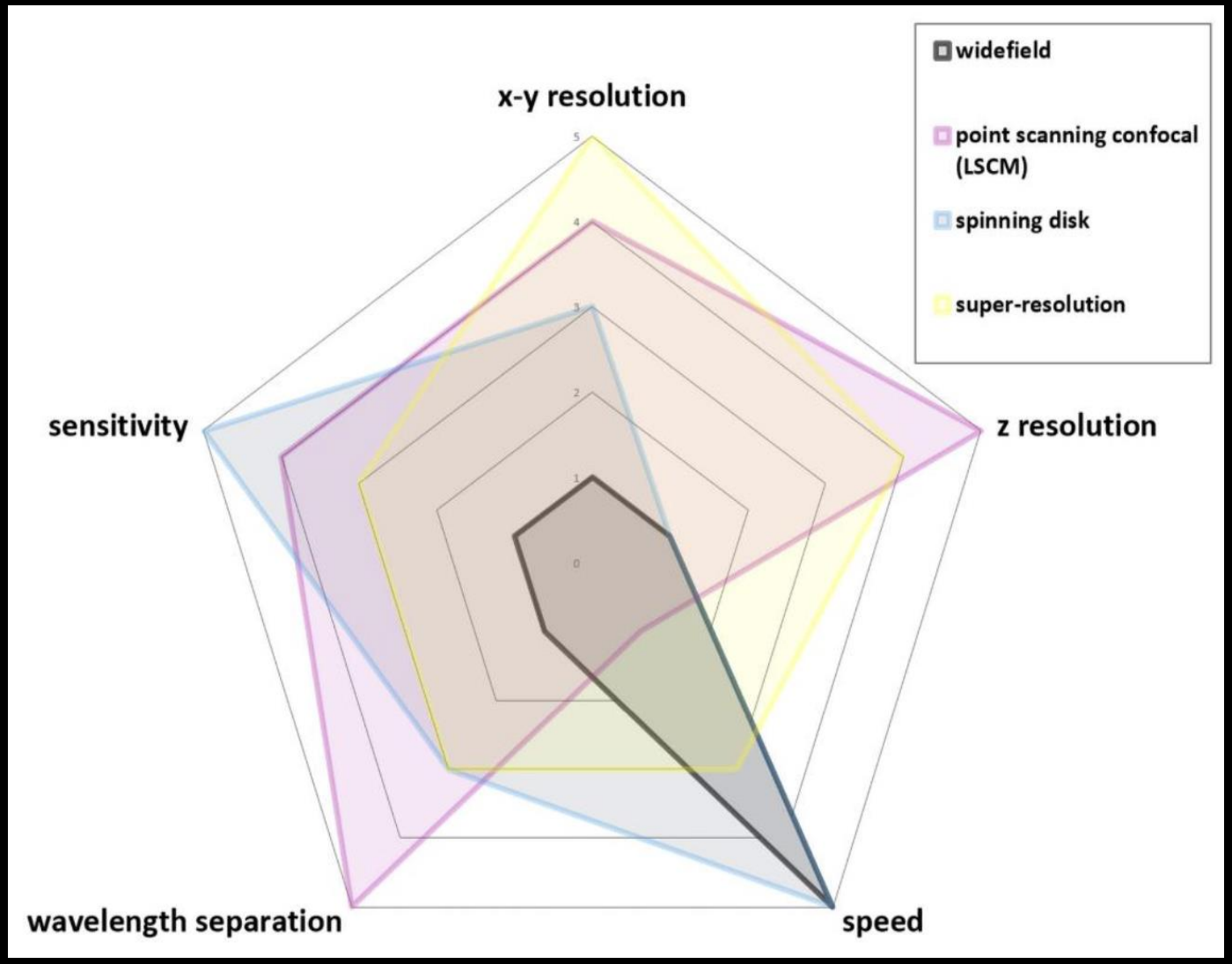
=



Reliable data

***Understanding your sample is just as important as the microscope***

# When choosing an imaging modality, it is all about compromise



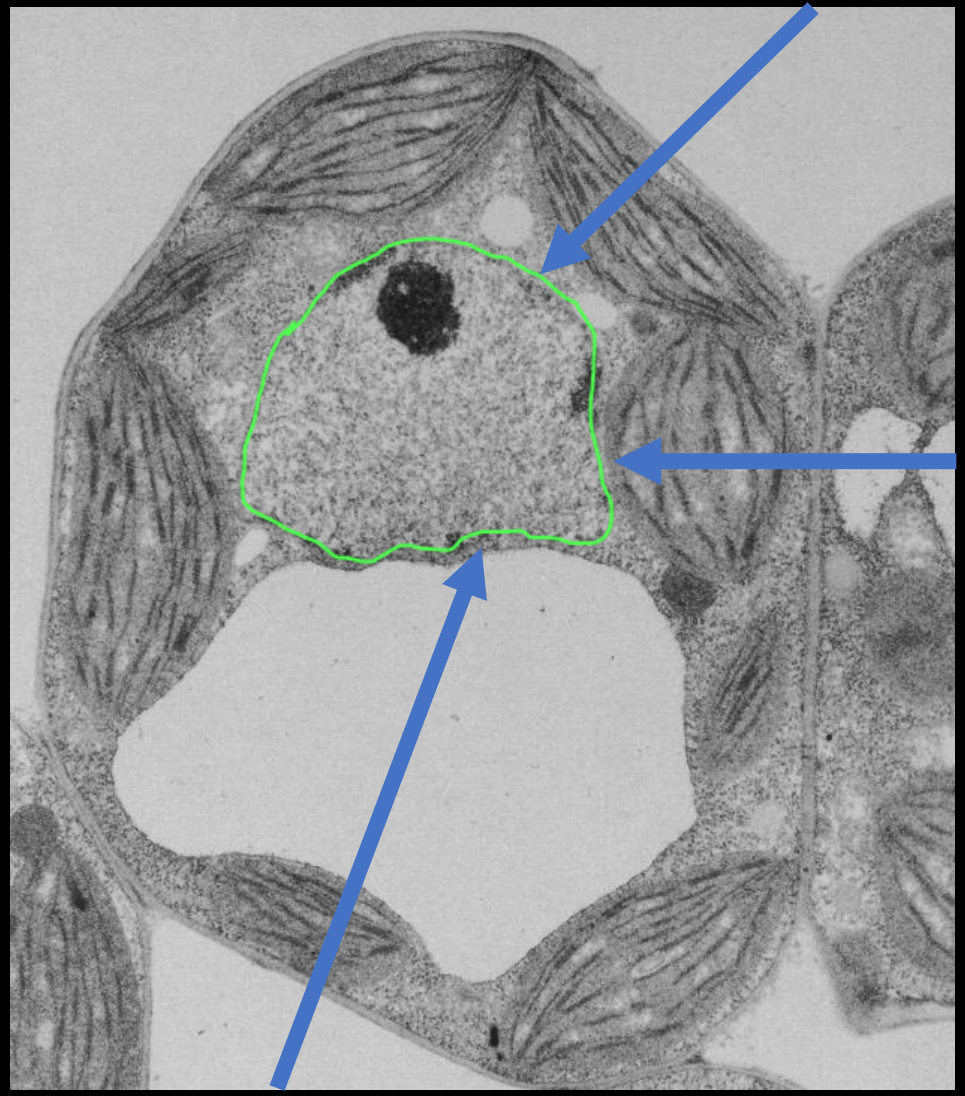

- The pentagon of pain describes how each imaging modality has compromise
- The plant sample and how it is mounted also has to be considered in addition to the criteria shown

***An excellent guide:***

**Best practices in plant fluorescence imaging and reporting: A primer**

Kirk J. Czymmek,[1,*] Yoselin Benitez-Alfonso,[2] Tessa Burch-Smith,[3] Luigi F. Di Costanzo,[4] Georgia Drakakaki,[5] Michelle Facette,[6] Daniel Kierzkowski,[7] Anastasiya Klebanovych,[1] Ivan Radin,[8] Suruchi Roychoudhry,[2] Heather E. McFarlane[9,*]

# Imaging plants introduces inherent complications for microscopy



- If we want to image the nucleus in this plant cell, what might be causing an issue
  - Cell wall > *refractive index mismatch, aberration*
  - Chloroplasts > *autofluorescence and scattering*
  - Vacuole > *distance from coverslip*
  - Imaging through neighbouring cells > *depth and scattering*
- These can cause light scattering, spherical aberrations or autofluorescence

***But! There are solutions to some of these***

# *Problem:* Every Interface Bends Light

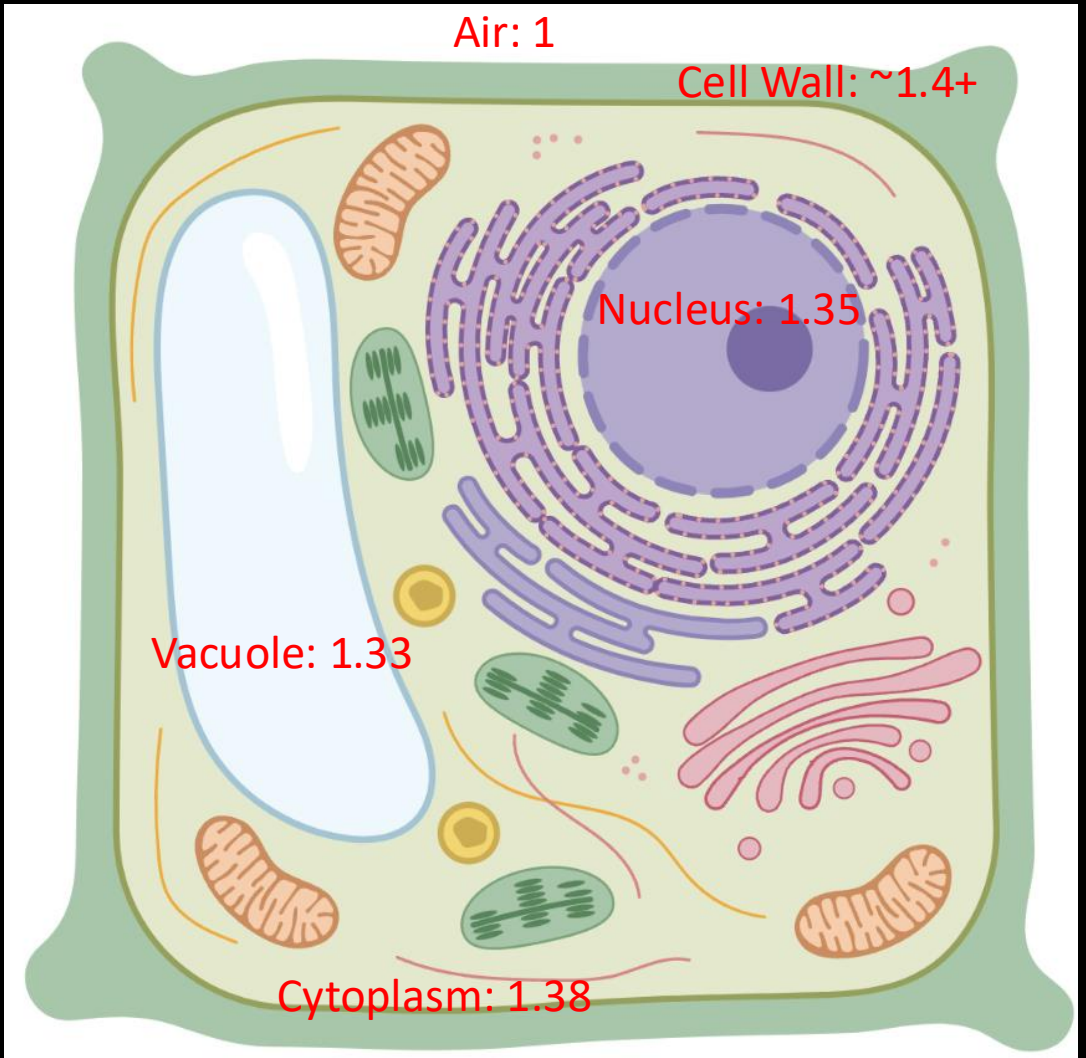



Image from biorender

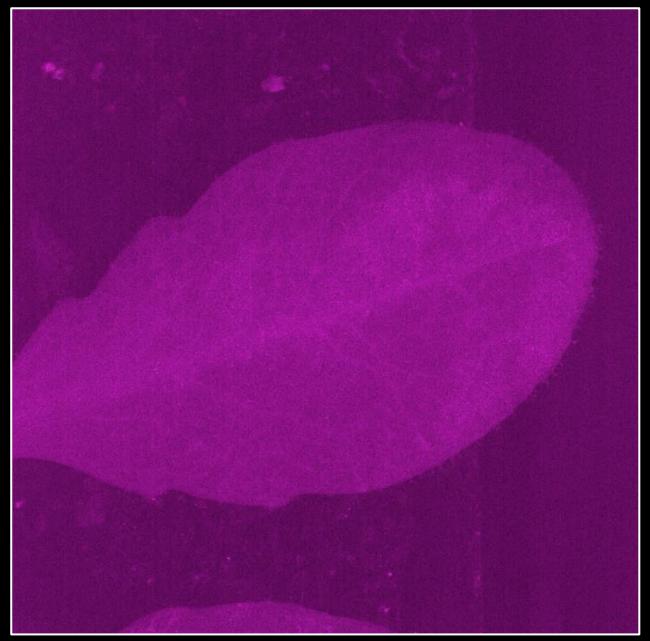
Objectives: Air: 1, Water: 1.33, Silicone Oil; 1.4, Oil; 1.518

- Light bends at every RI change, which blurs the image (spherical aberration)
- Chloroplast, Starch and phenolics scatter and absorb light, they also autofluoresce
- The deeper the nucleus sits, the more interfaces the light crosses

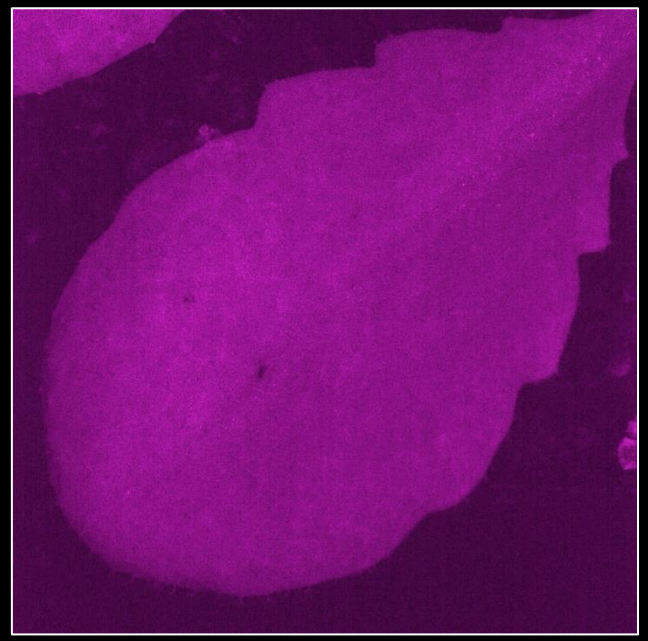
***Most sample preparation solutions are ways of reducing these mismatches***

*Problem:* Your sample is alive – and it notices you

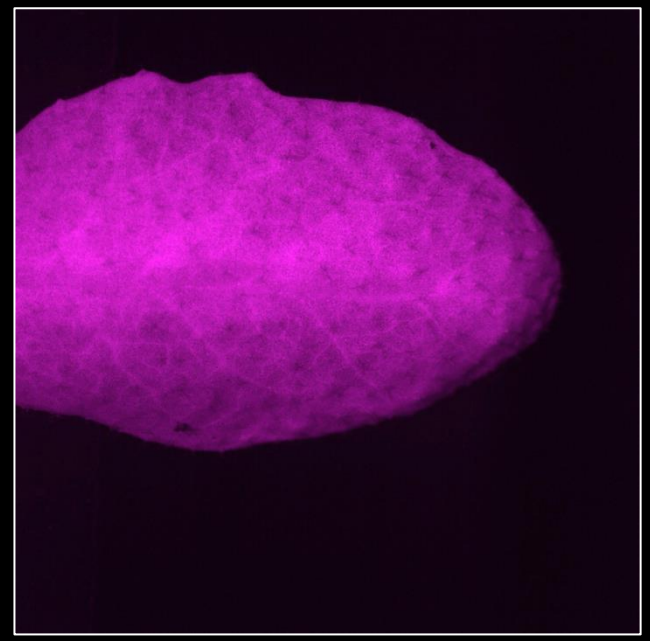
Cut



Macerated



Tickled



R-GECO Arabidopsis $Ca^{2+}$ reporter line

Collaboration with Grant lab, Warwick

- Touch and wounding trigger $Ca^{2+}$ and ROS signalling within seconds
- Under a coverslip, oxygen runs out and the sample heats up
- The laser is many times brighter than sunlight, so it causes photodamage and stress-induced autofluorescence
- For nuclei specifically: chromatin and nuclear shape respond to stress and mechanical force

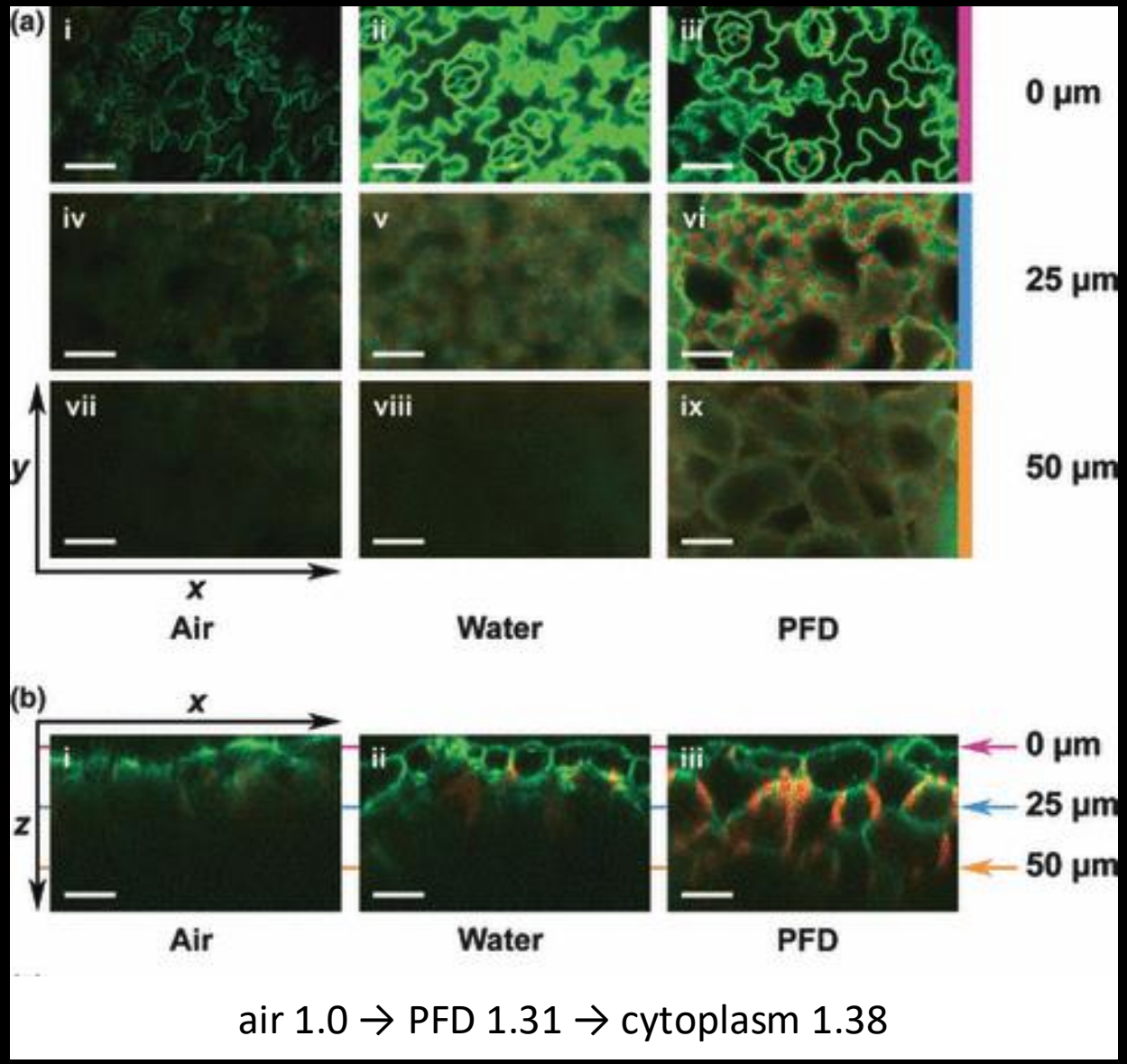
***If the prep changed the biology, the image is accurate, but it's of the wrong thing***

*DON’T PANIC – Keep calm and carry on*



*Don’t worry, there are workarounds for most of these issues*

# *Solution:* Removing air gaps - Perfluorodecalin

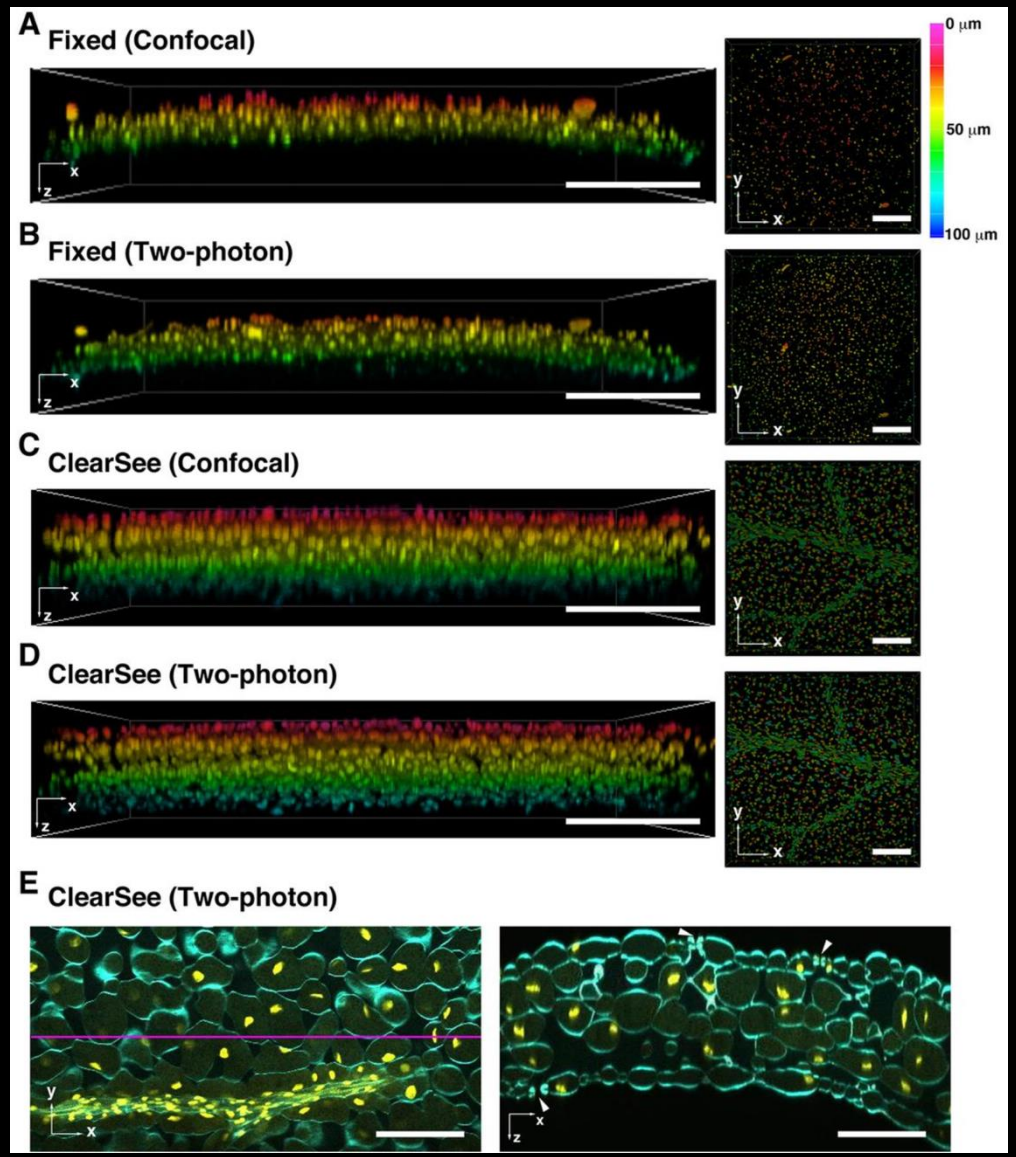


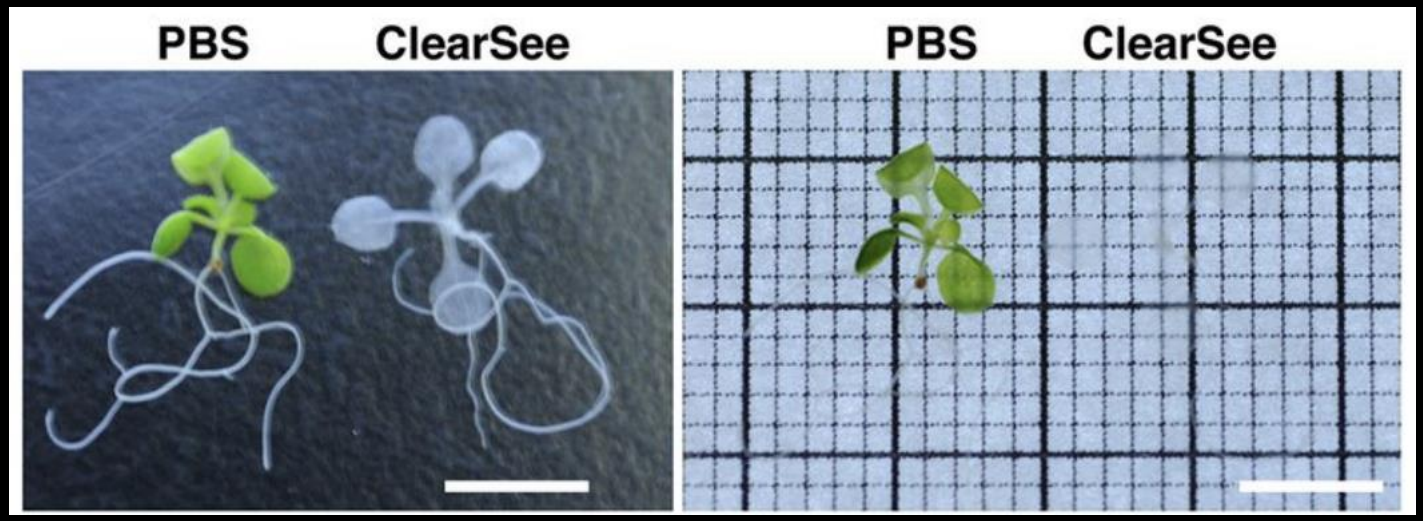

air 1.0 → PFD 1.31 → cytoplasm 1.38

- **What:** inert, $O_2$-rich perfluorocarbon (RI 1.31)
- **Optics:** floods air spaces → 2× imaging depth
- **Physiology:** less disturbance than water
- **Tips:** equilibrate first; best for leaves, not roots

Littlejohn et al., 2010, New Phytologist

# *Solution:* Removing the RI mismatch - ClearSee

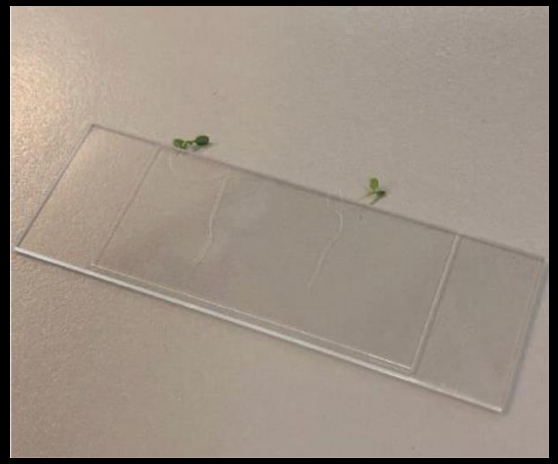


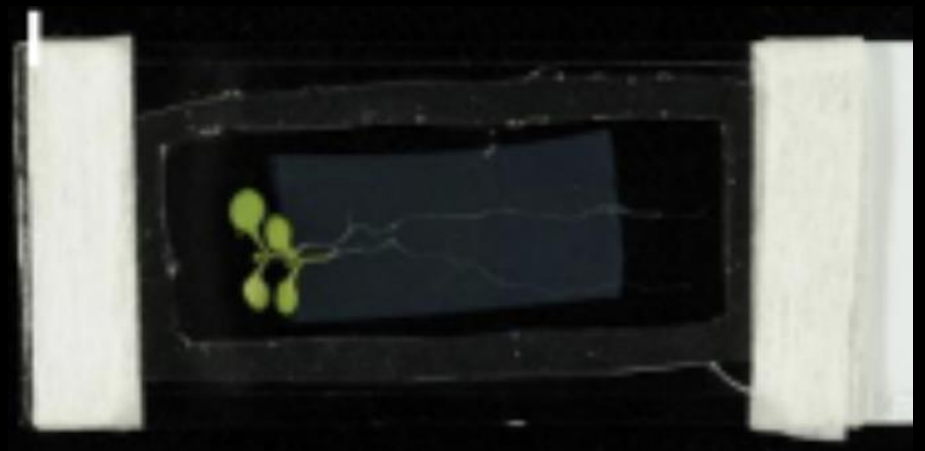

- **What:** clears tissue, removes chlorophyll, refractive index ~1.41
- **Keeps:** fluorescent proteins and dyes (SR2200, PI)
- **Enables:** whole-organ imaging of nuclei
- **Costs:** fixed samples only; days to weeks

Kurihara et al., 2015 Development

# *Solution:* Mount living samples gently



Simple mounting
Johnson et al., 2020, JCS



Agar pads
Kirchhelle & Moore, 2017, Jove



Vertical systems, 3i
von Wangenheim *et al.*
2017 *eLife*

- **Handle:** forceps on cotyledons, never the root
- **Support, don’t squash:** pads, spacers, chambers
- **Acclimate:** 5–10 minutes, then a fixed time window
- **Check health:** streaming? growing? If not, remount
- **Long imaging:** chambers, microfluidics, vertical stages

# *Solution:* How are you going to visualize your object of interest

| Label | Live? | Fixed? | Main sample-prep cost |
|---|---|---|---|
| Fluorescent proteins | Yes | Yes (PFA, not methanol) | Need transgenic lines; expression level and tag position can create artefacts |
| Self-labelling tags (HALO / SNAP) | Yes | Yes (if labeled first) | Dye must cross the cell wall and membrane; washing out unbound dye; background |
| Nanobody tags (ALFA) | Yes (nanobody-FP) or fixed | Yes (labelled nanobody) | Two transgenes; tiny tag, so minimal disruption |
| Dyes / stains (PI, FM4-64, SR2200, DAPI) | Some | Most (not FM4-64) | Penetration, specificity, toxicity over time |
| Antibodies / FISH | No | Yes: required | Fixation plus cell-wall digestion to get access; the only route to endogenous proteins or DNA |

*Your labelling choice decides whether your sample can stay alive, and which artefacts you have to control for.*

# *Tags / probes can alter the biological function of proteins*

Ketelaar et al., 2004, Plant Physiology

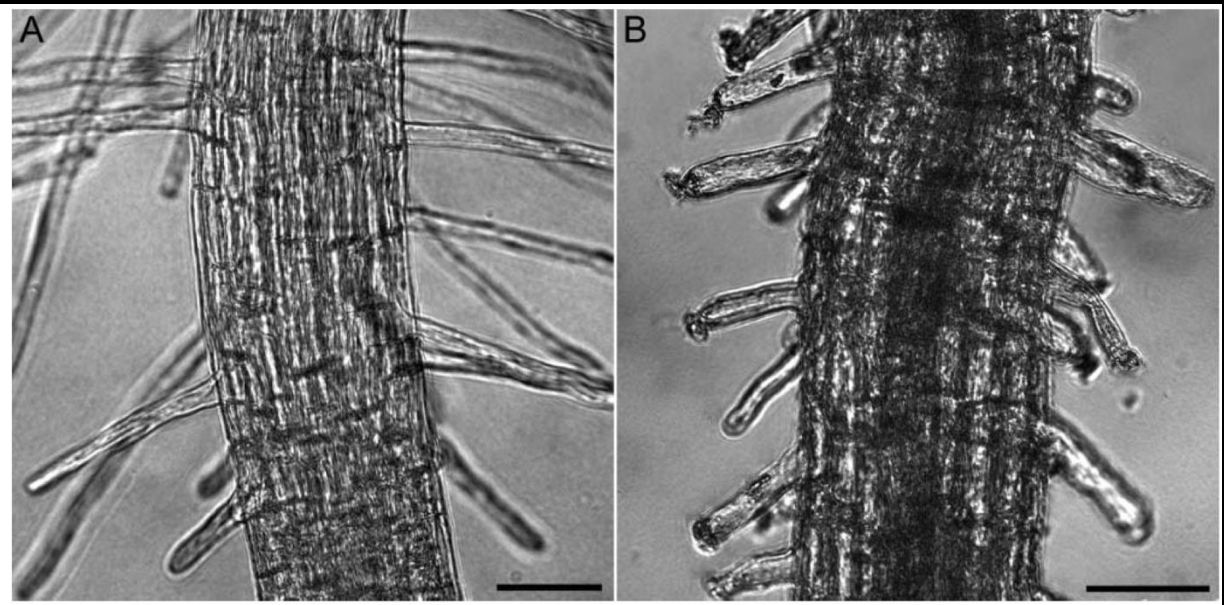



**Figure 3.** The response of growing wild-type and GFP-mTalin root hairs to induction with 1% ethanol. A is an image of the zone of growing root hairs of a wild-type plant, 2 h after induction with ethanol. B is an image of the zone of growing root hairs of a GFP-mTalin 2 h after induction with ethanol. Bars = 50 $\mu$m.

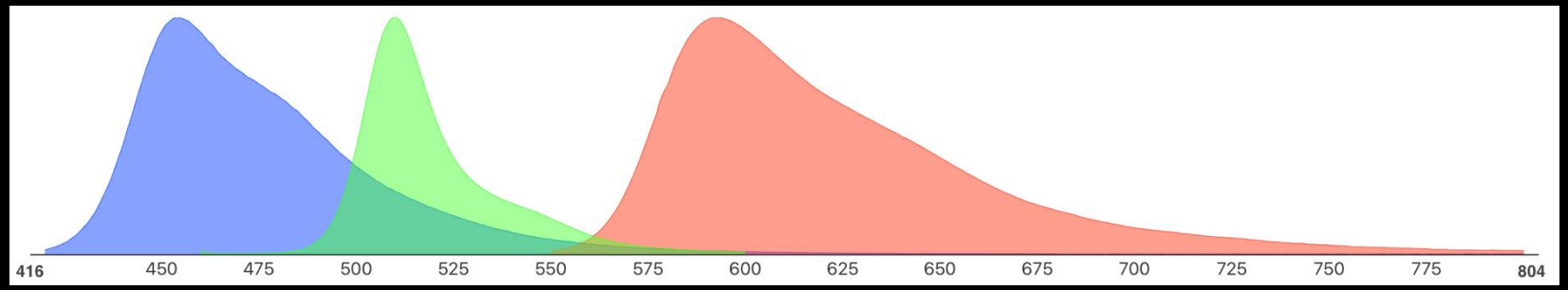
**Cautionary Notes on the Use of C-Terminal BAK1 Fusion Proteins for Functional Studies**

**Vardis Ntoukakis,[1] Benjamin Schwessinger,[1,2] Cécile Segonzac, and Cyril Zipfel[3]**
The Sainsbury Laboratory, Norwich NR4 7UH, United Kingdom

Ntoukakis *et al.* 2011 *Plant Physiol*

- Overexpression or inclusion of tags can affect biological function
- Labelling the actin cytoskeleton with different tags alters its dynamics and plant growth
- To control, generate rescue constructs and ensure protein function is rescued
- Alternatively, monitor biological processes (cytoplasmic streaming or cell division)

# *Solution: How do you choose the best fluorescent proteins?*




| Name | $\lambda_{ex}$ | $\lambda_{em}$ | Stokes | EC | QY | Brightness |
|---|---|---|---|---|---|---|
| mEGFP | 488 | 507 | 19 | 56,000 | 0.6 | 33.6 |
| mStayGold | 499 | 510 | 11 | 164,000 | 0.83 | 136.12 |

- New fluorescent proteins are announced almost daily! Consider:
  - ***Wavelength***
  - ***Brightness***
  - ***Maturation time***
  - ***Do you have the right imaging system?***
  - ***Monomeric***
  - ***Environmental stability***

**FPbase: a community-editable fluorescent protein database**

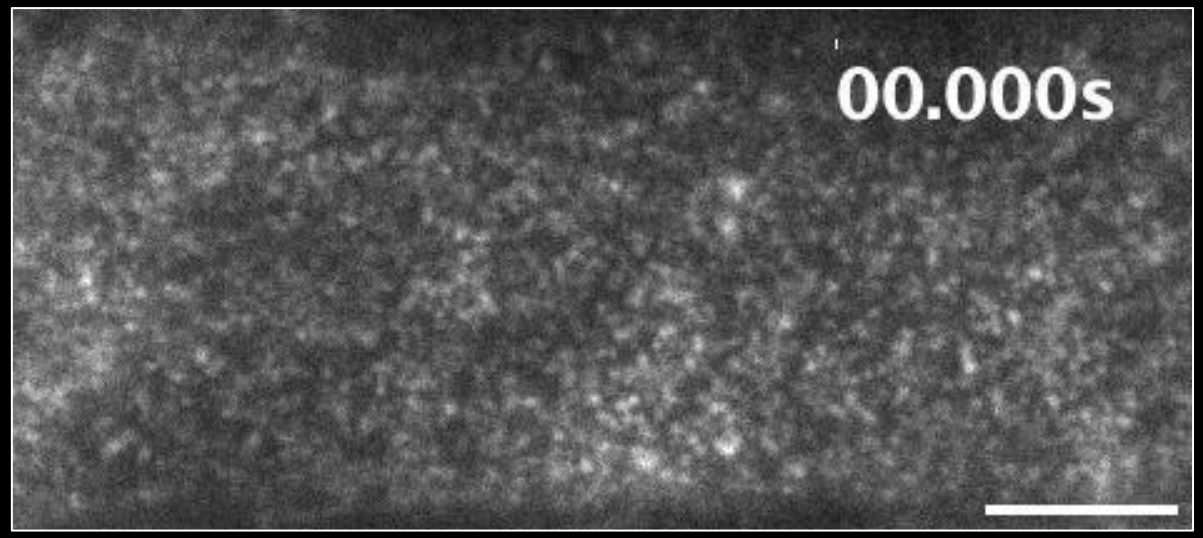
Talley J. Lambert

Choosing a Fluorescent Protein for Live-Cell Imaging: An Updated Guide

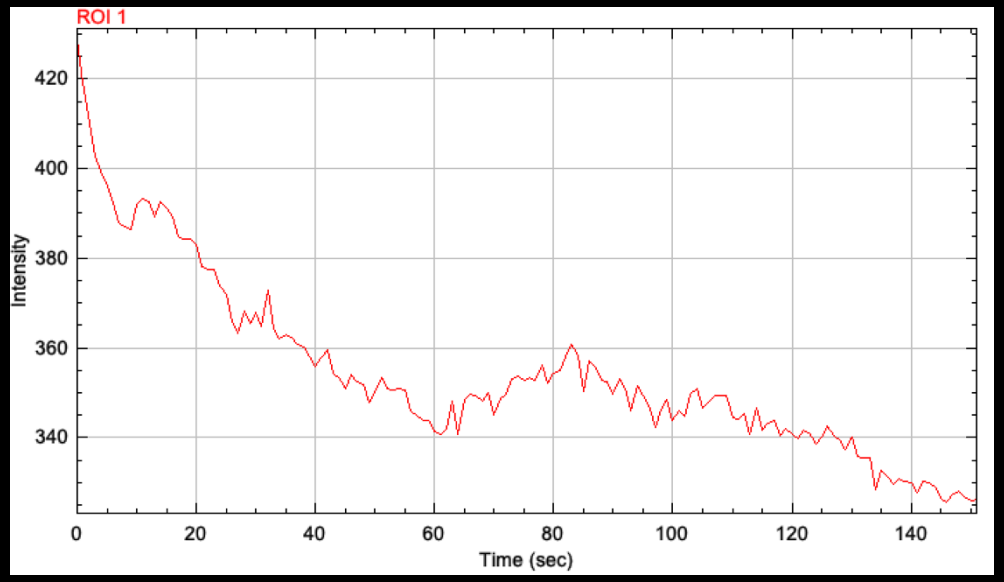
By Mike Lacy

# *Solution:* Limiting phototoxicity and photobleaching

pFER::FER-GFP

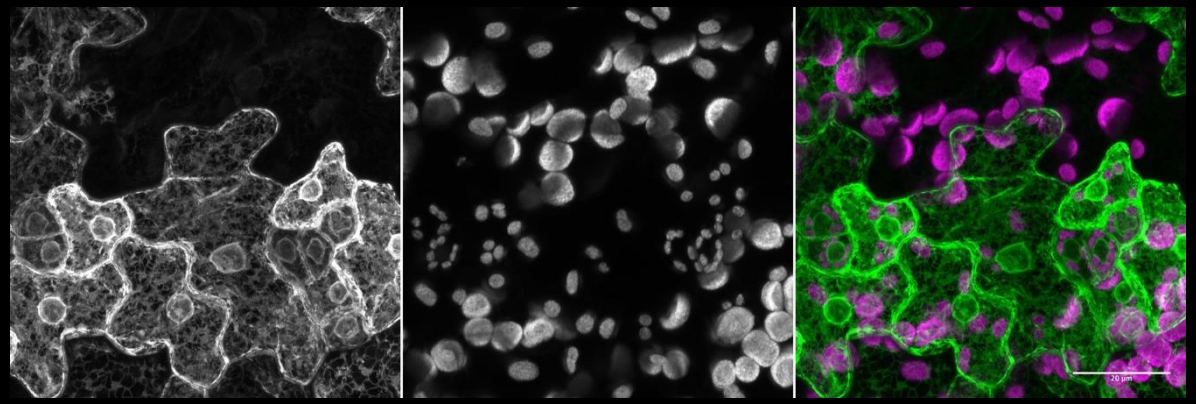


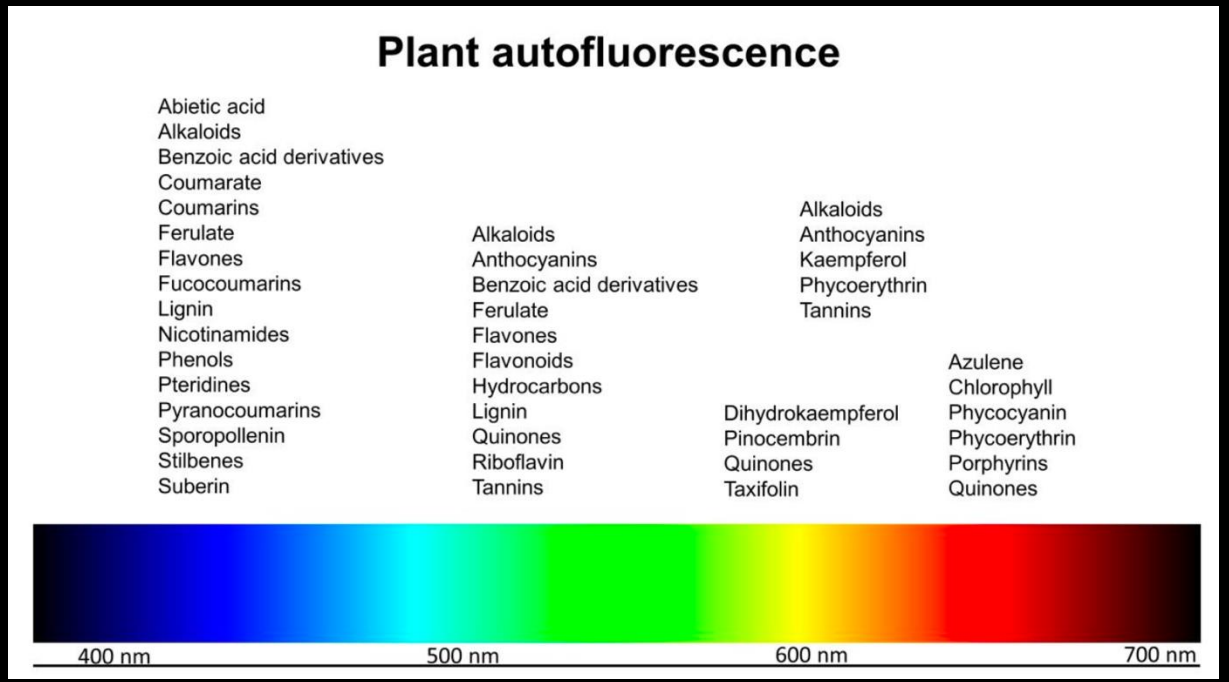

Intensity of fluorescence
Over entire region




- Phototoxicity and photobleaching are serious concerns with live cell dynamic imaging
  - *Can you image less?*
  - *Can you reduce laser power?*
  - *Can you choose different FPs?*
  - *Use a different imaging modality?*
- All of these come at costs, what compromise do you need to make? It will be sample specific

# *Solution:* Avoiding autofluorescence

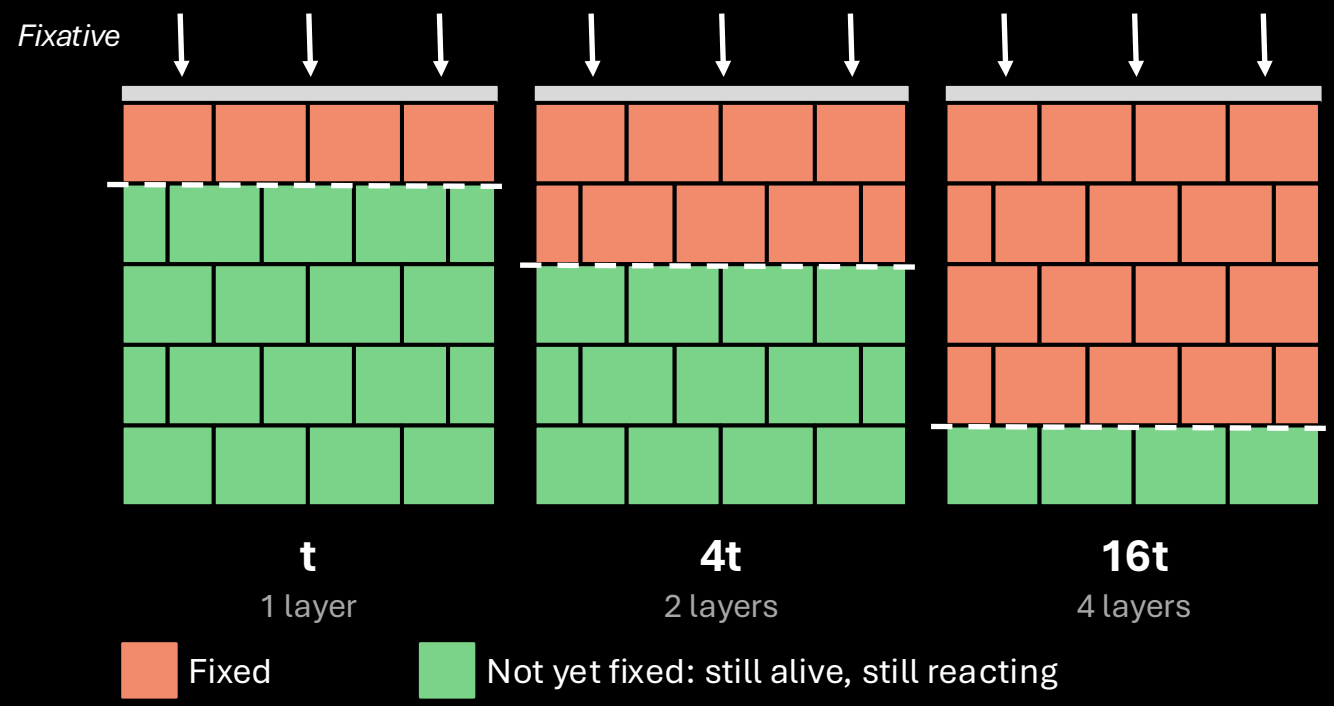





- Plants are incredibly autofluorescent, especially in the far red fluorescent spectrum with chlorophyll
  - Use good emission selection (<650nm)
  - Change the imaging modality
    - Lifetime imaging
  - Image cells which don't have chloroplasts
- Choose FPs wisely so they don't overlap with chlorophyll emission

Donaldson, 2020, Molecules

# *Problem:* Fixatives have to get through the wall

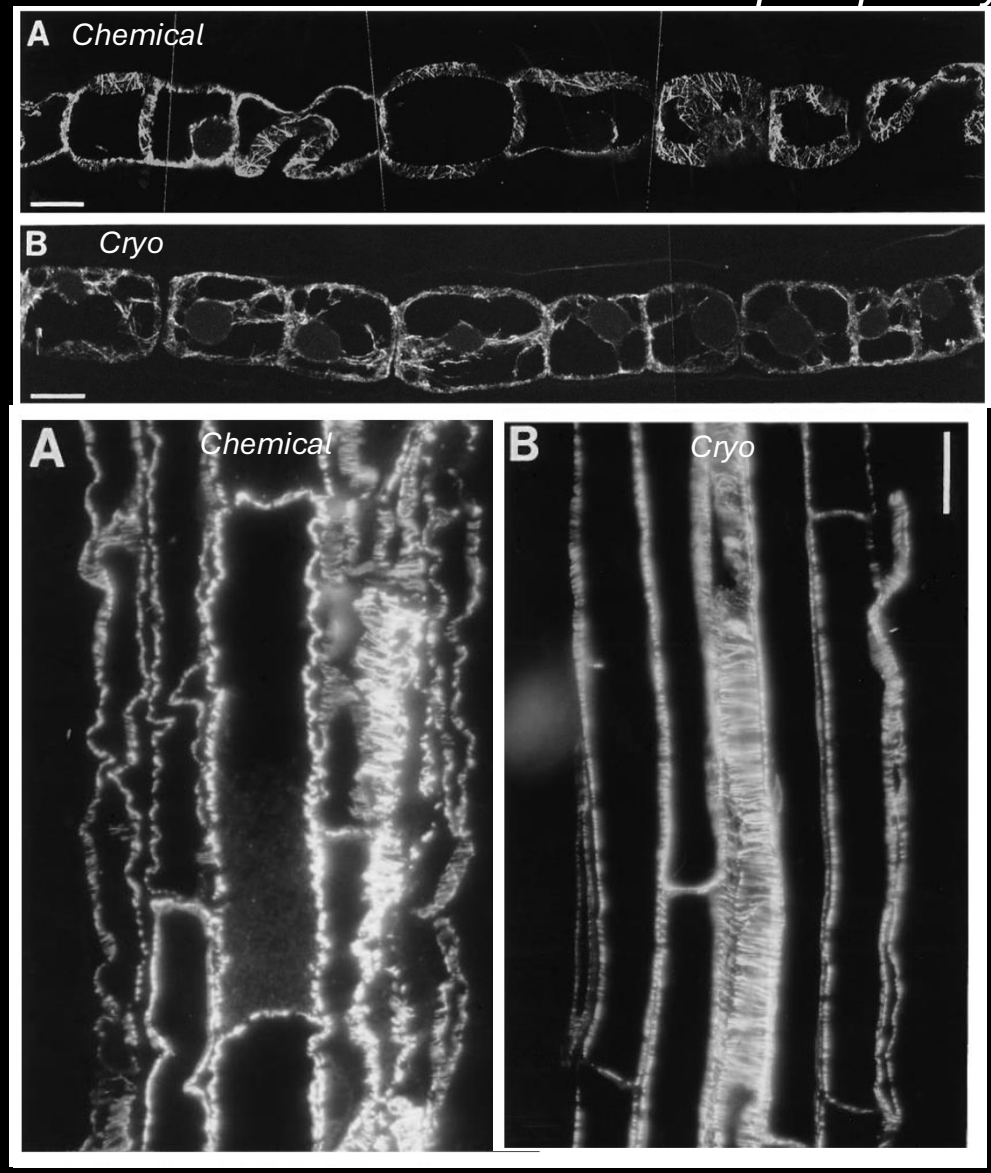



$d = K\sqrt{t}$

Doubling the depth takes four times as long (Medawar 1941)

- Cuticle, waxes and air spaces slow it further
- Inner cells keep changing until the fixative arrives
- Fix: small pieces, vacuum infiltration, fix fast

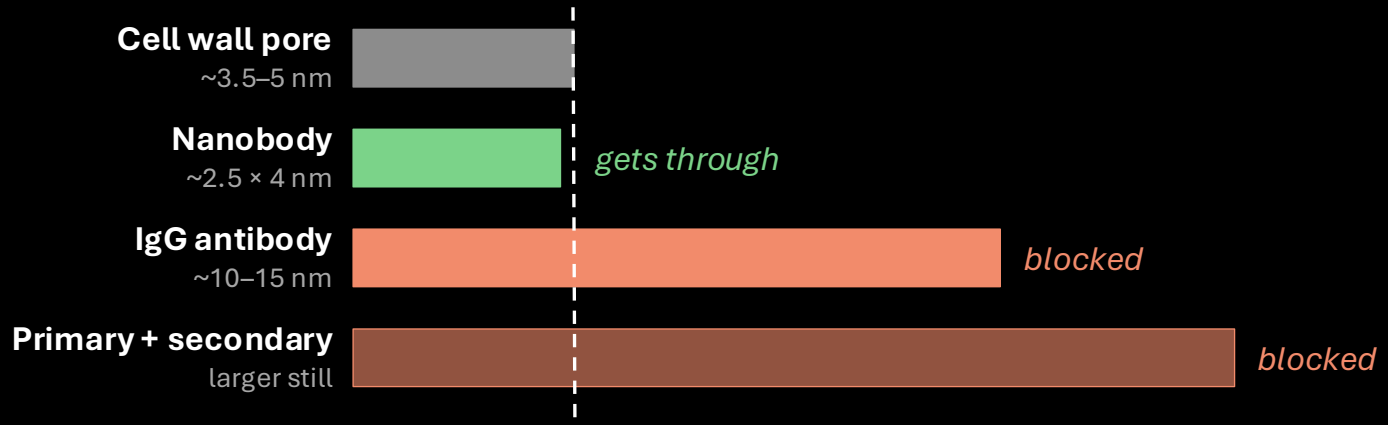
*Solution – Can freeze sample quickly*




Baskin et al. (1996) J. Microsc. 182:149–161

# *Solution:* Opening the wall for antibodies and FISH

## Size matters




## Digestion and permeabilisation: a trade-off

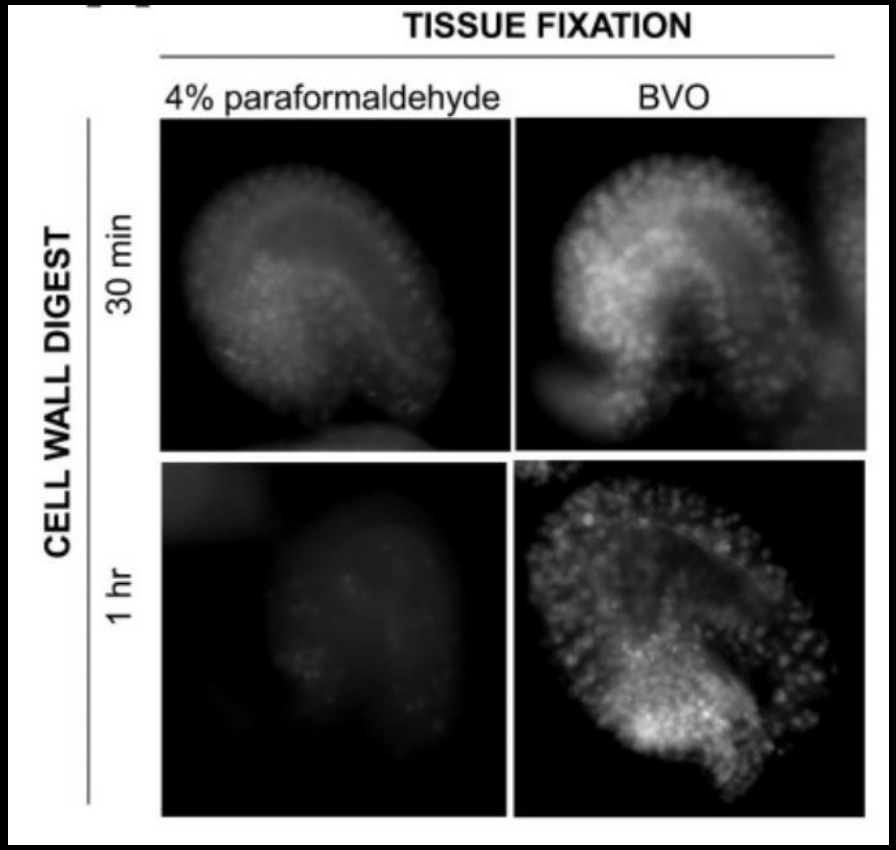
*Structure* ⟷ *Access*

**Too little**
Structure intact, but no signal inside

**Just enough**
Titrate for each tissue and species

**Too much**
Signal everywhere, but cells separate and structure is lost

## Nucleus signal lost if cell wall digested too long

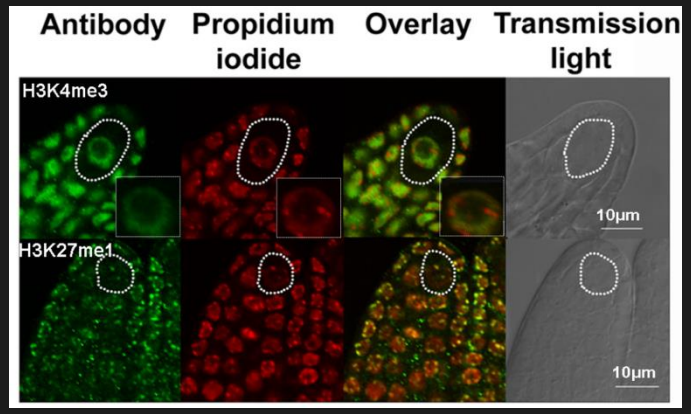



She et al. (2014) JoVE

Carpita et al. (1979) Science 205:1144 · Sauer et al. (2006) Nat. Protoc. 1:98 · Pasternak et al. (2015) Plant Methods · She et al. (2014) JoVE · Duncan et al. (2016) Plant Methods

# *Solution:* Isolated nuclei, squashes and spreads

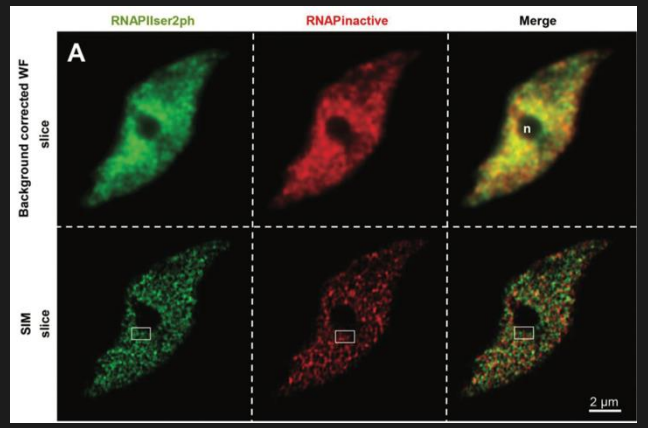
## Whole mount




**Keeps:** Tissue and cell-type context; true 3D nuclear shape
**Loses:** Hard to label and image deep inside
**Best for:** Nuclear organisation in specific cell types

She et al. (2014) JoVE

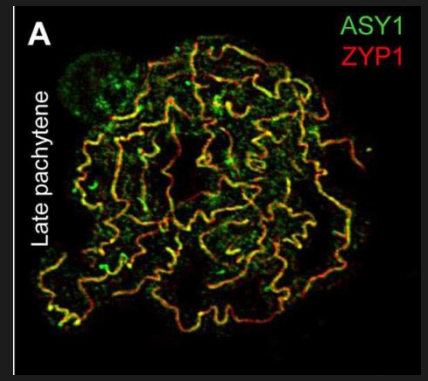
## Isolated nuclei




**Keeps:** No wall: probes get in easily; flat on the coverslip; sort by ploidy
**Loses:** Which cell each nucleus came from
**Best for:** Chromatin detail, SIM and single-molecule imaging

Schubert & Weisshart (2015) J. Exp. Bot

## Squashes and spreads



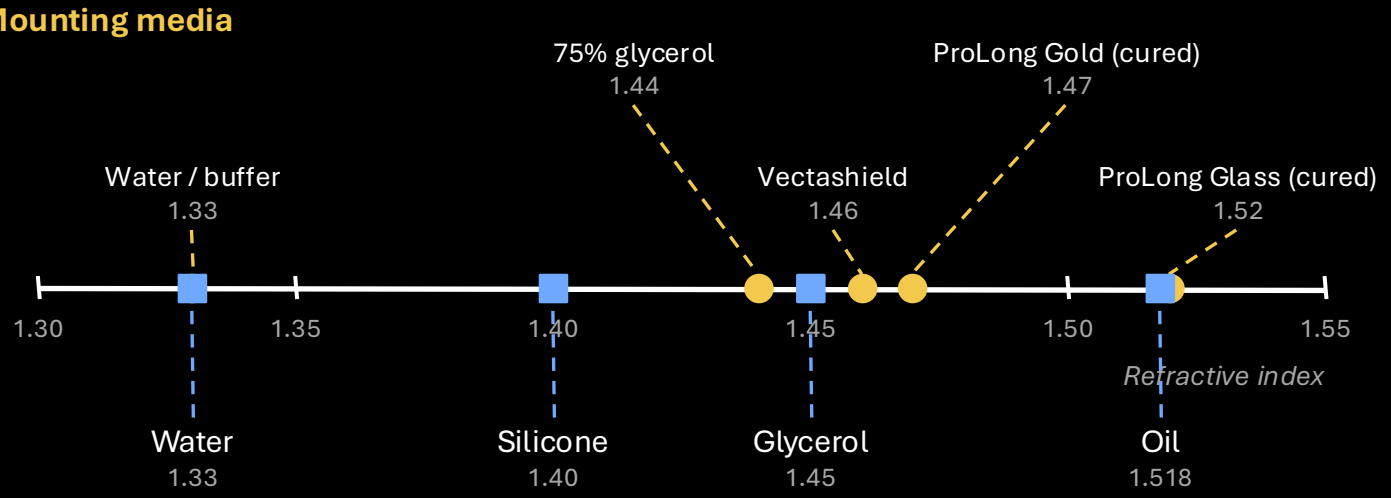

**Keeps:** Best chromosome morphology
**Loses:** Tissue context and 3D shape (flattened on purpose)
**Best for:** Counting and mapping chromosomes; FISH karyotyping

Hesse et al., (2019), Frontiers in plant science

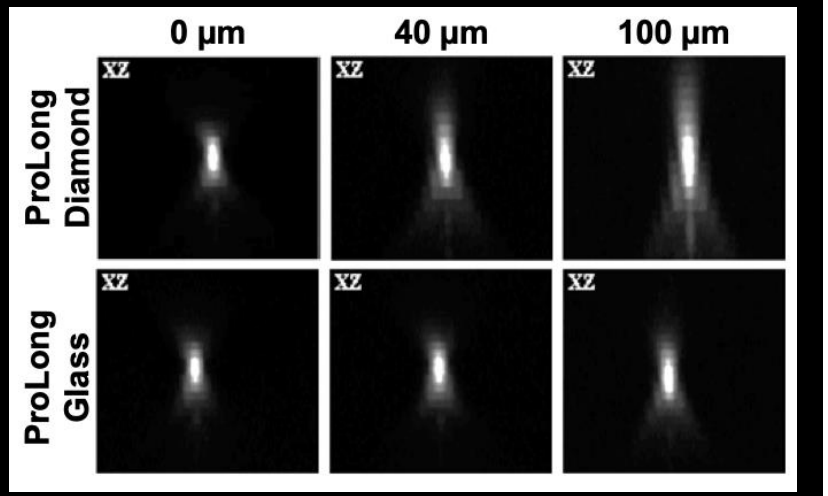
**Choose by the question:** more resolution and easier labelling ↔ less biological context

# *Solution:* Mounting fixed samples for high resolution

## Match your mounting medium to your objective

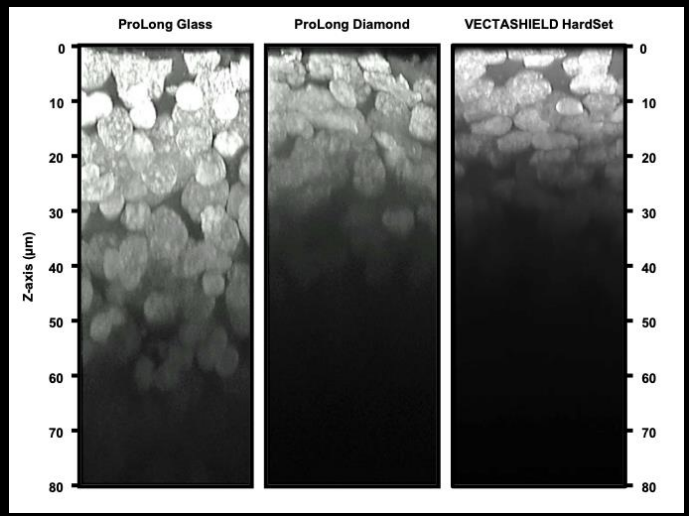



**Mismatch = spherical aberration that grows with depth.** Matching ProLong Glass to oil improved axial resolution by up to 75% beyond 100 µm deep.

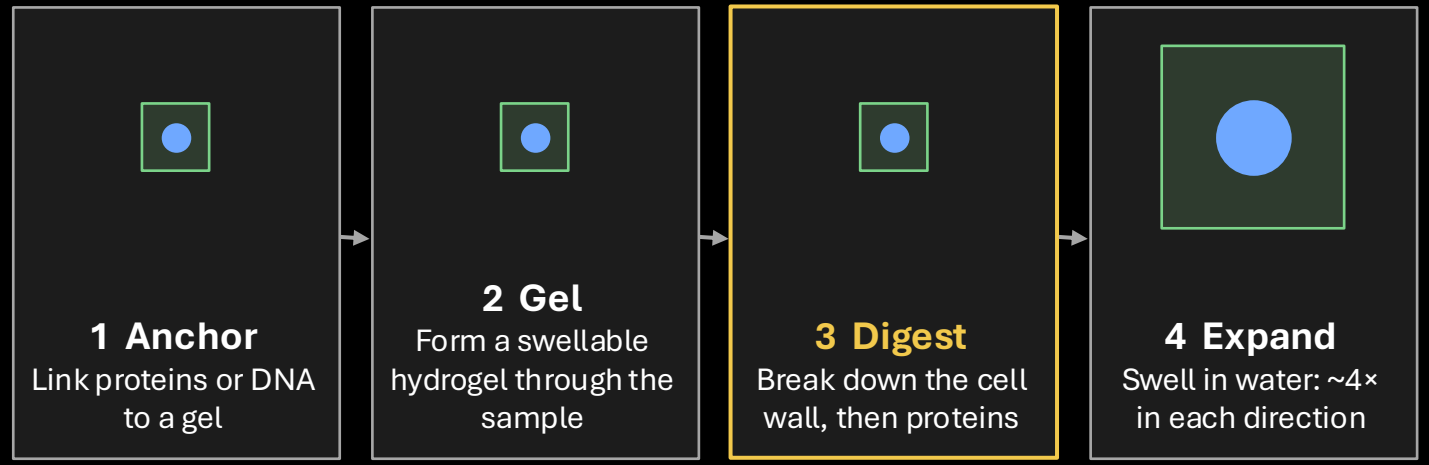



## Before you image

- #1.5H ***clean*** coverslips (170 µm)
- Thin, flat, and close to the coverslip
- Spacers for whole mounts, so you don't squash them
- Let hardening mountants cure: RI changes as they set
- Use an antifade and seal the edges
- Set the correction collar on glycerol and silicone lenses



# *Where next:* Expansion microscopy in plants



**1 Anchor**
Link proteins or DNA to a gel

**2 Gel**
Form a swellable hydrogel through the sample

**3 Digest**
Break down the cell wall, then proteins

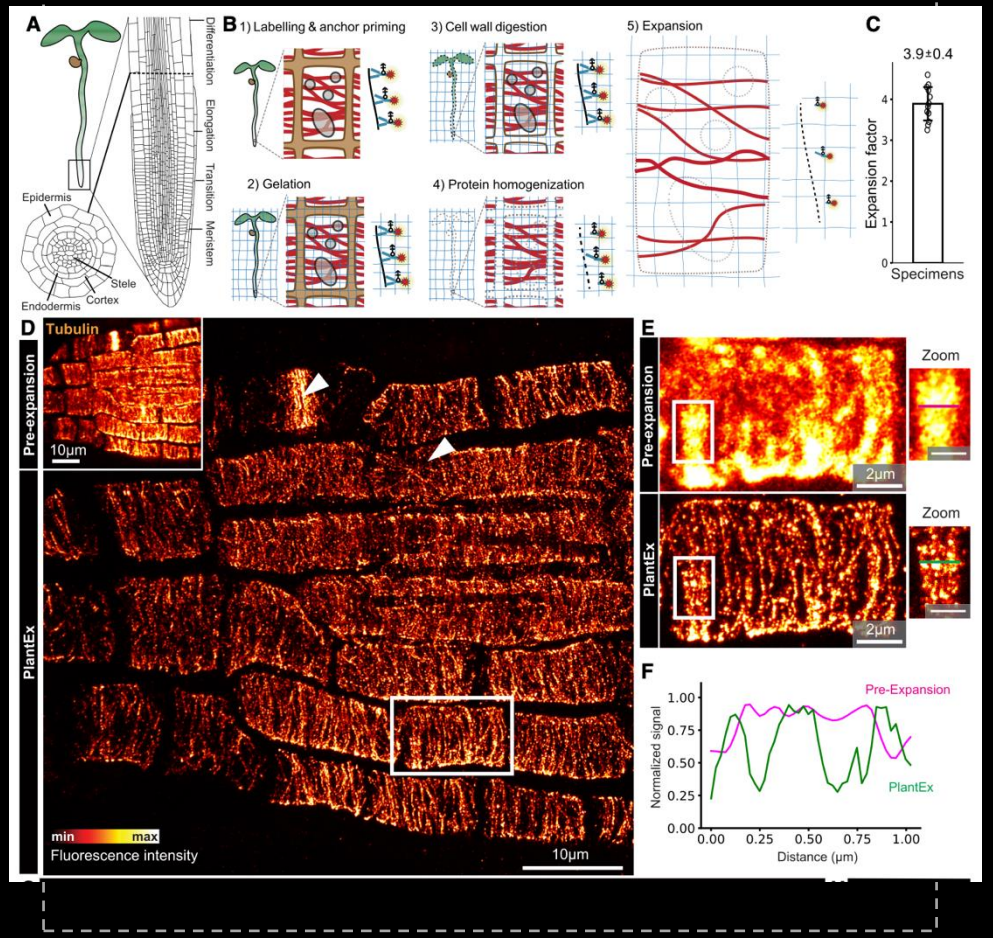
**4 Expand**
Swell in water: ~4× in each direction

## What you gain

- ~4× resolution on a standard confocal
- Cleared, near-RI-matched sample (mostly water)
- No super-resolution microscope needed
- Can be combined with STED for more (PlantEx)

## What to watch

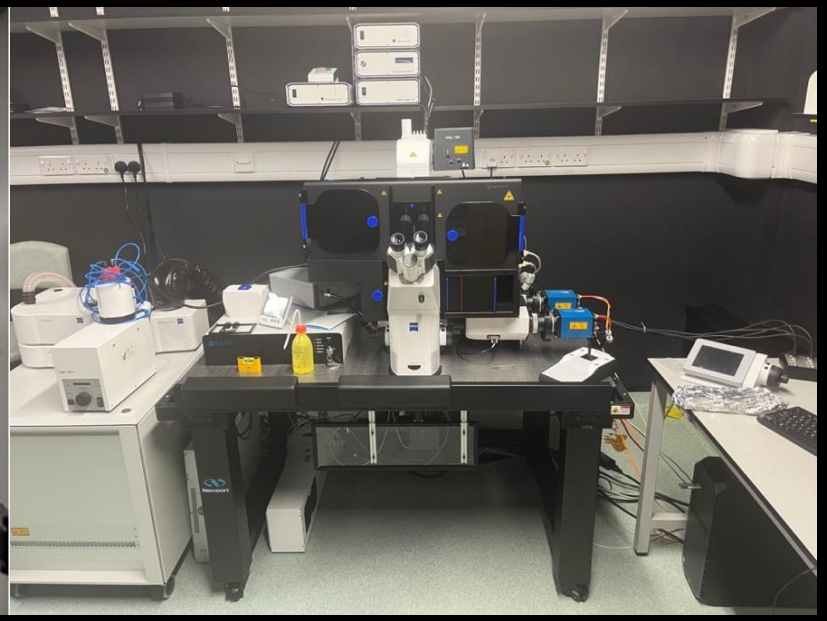
- Fixed tissue only; calibrate for each tissue
- Is expansion even? Image the same structure before and after
- Chromatin: barley nuclei expanded ~4.2×, but fine structure only partly preserved
- Signal is diluted ~64× by volume




Gallei et al. (2025) Plant Cell 37:koaf006 (PlantEx) · Kubalová et al. (2020) Chromosome Res. · Chen, Tillberg & Boyden (2015) Science

# *What we don't talk about*







- What are the conditions in your imaging suite?
  - *Temperature? Light? Humidity*
- What time of day do you image?
  - Circadian rhythms have large effect
- How has your sample been handled before you begin mounting?
  - Poor handling, drought experience, light quality
- Differences in sample processing between different experimenters?
  - Sounds like can be easily controlled but bigger than you think!

# *Summary:* Matching the prep to the question

## Do you need your sample alive?

### YES → LIVE

Which tissue, and for how long?

*Leaf, air spaces*

**Mount in PFD**

Fills air spaces; keeps tissue oxygenated

*Root or seedling, short imaging*

**Gentle mount**

Agar pad or chamber; acclimate 5–10 min

*Long time-lapse*

**Chambers, vertical stage, light sheet**

Keep roots growing with gravity

### NO → FIXED

What do you need to see?

*Deep cells, whole organ (FP or dye)*

**ClearSee**

Clears tissue, keeps FPs; days to weeks

*Endogenous protein, RNA or DNA*

**Fix + open the wall**

Whole-mount immuno or (sm)FISH

*Chromatin detail, chromosomes, SIM*

**Isolated nuclei and spreads**

Flat and accessible; loses tissue context

**Every route:** choose your label wisely · image a wild-type control with identical settings · check for artefacts before you quantify

# Your sample-prep checklist

**1 Grow**
Same age, media, conditions and time of day

**2 Choose**
Tissue, cell type and zone decided before you look; include wild type

**3 Prepare**
**Live:** support, don't squash
**Fixed:** fix fast; minimum digestion

**4 Acclimate**
**Live:** 5–10 min, then a fixed time window
**Fixed:** equilibrate in mounting medium

**5 Check**
Healthy and intact?

YES

**6 Image**
Locked settings, no saturation, controls on the same day

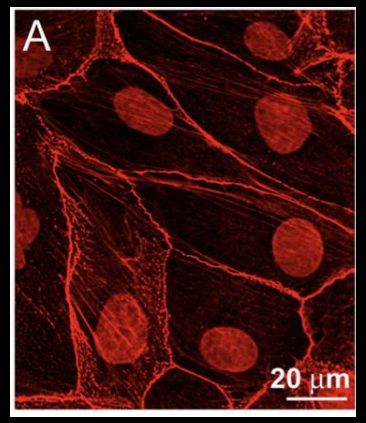
**7 Record**
Raw files, metadata, and exactly what you did to the sample

NO

**Back to step 3:** remount the sample

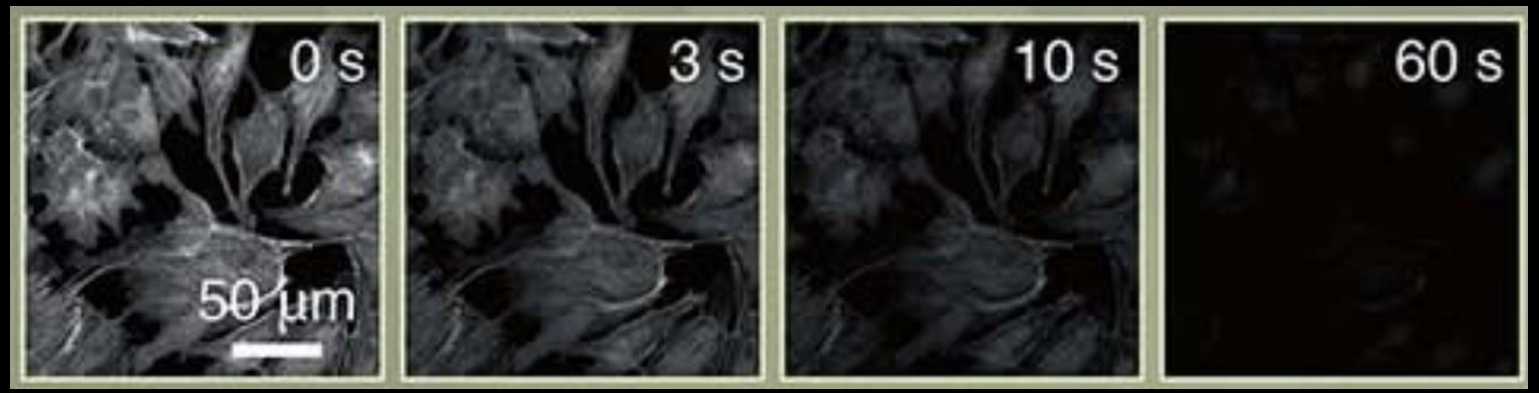
# *What went wrong?*

How many things are labelled?



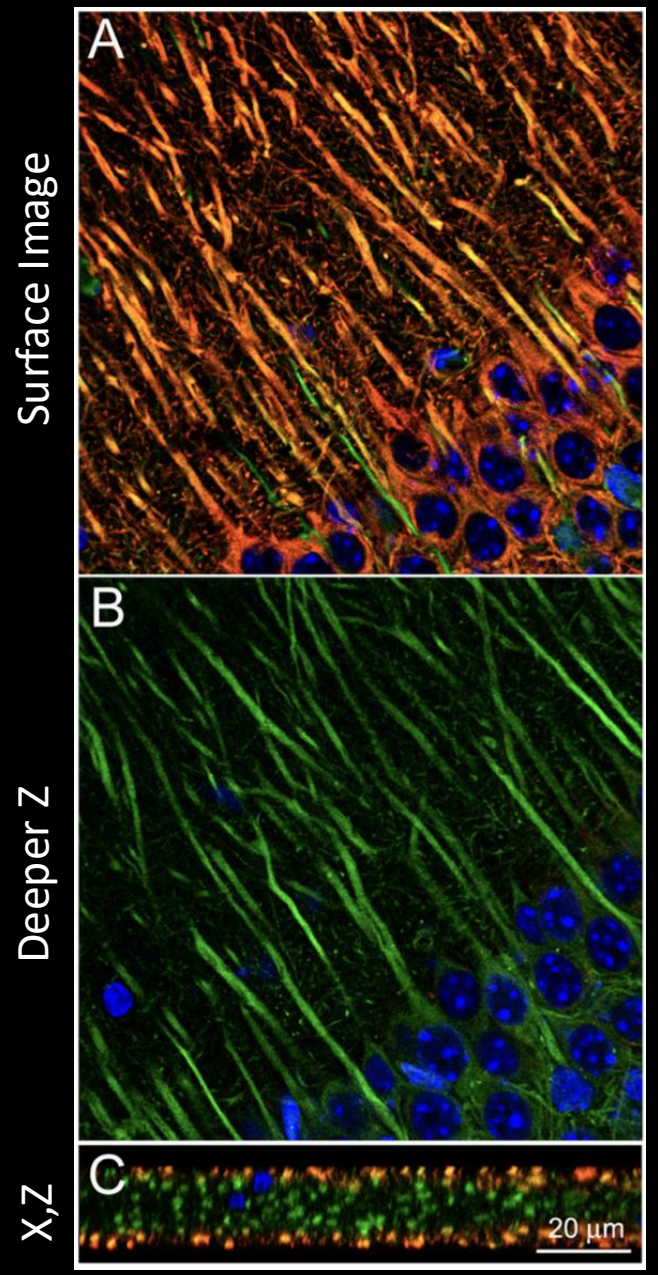

North, 2006, JCB

Where does the signal go?




Jonkman et al., 2020, Nature Protocols

Is staining uniform?




North, 2006, JCB

# Thanks for your time – Questions?

***Understanding your sample is just as important as the microscope***

***Good imaging is about compromise with the microscope <u>AND the sample</u>***

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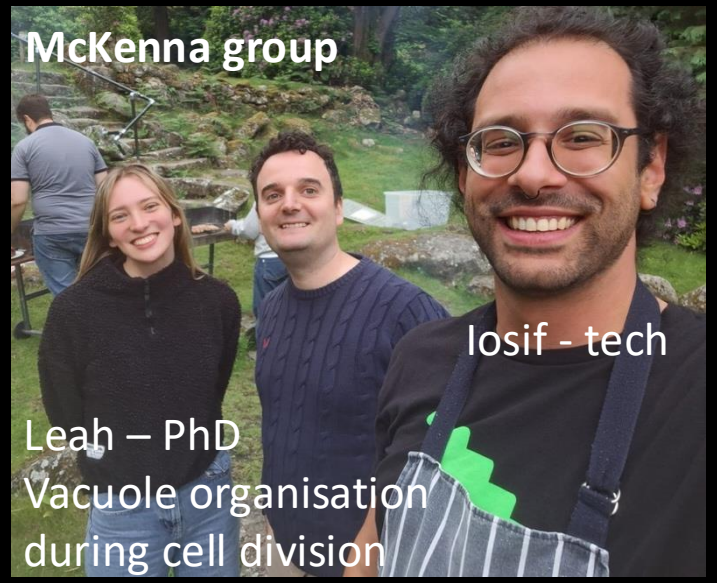


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# Reading list – *Don't read them all!!!!*

**Start here**

- Czymmek KJ *et al.* (2025) Best practices in plant fluorescence imaging and reporting: a primer. *The Plant Cell* 37: koaf143. doi:10.1093/plcell/koaf143
- North AJ (2006) Seeing is believing? A beginners' guide to practical pitfalls in image acquisition. *J. Cell Biol.* 172: 9–18. doi:10.1083/jcb.200507103
- Jonkman J, Brown CM, Wright GD, Anderson KI, North AJ (2020) Tutorial: guidance for quantitative confocal microscopy. *Nature Protocols* 15: 1585–1611. doi:10.1038/s41596-020-0313-9

**Optics of plant tissue and mounting live samples**

- Littlejohn GR, Gouveia JD, Edner C, Smirnoff N, Love J (2010) Perfluorodecalin enhances *in vivo* confocal microscopy resolution of *Arabidopsis thaliana* mesophyll. *New Phytologist*. doi:10.1111/j.1469-8137.2010.03244.x
- Kirchhelle C, Moore I (2017) A simple chamber for long-term confocal imaging of root and hypocotyl development. *J. Vis. Exp.* 123: e55331
- von Wangenheim D *et al.* (2017) Live tracking of moving samples in confocal microscopy for vertically grown roots. *eLife* 6: e26792. doi:10.7554/eLife.26792
- Johnson *et al.* (2020) *J. Cell Sci.*

**Clearing**

- Kurihara D, Mizuta Y, Sato Y, Higashiyama T (2015) ClearSee: a rapid optical clearing reagent for whole-plant fluorescence imaging. *Development* 142: 4168–4179. doi:10.1242/dev.127613

**Reporters, fluorescent proteins and autofluorescence**


- Ketelaar T, Anthony RG, Hussey PJ (2004) Green fluorescent protein–mTalin causes defects in actin organization and cell expansion in Arabidopsis and inhibits actin depolymerizing factor's actin depolymerizing activity in vitro. *Plant Physiology* 136: 3990–3998
- Ntoukakis V, Schwessinger B, Segonzac C, Zipfel C (2011) Cautionary notes on the use of C-terminal BAK1 fusion proteins for functional studies. *Plant Physiology*
- Lambert TJ (2019) FPbase: a community-editable fluorescent protein database. *Nature Methods* 16: 277–278
- Lacy M. Choosing a fluorescent protein for live-cell imaging: an updated guide. -https://blog.addgene.org/choosing-a-fluorescent-protein-for-live-cell-imaging-an-updated-guide
- Donaldson L (2020) Autofluorescence in plants. *Molecules* 25: 2393. doi:10.3390/molecules25102393


**Fixation and opening the wall**


- Medawar PB (1941) The rate of penetration of fixatives. *J. R. Microsc. Soc.* 61: 46–57
- Baskin TI, Miller DD, Vos JW, Wilson JE, Hepler PK (1996) Cryofixing single cells and multicellular specimens enhances structure and immunocytochemistry for light microscopy. *J. Microscopy* 182: 149–161
- Carpita N, Sabularse D, Montezinos D, Delmer DP (1979) Determination of the pore size of cell walls of living plant cells. *Science* 205: 1144–1147
- Sauer M, Paciorek T, Benková E, Friml J (2006) Immunocytochemical techniques for whole-mount in situ protein localization in plants. *Nature Protocols* 1: 98–103. doi:10.1038/nprot.2006.15
- Pasternak T *et al.* (2015) Protocol: an improved and universal procedure for whole-mount immunolocalization in plants. *Plant Methods* 11: 50. doi:10.1186/s13007-015-0094-2
- Duncan S *et al.* (2016) A method for detecting single mRNA molecules in *Arabidopsis thaliana*. *Plant Methods* 12: 13

**Nuclei, squashes and spreads**


- She W, Grimanelli D, Baroux C (2014) An efficient method for quantitative, single-cell analysis of chromatin modification and nuclear architecture in whole-mount ovules in Arabidopsis. *J. Vis. Exp.* e51530
- Schubert V, Weisshart K (2015) Abundance and distribution of RNA polymerase II in Arabidopsis interphase nuclei. *J. Exp. Bot*.
- Hesse S, Zelkowski M, Mikhailova EI, Keijzer CJ, Houben A, Schubert V (2019) Ultrastructure and dynamics of synaptonemal complex components during meiotic pairing and synapsis of standard (A) and accessory (B) rye chromosomes. *Front. Plant Sci.* 10: 773. doi:10.3389/fpls.2019.00773


**Mounting fixed samples**


- Thermo Fisher Scientific. Matching refractive index mounting media improves axial resolution and image quality in 3D biological samples (poster; manufacturer data).


**Expansion microscopy**


- Chen F, Tillberg PW, Boyden ES (2015) Expansion microscopy. *Science* 347: 543–548. doi:10.1126/science.1260088
- Gallei MC *et al.* (2025) Super-resolution expansion microscopy in plant roots. *The Plant Cell* 37: koaf006. doi:10.1093/plcell/koaf006
- Kubalová I *et al.* (2020) Prospects and limitations of expansion microscopy in chromatin ultrastructure determination. *Chromosome Research*. doi:10.1007/s10577-020-09637-y

**Further reading (mentioned in the talk or useful for the practicals)**


- Grison MS *et al.* (2025) Root expansion microscopy: a robust method for super resolution imaging in Arabidopsis. *The Plant Cell* 37(4) (ROOT-ExM)
- Laissue PP *et al.* (2017) Assessing phototoxicity in live fluorescence imaging. *Nature Methods* 14: 657–661. doi:10.1038/nmeth.4344
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