AGILE

Funded by the European Union

AGILE Training Event #2
IPK Gatersleben
18-20/11/2026

Designing an informed imaging workflow

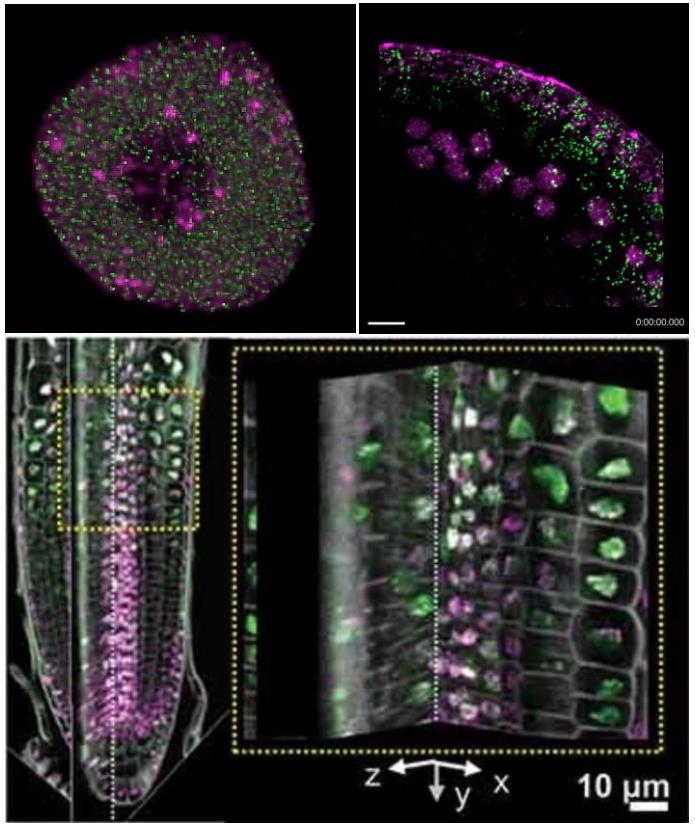
from sample preparation to image acquisition

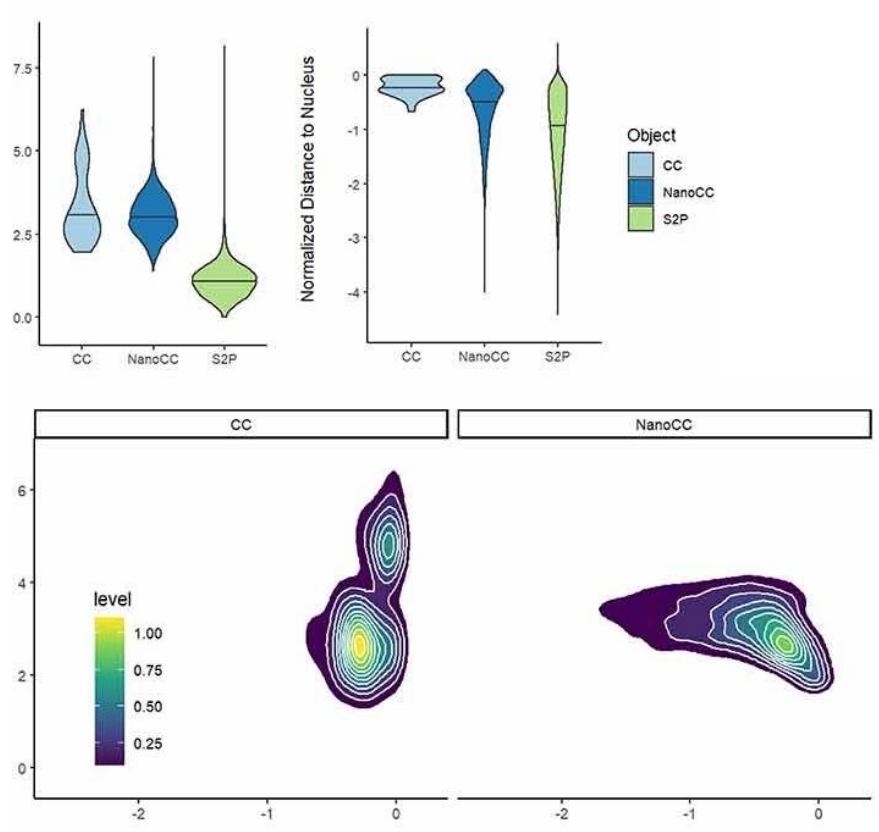
University of Zurich[UZH]

Célia Baroux
Department of Plant and Microbial Biology
University of Zürich, Switzerland

Transforming ideas into data



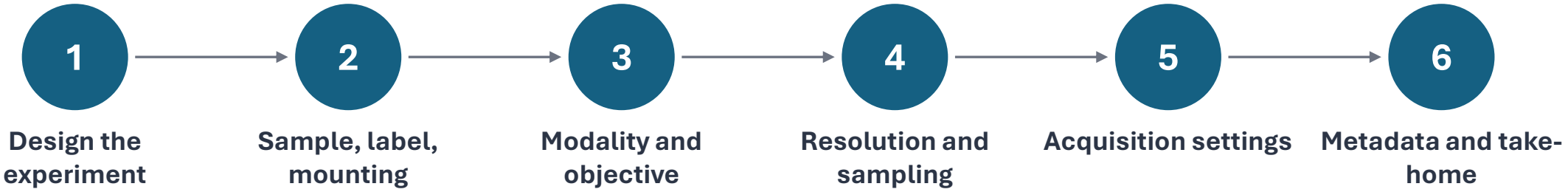




iconscout.com/illustration/biologist-studies-plant-cell-with-microscope-5226652

andall et al, Nucleus 2022
Dumur et al. Nucleus 2019
Cromer et al. Nature 2024s

Roadmap



A quiz closes each part

Q1 design · Q2–Q3 sample and mounting · Q4–Q5 objective, sampling, live imaging · Q6 detector settings

Survey before and after

This session covers workflow steps 1–3 (design, sample, acquisition). Processing, analysis and data follow in Training Events #3 and #5.

Hidden to fit 50 min: background literature – cite it on slide 2 instead

2019

NUCLEUS
2019, VOL. 10, NO. 1, 181–212
https://doi.org/10.1080/19491034.2019.1644592

Taylor & Francis
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REVIEW

OPEN ACCESS Check for updates

Probing the 3D architecture of the plant nucleus with microscopy approaches: challenges and solutions

Tao Dumur[a], Susan Duncan [b], Katja Graumann [c], Sophie Desset [d], Ricardo S Randall [e], Ortrun Mittelsten Scheid[a], Hank W Bass [f], Dimiter Prodanov [g], Christophe Tatout [d], and Célia Baroux [e]

[a]Gregor Mendel Institute (GMI) of Molecular Plant Biology, Austrian Academy of Sciences, Vienna Biocenter (VBC), Vienna, Austria; [b]Norwich Research Park, Earlham Institute, Norwich, UK; [c]Department of Biological and Medical Sciences, Oxford Brookes University, Oxford, UK; [d]GReD, Université Clermont Auvergne, CNRS, INSERM, Clermont–Ferrand, France; [e]Department of Plant and Microbial Biology, Zürich-Basel Plant Science Center, University of Zürich, Zürich, Switzerland; [f]Department of Biological Science, Florida State University, Tallahassee, FL, USA; [g]Environment, Health and Safety, Neuroscience Research Flanders, Leuven, Belgium

Checklist for a good imaging design

At the start of each imaging experiment, it is important to careful evaluate (i) the choice of fluorophore, (ii) sample preparation, (iii) the optical/microscopy setup and (iv) the image acquisition parameters. We refer to this collectively as *imaging design*. A good

- Selection of fluorophores
- Sample preparation
- Choice of objective
- Image acquisition setup

2020

© 2020. Published by The Company of Biologists Ltd | Journal of Cell Science (2020) 133, jcs250027. doi:10.1242/jcs.250027

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OPINION

SUBJECT COLLECTION: IMAGING

Hypothesis-driven quantitative fluorescence microscopy – the importance of reverse-thinking in experimental design

Eric C. Wait, Michael A. Reiche and Teng-Leong Chew*

ABSTRACT

One of the challenges in modern fluorescence microscopy is to reconcile the conventional utilization of microscopes as exploratory instruments with their emerging and rapidly expanding role as a quantitative tools. The contribution of microscopy to observational biology will remain enormous owing to the improvements in acquisition speed, imaging depth, resolution and biocompatibility of modern imaging instruments. However, the use of fluorescence microscopy to facilitate the quantitative measurements necessary to challenge hypotheses is a relatively recent concept, made possible by advanced optics, functional imaging probes and rapidly increasing computational power. We argue here that to fully leverage the rapidly evolving application of microscopes in hypothesis-driven biology, we not only need to ensure that images are acquired quantitatively but must also re-evaluate how microscopy-based experiments are designed. In this Opinion, we present a reverse logic that guides the design of quantitative fluorescence microscopy experiments. This unique approach starts from identifying the results that would quantitatively inform the hypothesis and map the process backward to microscope selection. This ensures that the quantitative aspects of testing the hypothesis remain the central focus of the entire experimental design.

2023

Received: 21 December 2022 | Revised: 25 May 2023 | Accepted: 26 May 2023
DOI: 10.1111/jmi.13208

Journal of Microscopy RMS

INVITED REVIEW

Made to measure: An introduction to quantifying microscopy data in the life sciences

Siân Culley[1] | Alicia Cuber Caballero[1] | Jemima J Burden[2] | Virginie Uhlmann[3]

adversely influence the quantitative output. The first step consists of defining the goal of the imaging experiment (what quantitative property is at the centre of the question being investigated) prior to acquiring data and using this information to guide experimental design.[11] Once

In order to be able to formulate what one *wants* to measure, one must first understand what *can* be measured.

2025

THE PLANT CELL

The Plant Cell, 2025, **37**, koaf143
https://doi.org/10.1093/plcell/koaf143
Advance access publication 23 July 2025
Review

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,*]

Abstract

Microscopy is a fundamental approach for plant cell and developmental biology as well as an essential tool for mechanistic studies in plant research. However, setting up a new microscopy-based experiment can be challenging, especially for beginner users, when implementing new imaging workflows or when working in an imaging facility where staff may not have extensive experience with plant samples. The basic principles of optics, chemistry, imaging, and data handling are shared among all cell types. However, unique challenges are faced when imaging plant specimens due to their waxy cuticles, strong/broad spectrum autofluorescence, recalcitrant cell walls, and air spaces that impede fixation or live imaging, impacting sample preparation and image quality. As expert plant microscopists, we share our collective experience on best practices to improve the quality of published microscopy results and promote transparency, reproducibility, and data reuse for meta-analyses. We offer plant-specific advice and examples for microscope users at all stages of fluorescence microscopy workflows, from experimental design through sample preparation, image acquisition, processing, and analyses, to image display and methods reporting in manuscripts. We also present standards for methods reporting that will be valuable to all users and offer tools to improve reproducibility and data sharing.

Reverse thinking: start from measurement, not the microscope



From the biological question, decide which quantification you need - then work backwards:

- Which measurement (signal intensity, distances, shapes, velocity..)
- Which resolution (spatial, temporal)
- Which sampling (2D, 3D, 4D)
- Which sample and label can deliver it.
    - Including a secondary label for signal normalisation
    - Including a control for measuring false positives, biological noise
    - Including a reference staining for normalizing fluorescence intensity

From question to measurable feature

Question	Feature to measure	What the images must provide
Does the protein relocate within the nucleus?	Position relative to the periphery or a landmark	3D stack, good axial resolution, a reference channel
Is chromatin more compact upon treatment?	Volume, intensity, number of chromocenters or nuclear speckles	Linear intensity, no saturation
Are nuclear bodies (speckles, photobodies) cyclically assembled and disassembled ?	Number, size and shape of nuclear bodies per nucleus	• 3D stack – moderate axial resolution enough, specific speckle label, • reference signal for the nucleus (DNA or boundary)
How fast does it happen?	Change over time, velocity	Live imaging, time resolution prioritized over spatial resolution, low light dose
How consistent is the response across a tissue – how many cells respond?	Counts across a tissue	Many nuclei per sample, moderate resolution is enough High working distance necessary, Multiphoton preferred

The feature sets the **dimensions** (2D, 3D, 4D), the **resolution**, the imaging **modality**, the type and **number** of samples.

Imaging design checklist

1 Design

- ❑ Measurable feature and dimensions (2D, 3D, 4D)
- ❑ Biological replicates: n per condition
- ❑ Positive, negative, unlabelled controls
- ❑ Blinding and randomised imaging order
- ❑ Pilot run: variability, settings, n

2 Sample preparation

- ❑ Fixed or live; whole-mount or isolated nuclei
- ❑ Label and fluorophore (lasers, filters)
- ❑ Fixation and permeabilisation
- ❑ Clearing and autofluorescence
- ❑ Mounting medium and refractive index
- ❑ Controls and reference signal

3 Acquisition

- ❑ Modality: resolution, speed, depth, dose
- ❑ Objective: NA, immersion, WD
- ❑ Sampling: pixel, z-step, Δt (Nyquist)
- ❑ Speed, averaging vs sum-up
- ❑ Detector: photon count, gain/ offset, bit depth
- ❑ Crosstalk and chromatic shift
- ❑ Light dose and bleaching

Document from day one samples and images in the **AGILE registry**.

Design the experiment before imaging

Replicates

Biological replicates (plants, batches) are not technical ones (nuclei, fields). Plan n per condition.

Controls

Positive, negative and unlabelled controls, imaged with the same settings.

Reference staining for normalizing fluorescence intensity

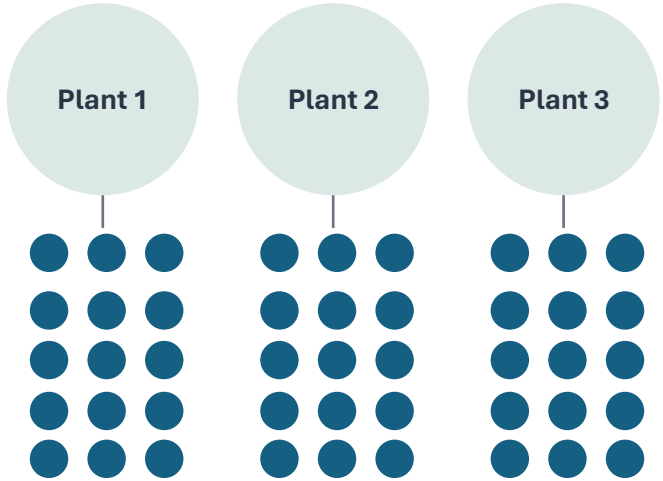
Blinding

Code the samples so their identity stays unknown during acquisition; randomise the imaging order.

Pilot

A small first run estimates variability, fixes the settings and tells you how many samples you need.

What is n?



n = 3 independent plants

15 nuclei per plant come from different cells, so they are also biological measurements.

Pool nuclei across plants only after checking replicability between plants. Always record the plant of origin * for every dataset.

*incl growth conditions, time of sample prep/image acquisition

Choosing the biological sample

Fixed or live

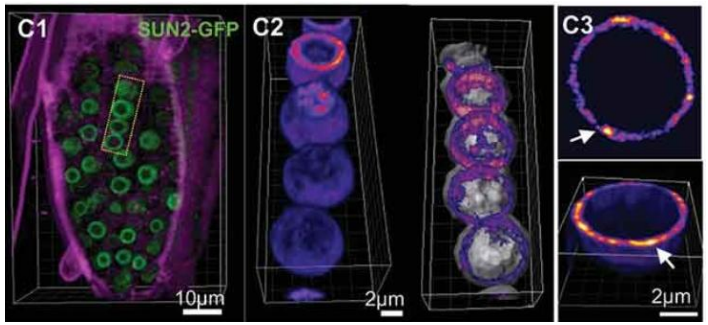
- Fixed: antibodies, FISH, highest spatial resolution.
- Live, static: one time point, no fixation artefacts, limited spatial resolution
- Live, dynamic: time-lapse, limited light dose, limited spatial resolution

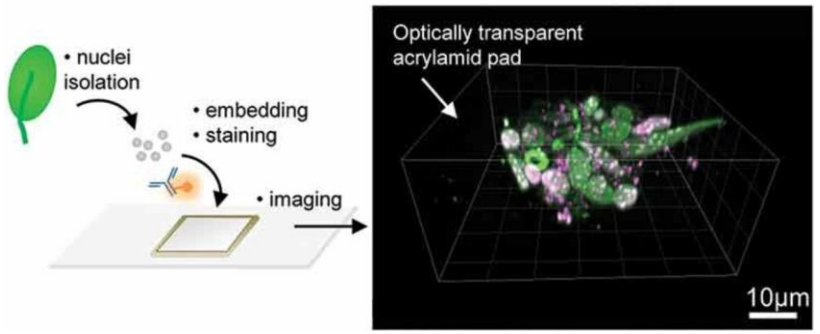
Whole-mount or isolated nuclei

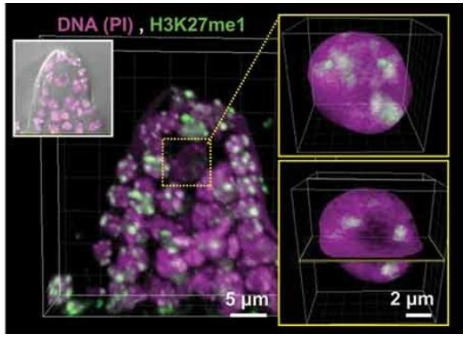
- Whole-mount keeps tissue context and cell identity.
- Isolated or spread nuclei: better labelling and resolution, but context is lost.

Cell-type specificity

- Target the cells that matter:
  - tissue landmarks/position
  - cell-type specific markers.
- Or sort nuclei by cell type: FACS, INTACT.







Dumur T et al. (2019) Nucleus 10: 181–212

Labelling strategy

Strategy	Best for	Watch out for
Fluorescent protein reporter	Live imaging, endogenous context, no permeabilisation	Expression level, tag interference, maturation, brightness
Immunostaining	Endogenous proteins and modifications (e.g. histone marks)	Antibody specificity, penetration in tissue, fixation artefacts
Fluorescent In Situ Hybridization (FISH)	DNA or RNA loci, chromosome territories	Harsh denaturation alters structure; probe access
DNA dyes (DAPI, PI, Abberior Live dyes)	Nuclear volume, chromatin domains, counterstain	Spectral overlap, staining saturation, dye-specific bias

- **Choose fluorophores together with the microscope's lasers and filters, away from autofluorescence and well separated from other fluorophores.**
- or use spectral separation or life time-based separation (Tau gating)
- **FPbase helps compare fluorescent proteins.**

Choosing a fluorophore: key specs

Excitation / emission maxima
Match your lasers and filters.

Stokes shift
Distance between excitation and emission peaks.

Extinction coefficient (ε)
How well it absorbs light (M^{-1} cm^{-1}).

Quantum yield (QY)
Fraction of absorbed photons emitted.

Brightness = ε × QY
e.g. EGFP 33.5, mCherry 15.8.

(x1000)

Photostability
How fast it bleaches under your illumination.

pKa
pH at which half the fluorescence is lost.

Maturation time
Time for an FP chromophore to form.

Oligomeric state
Use monomeric FPs for fusions.

Fluorescence lifetime
Basis of FLIM and FRET readouts.

Two-photon cross-section
Brightness under 2P excitation (GM).

Photoswitching, blinking
Required for SMLM (STORM, PALM).

Values are measured in vitro (FPbase: EGFP ε 55,900, QY 0.60, pKa 6.0; mCherry ε 72,000, QY 0.22, pKa 4.5). In your cells, pH, fixation and mounting can change them.

Comparing fluorescent proteins

Protein	Ex / Em (nm)	ε (M^{-1} cm^{-1})	QY	Brightness	pKa	Maturation	State	Photostability
EGFP	488 / 507	55,900	0.60	33.5	6.0	25 min	Weak dimer	Moderate
StayGold	496 / 505	159,000	0.93	147.9	4.0	14 min	Dimer	Very high
mStayGold	499 / 510	164,000	0.83	136.1	4.8	n.d.	Monomer	Higher than StayGold
mCherry	587 / 610	72,000	0.22	15.8	4.5	15 min	Monomer	Moderate
TagRFP-T	555 / 584	81,000	0.41	33.2	4.6	100 min	Monomer	High

How brightness is calculated

ε × QY, divided by 1000 for readability. EGFP: 55,900 × 0.60 = 33,540 → 33.5.

Read with care

In vitro values. Effective brightness in cells also depends on maturation, expression and pH. StayGold is a dimer: use mStayGold for fusions.

FPbase.org · Hirano et al. 2022 Nat Biotechnol (StayGold) · Ando et al. 2024 Nat Methods 21: 648 (mStayGold) · Shaner et al. 2008 Nat Methods 5: 545 (TagRFP-T)

Comparing organic dyes

Range	Dye	Ex / Em (nm)	ε (M^{-1} cm^{-1})	QY	Brightness	Best for
Green	Alexa Fluor 488	495 / 519	73,000	0.92	67	Confocal workhorse, pH-insensitive
Green	ATTO 488	501 / 523	90,000	0.80	72	Confocal, STED (590–610 nm)
Green	abberior STAR GREEN	493 / 519	75,000	0.84	63	STED (590–610 nm)
Orange	Cy3	554 / 568	150,000	0.15	23	FISH; QY depends on environment
Orange	Alexa Fluor 568	578 / 603	88,000	0.69	61	Confocal
Orange	abberior STAR 580	587 / 607	85,000	0.90	77	STED (750–800 nm), replaces Cy3
Orange	abberior STAR ORANGE	589 / 616	95,000	0.55	52	STED, 2-colour with STAR RED
Far-red	Cy5	650 / 669	250,000	0.27	68	dSTORM (blinks in thiol buffer)
Far-red	Alexa Fluor 647	650 / 668	239,000	0.33	79	dSTORM reference dye
Far-red	ATTO 647N	646 / 664	150,000	0.65	98	STED (775 nm), stable pH 2–11
Far-red	abberior STAR RED	638 / 655	120,000	0.90	108	STED (750–800 nm)

Brightness = ε × QY / 1000. Vendor values for the free dye in buffer; conjugation changes QY (Cy3 most). Dyes (~1 kDa) are far more photostable than FPs but need antibodies or tags (HaloTag, SNAP).

Thermo Fisher Alexa Fluor dye table · ATTO-TEC catalogue · abberior product data · AAT Bioquest cyanine data · Dempsey et al. 2011 Nat Methods 8: 1027

Match the fluorophore to the method

Live imaging	Multiphoton	STED	STORM, PALM
• photostability sets how long you can image. • Bleaching can mimic a biological decrease. • Photostable FPs: mStayGold, TagRFP-T. • Mind maturation time.	• 2P spectrum is not simply 2× the 1P spectrum: often blue-shifted and broader. • Check the 2P cross-section (GM) at your laser line. • Not all FPs respond to 2P ! (eg mCherry).	• Very photostable dyes that survive the depletion beam. • Emission tail must overlap the depletion line (often 775 nm). • Far-red organic dyes (e.g. ATTO 647N, STAR RED) outperform FPs.	• Needs photoswitching: many photons per switch, low duty cycle. • dSTORM: Alexa Fluor 647 or Cy5 in a thiol blinking buffer. • PALM: photoconvertible FPs (mEos3.2, Dendra2).

At the nanoscale, label size matters: an FP adds ~27 kDa; primary + secondary antibodies can place the dye 10–20 nm from the target, as large as SMLM resolution.

Controls for labelling

1

Unlabelled

Reveals autofluorescence in each channel.

2

Single labels

Reveal bleed-through between channels.

3

Secondary only

Reveals non-specific antibody binding.

4

Biological negative

A mutant or knockdown tests the label's specificity.

5

Reference signal

A stable reference (DNA stain, constitutive protein): report the target as a ratio, like actin in RT-qPCR.

Image every control with exactly the settings of the real samples, and keep these images in the registry too.

Fixation, permeabilisation and clearing

Get the fixative in

Air spaces and cuticles block penetration: vacuum infiltration, enough time, small tissue pieces.

Open the cell wall

Enzymatic digestion and detergents let antibodies and probes in, at the cost of some structure.

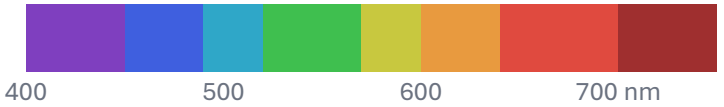
Clear the tissue

Plant clearing reagents (e.g. ClearSee) reduce scattering and chlorophyll autofluorescence while keeping FPs.

Know the artefacts

Shrinkage, flattening, altered chromatin compaction. Validate against a live reporter where possible.

Plant autofluorescence



Cell walls

Chlorophyll

Stress compounds

phenolics, flavonoids

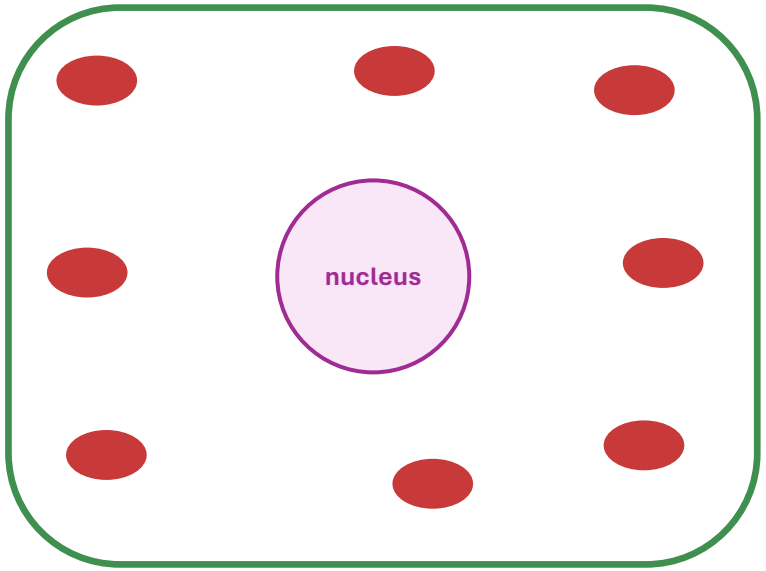
Choose fluorophores away from these bands; stress raises the green-yellow background.

Always image an unlabelled sample with the same settings.

Approximate ranges; they depend on tissue and fixation.

Donaldson L (2020) Molecules 25

Quiz 2 · Sample preparation



Red signal in nucleus and chloroplasts

Your mCherry reporter in leaf nuclei seems bright everywhere, including in chloroplasts.

Which control do you image first?

- ❑ Secondary antibody only
- ❑ Unlabelled wild type, same settings
- ❑ Single-label sample
- ❑ A brighter reporter line

What reduces chlorophyll autofluorescence while keeping the fluorescent protein?

- ❑ Methanol extraction
- ❑ ClearSee clearing
- ❑ Harder mounting medium
- ❑ Higher detector gain

Mounting medium and refractive index

A refractive-index mismatch between immersion medium, mounting medium and sample causes spherical aberration: dimmer signal, blurred and stretched or compressed z, worse with depth.

Match the objective

Oil for high-index media, water or silicone for live and aqueous samples, glycerol for intermediate media.

Know your medium

In our hands: ½ MS, Immersol, ProLong Gold, Vectashield, Renaissance SR2200. Each has its own RI and curing behaviour.

Check the z-axis

Image a known object (beads, a round nucleus) to see whether z distances are distorted.

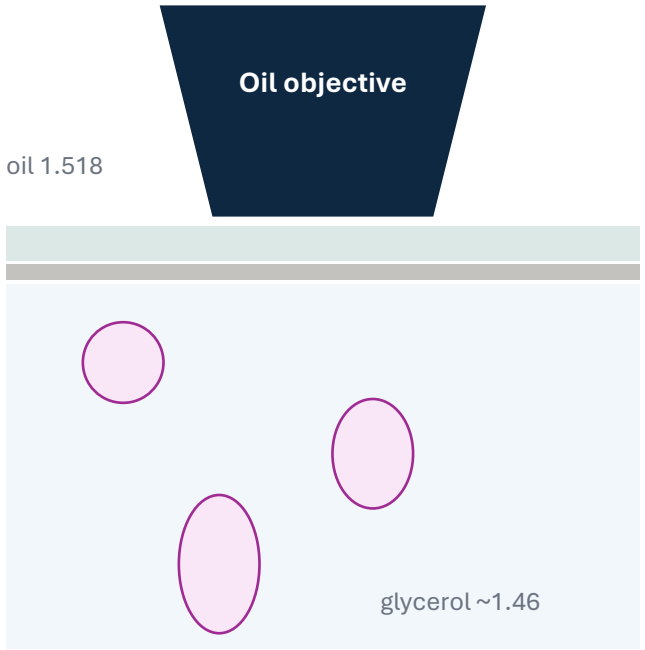
Immersion	n	Suited to
Water (Immersol W)	1.334	Live, aqueous samples
Silicone (Immersol Sil 406)	1.406	Live tissue, deeper imaging
Glycerol (Immersol G)	1.456	Cleared or glycerol-mounted
Oil (Immersol 518 F)	1.518	Fixed, hardening media

Tunable RI: mix the sample medium to the objective, e.g. TDE in water from 1.33 to oil (1.52).

Check the RI of your mounting medium; hardening media change RI as they cure.

ZEISS Immersol data (RI at 23 °C) · Staudt T et al. (2007) Microsc Res Tech 70: 1–9 (TDE) · Jonkman J et al. (2020) Nat Protoc 15: 1585–1611, Fig. 4 · Czymmek KJ et al. (2025) Plant Cell, Fig. 5

Quiz 3 · Mounting and refractive index



Nuclei stretch and dim with depth

You image a cleared, glycerol-mounted root with an oil objective (n = 1.518). Round nuclei look stretched in z and get dimmer with depth.

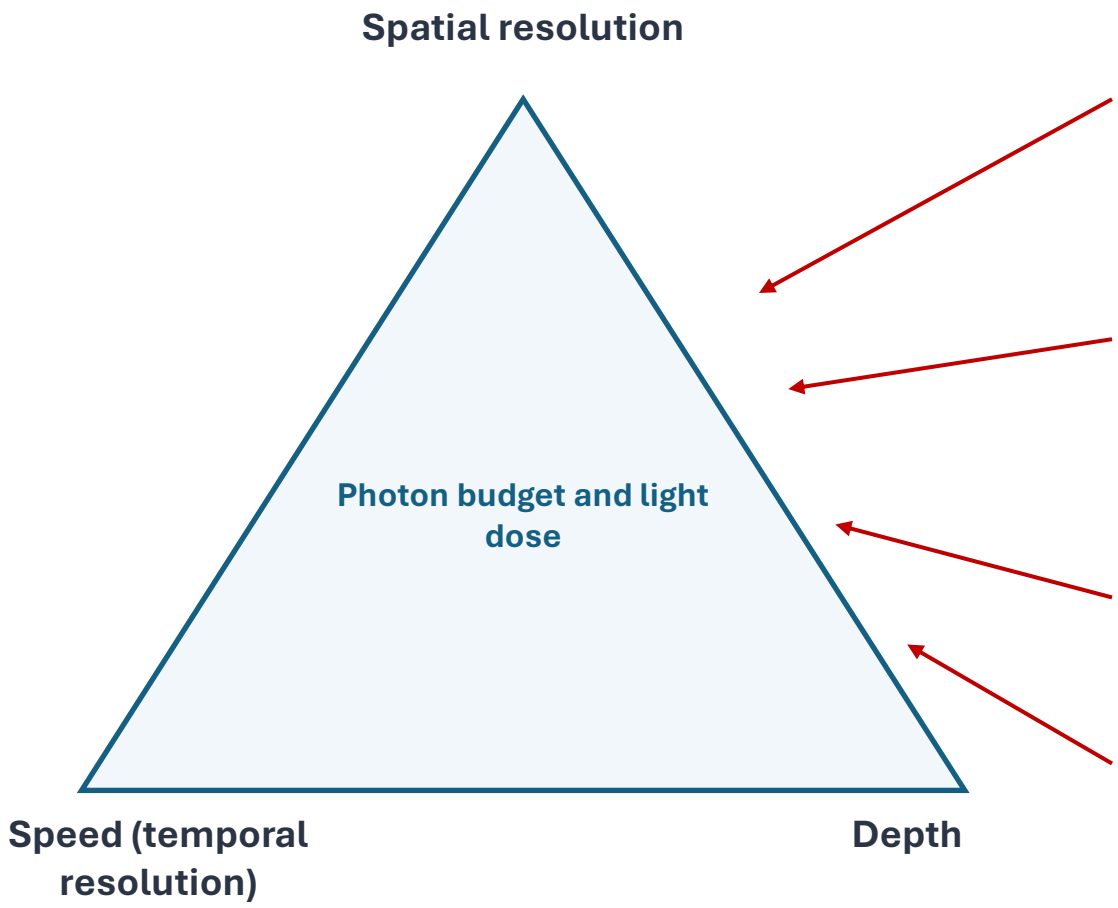
What is the most likely cause?

- ❑ z-step too large
- ❑ Refractive-index mismatch
- ❑ Photobleaching
- ❑ Chromatic shift

Which change helps most?

- ❑ Higher laser power at depth
- ❑ Glycerol or silicone objective, or RI-matched medium
- ❑ Smaller pixel size
- ❑ Longer exposure

The imaging trade-off triangle



Where do they map? -> assignment

STED, SMLM, MINFLUX

Highest resolution, paid in speed and light dose.

Spinning disk, light sheet

Speed and low dose, at moderate resolution.

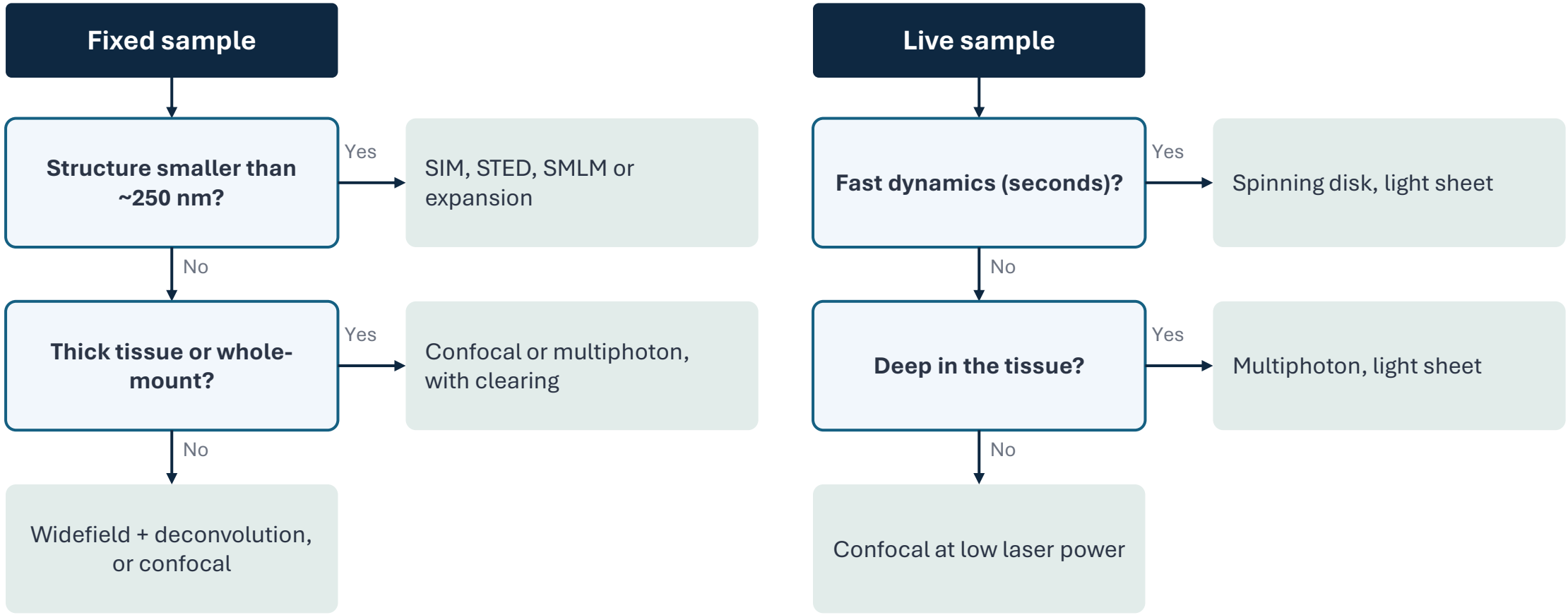
Multiphoton

Depth in tissue, at lower resolution.

Your sample

Decide which corner the measurement needs, and accept the cost elsewhere.

Decision path



Adapted from Dumur T et al. (2019) Nucleus 10: 181–212

Choosing the objective

Numerical aperture

Sets resolution and brightness

Immersion

Match the refractive index of the sample.

Working distance

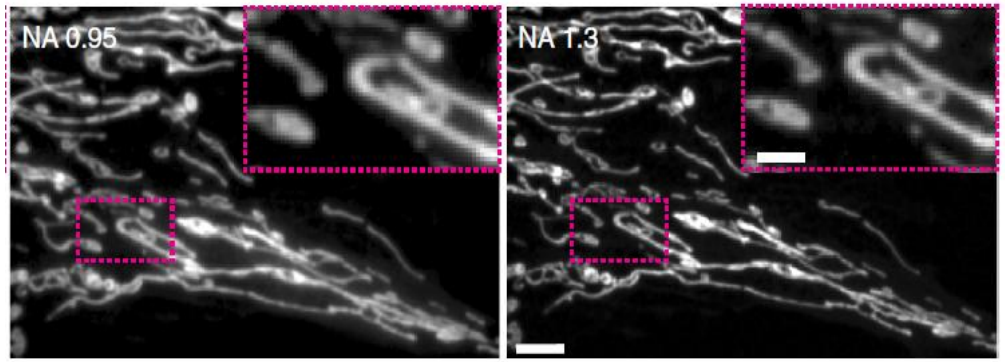
Thick samples need longer WD, often at lower NA.

Correction collar

Adjust for coverslip thickness (0.17 mm) and temperature.

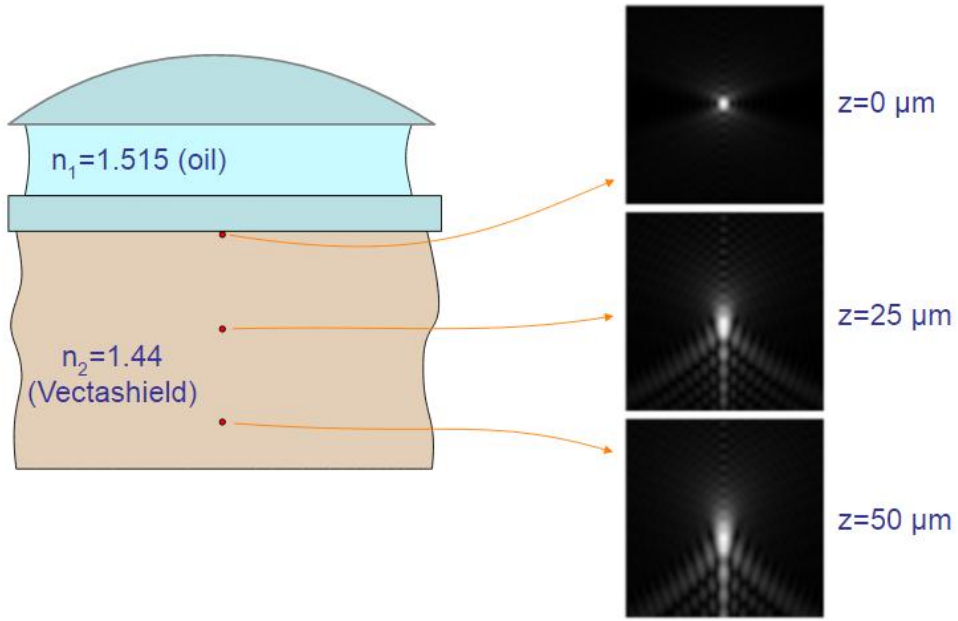
Choosing the objective

The NA, not the magnification determines the resolution



Montero Llopis P et al. (2021) Nat Methods 18: 1463–1476

Matching RI limits spherical abberations



Resolution: what can you resolve?

Lateral

$d \approx 0.61\,\lambda / NA$

λ = 520 nm, NA 1.4 → about 230 nm

Axial

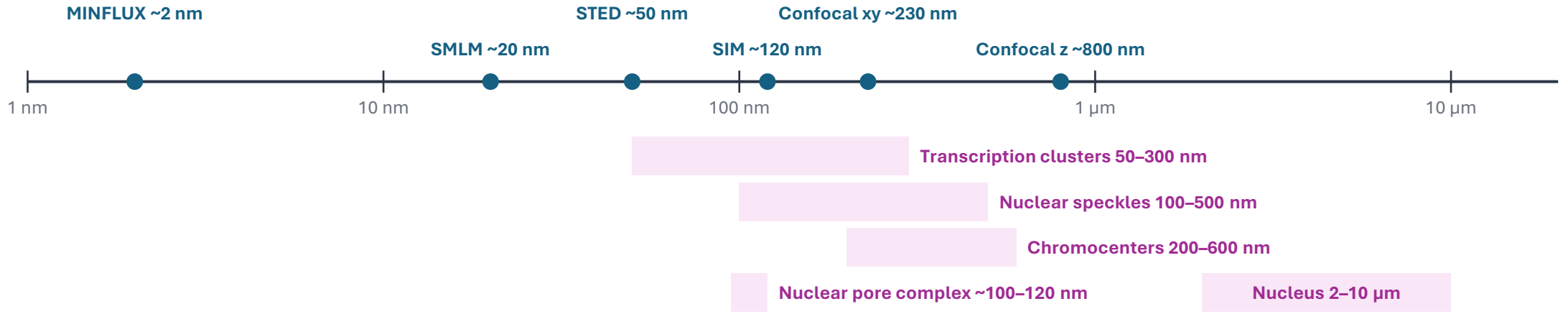
$d \approx 2\,\lambda\,n / NA^2$

Same objective, oil (n = 1.52) → about 800 nm

Axial resolution is 3–4 times worse than lateral.

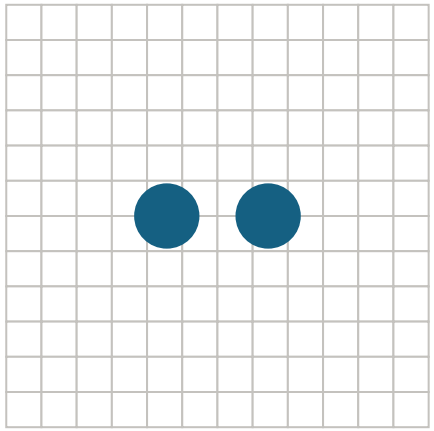
Plan 3D measurements (distances, volumes) accordingly.

Length scales in the plant nucleus (log scale)

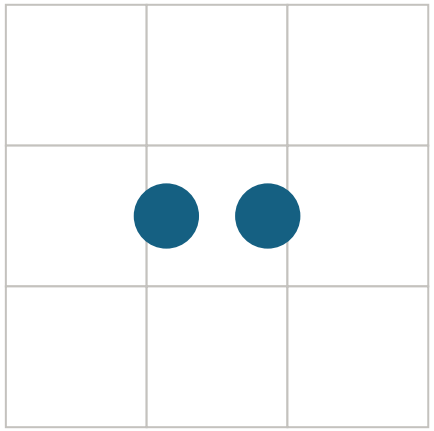


Jonkman J et al. (2020) Nat Protoc 15, Fig. 3 · plant NPC ~105 nm: Fiserova J et al. (2009) Plant J 59: 243–255 · MINFLUX: Balzarotti F et al. (2017) Science 355: 606–612

Sampling: Nyquist in x, y, z and time



Pixel ≤ ½ resolution: both objects resolved



Pixel too large: the two objects merge

Dimension	Resolution	Sample at
x, y (pixel)	~230 nm	≤ 100 nm
z (step)	~800 nm	≤ 350 nm
time (Δt)	event lasts ~60 s	≤ 30 s

In time, the “resolution” is how fast the structure changes: an object must not move more than about one resolution unit between frames.

Undersampling loses detail for good.

Oversampling costs time and light dose, but helps deconvolution.

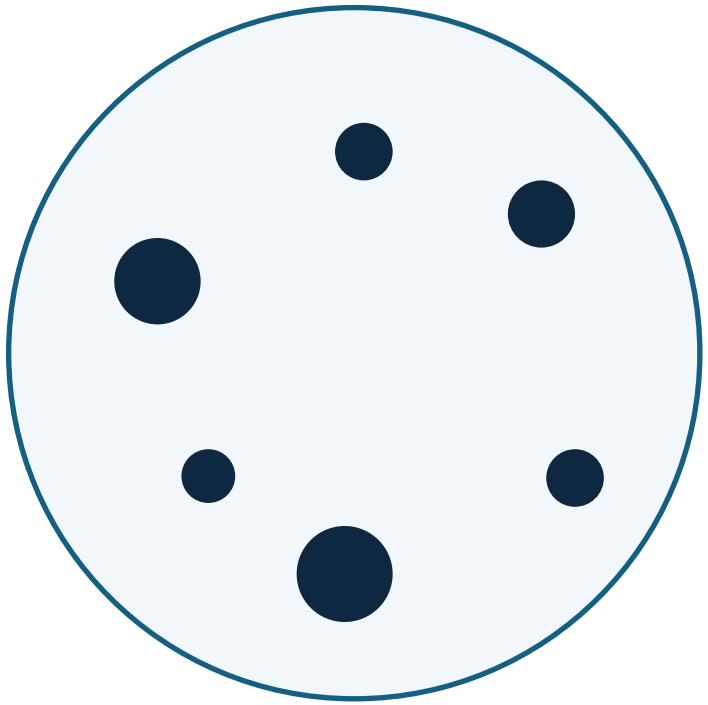
How to adjust voxel size?

xy: format + zoom

z: step size

Czymmek KJ et al. (2025) Plant Cell 37: koaf143, Fig. 6A–B (Nyquist vs undersampled) · Pawley JB (2006) Handbook of Biological Confocal Microscopy

Quiz 4 · Objective and sampling



Nucleus (2–10 µm) with chromocenters (200–600 nm)

You want to resolve the distance between chromocenters (200–600 nm diameter) and the nuclear periphery (Arabidopsis nucleus 2–10 µm).

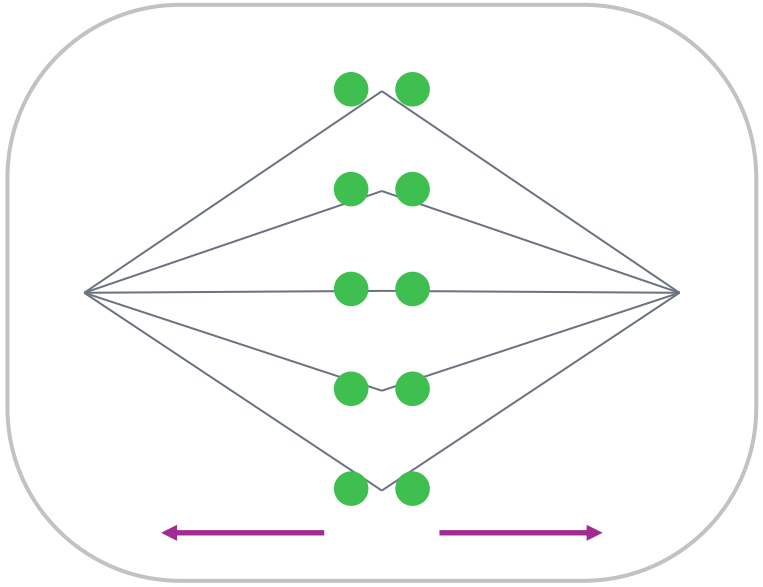
Which objective do you select?

- ❑ 40x NA 0.9
- ❑ 40x NA 1.3
- ❑ 63x NA 1.25
- ❑ 100x NA 1.1

Which z-step do you set for the z-stack?

- ❑ 600 nm
- ❑ 300 nm
- ❑ 1 µm
- ❑ 100 nm

Quiz 5 · Live imaging



Sister centromeres separating in anaphase

You want to measure the velocity of centromeres (CENH3-GFP) during mitosis in the root tip. Assume they move about 1 µm per minute.

Which imaging modality do you select?

- ❑ Point-scanning confocal, high averaging
- ❑ Spinning disk or light sheet
- ❑ STED
- ❑ Widefield, single plane

Which time interval (Δt) do you set?

- ❑ 2 min
- ❑ 30 s
- ❑ 10 s
- ❑ 1 s

Getting the signal right

Signal-to-noise

Enough photons for the measurement; averaging or **accumulation** helps.

Detector

PMT, hybrid (HyD, GaAsP) or camera; **photon counting** for linear quantification.

Gain and offset

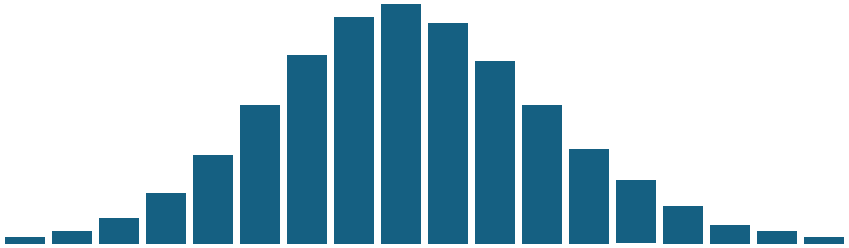
Full dynamic range: no saturated pixels, no clipped background. Check with a range LUT.

Prefer photon counting!

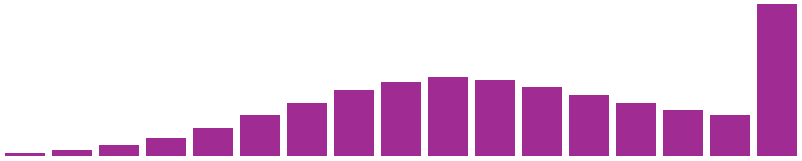
Bit depth

Acquire and save in 12 or 16 bit, in the raw file format.

Well exposed: full range, no clipping

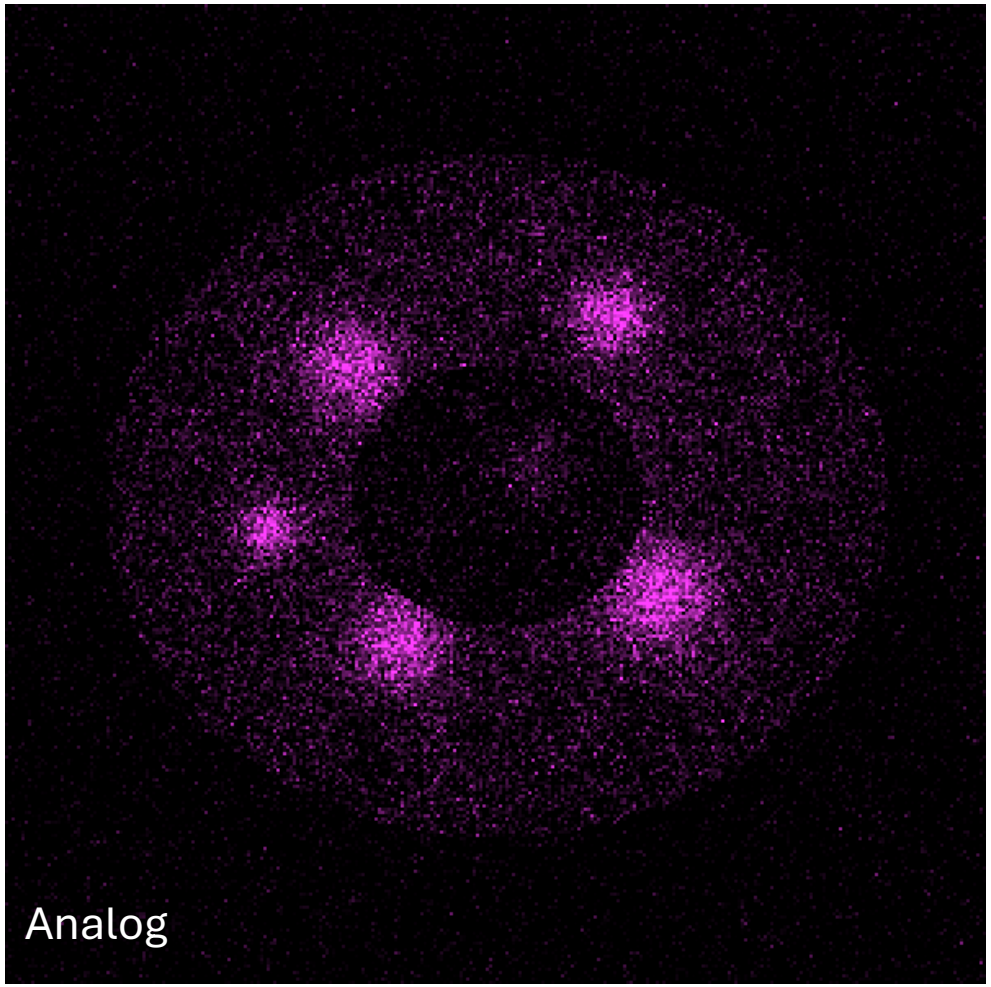


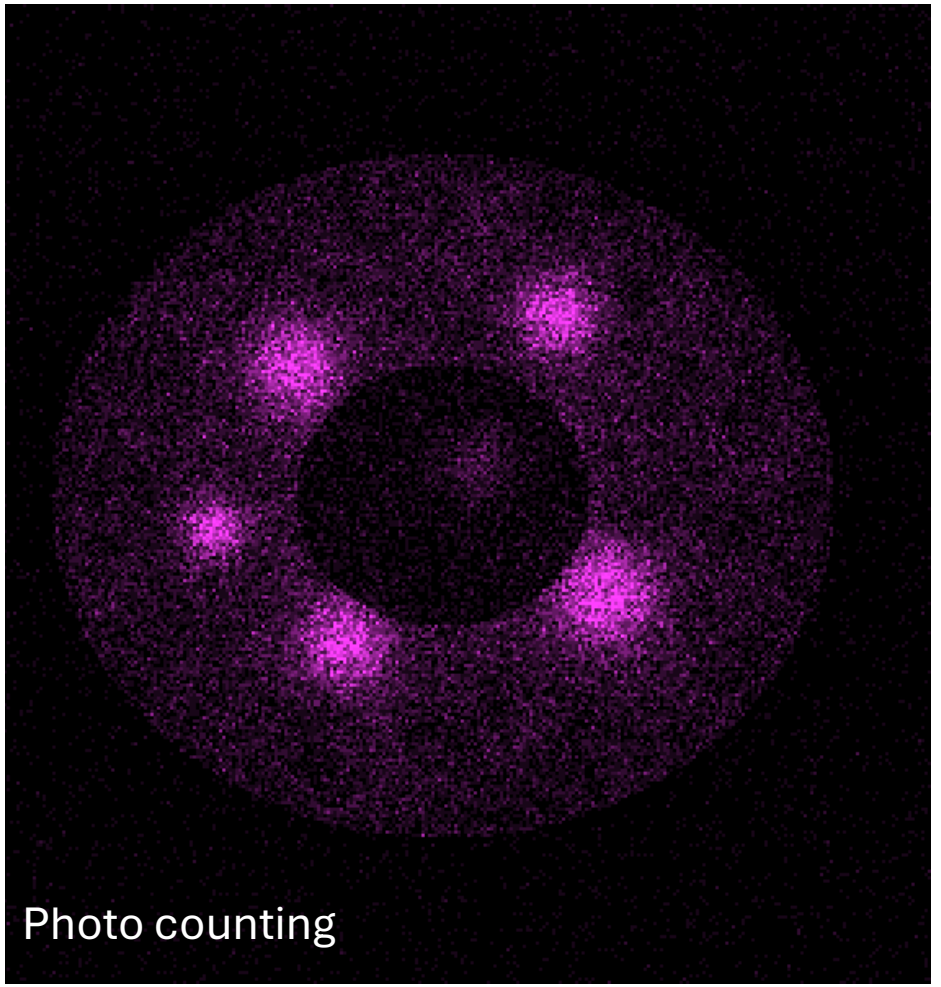
Saturated: spike at the maximum value



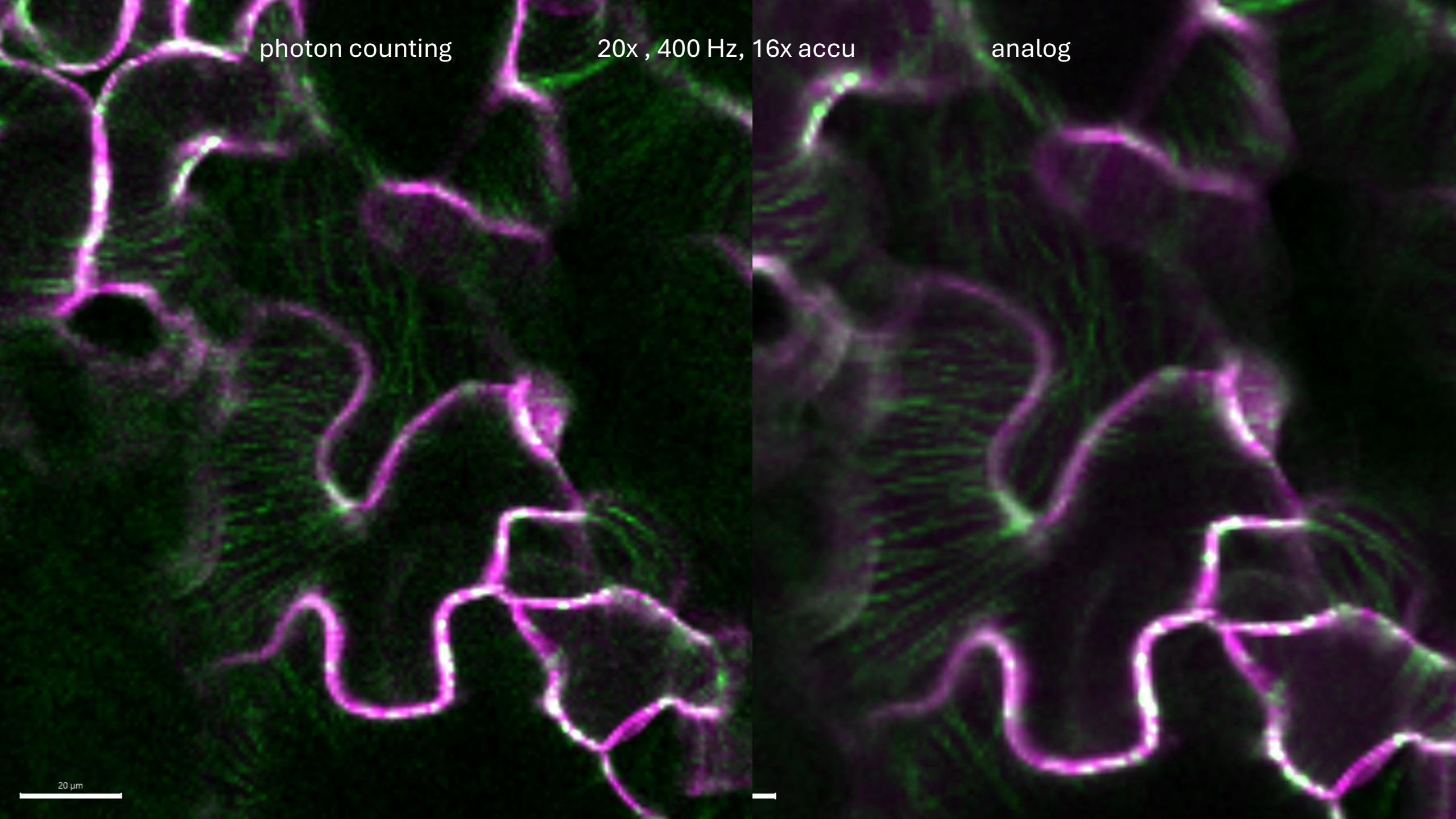
Intensity histograms (schematic): dark → bright

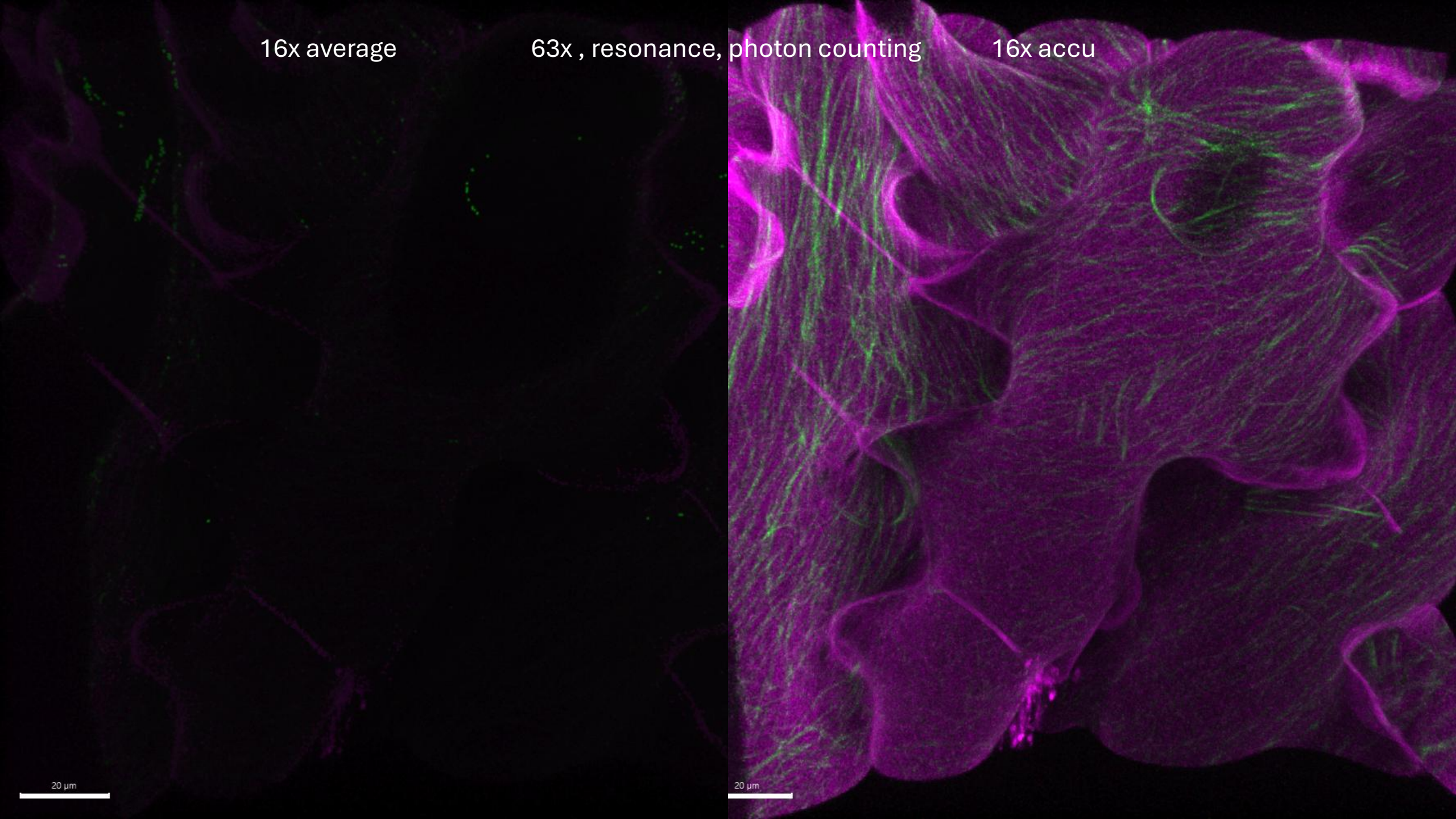
Photon counting beats analog averaging



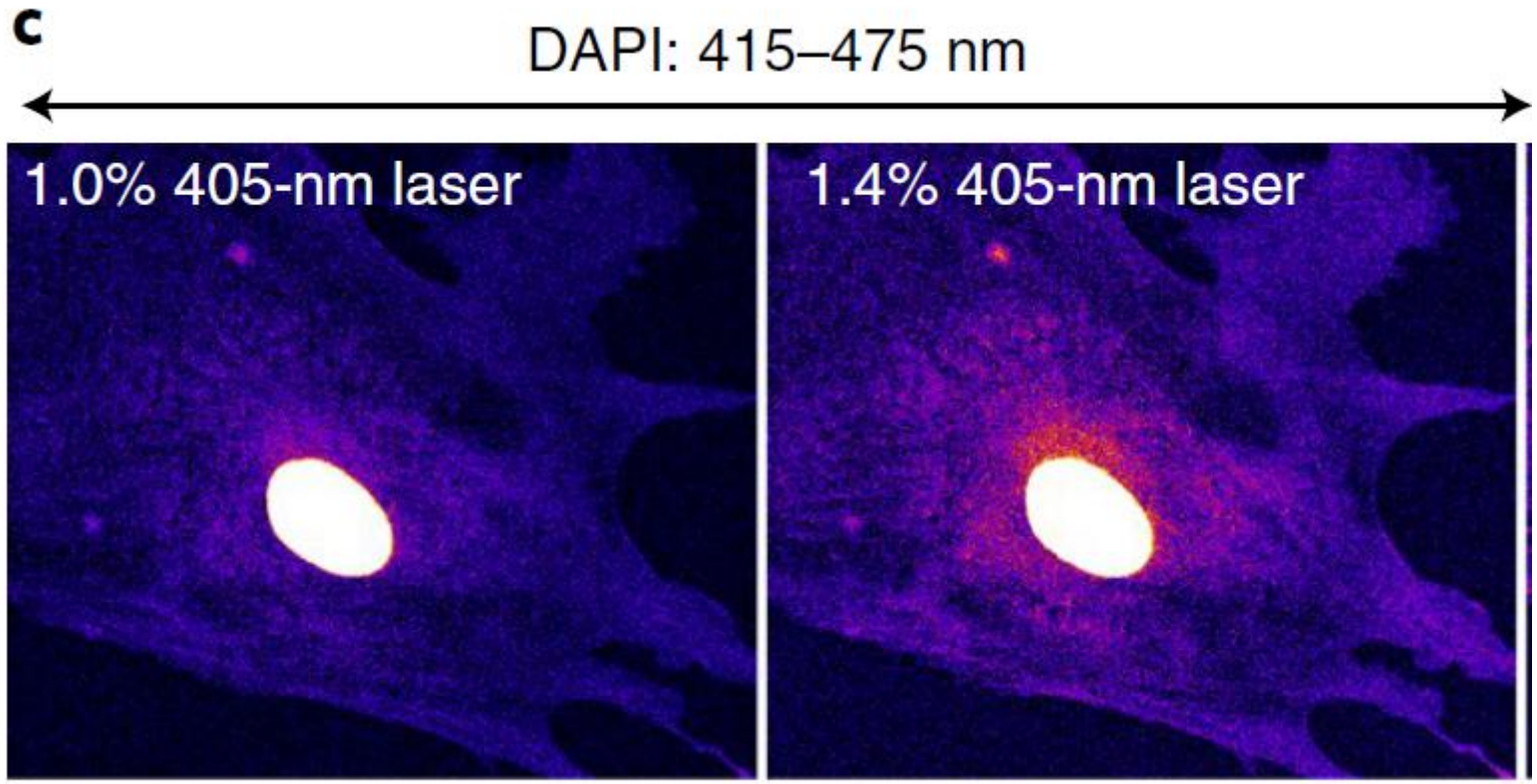


Same nucleus, same photons (simulated images)


photon counting
20x , 400 Hz, 16x accu
analog
20 µm

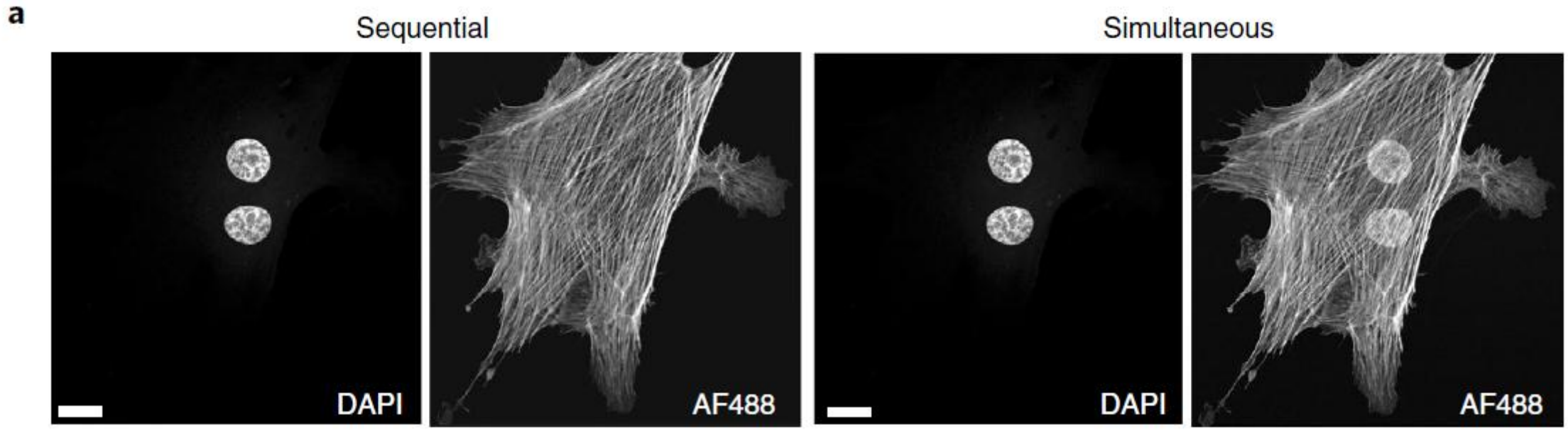

16x average
63x , resonance, photon counting
16x accu
20 µm
20 µm

Laser power: less is better



Montero Llopis P et al. (2021) Nat Methods 18: 1463–1476

Sequential scanning prevents cross-talk

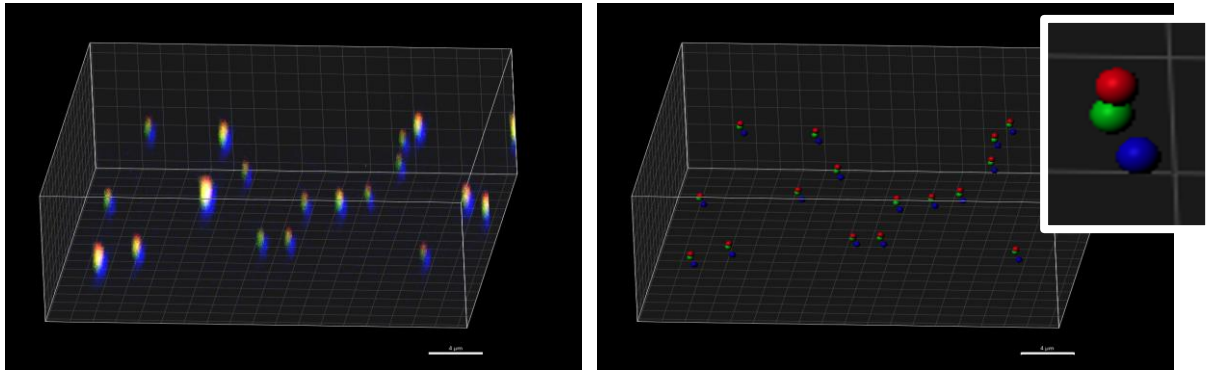


Montero Llopis P et al. (2021) Nat Methods 18: 1463–1476

Limit chromatic shifts – or correct

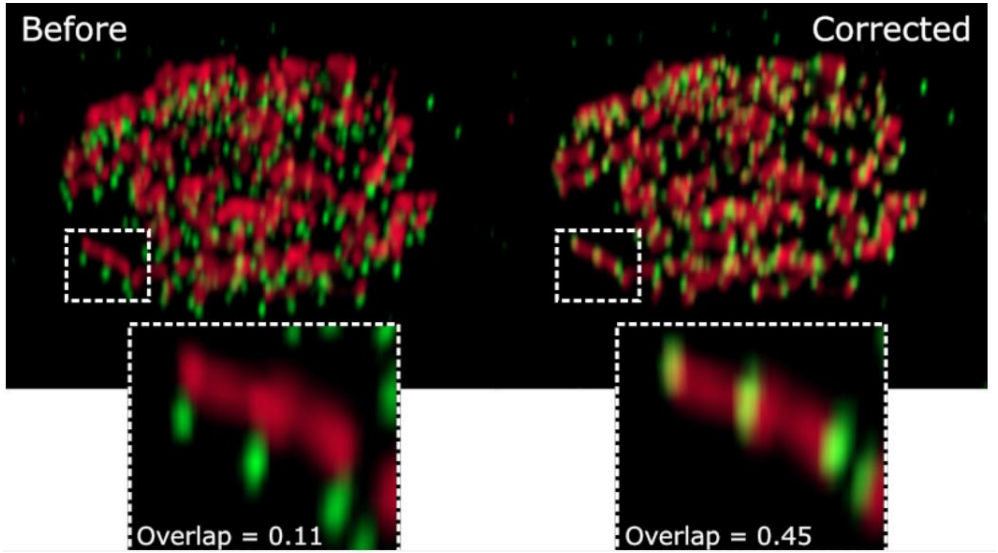
Chromatic shift

Channels offset in x, y and especially z. Measure with multicolour beads and correct.



Misregistration changes colocalisation, distances and intensity ratios.

-> Correction needed

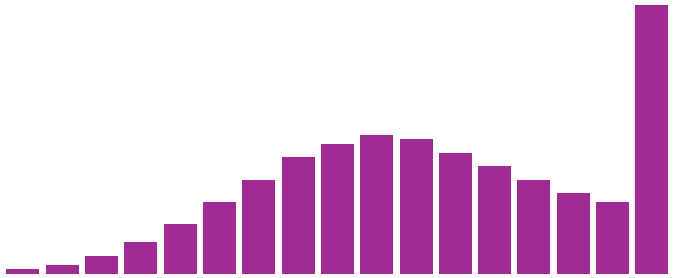


Red: Aurora kinase
Green: proteinx

https://svi.nl/Chromatic-Aberration-Corrector-Software

Quiz 6 · Acquisition settings

12-bit image: histogram



Spike at 4095

You compare chromatin compaction (DAPI intensity) between two genotypes. The histogram of the mutant shows a spike at 4095.

What do you do?

- ❑ Keep going, the image looks good
- ❑ Lower gain or laser power for this sample only
- ❑ Re-image both genotypes with one non-saturating setting
- ❑ Save as 8-bit JPEG to reduce the range

The red channel shows faint GFP signal. Most likely cause?

- ❑ Chromatic shift
- ❑ Crosstalk (bleed-through)
- ❑ Photobleaching
- ❑ Undersampling

Record the metadata from day one

Sample

- Genotype, age, growth conditions
- Treatment
- Labelling, fixation, clearing
- Mounting medium

Microscope

- Instrument, objective (mag, NA, immersion)
- Lasers, filters, detector
- Voxel size, z-step, Δt
- Gain, offset, power, averaging

17%

of 240 research articles passed a test for the minimal imaging information needed to repeat the experiment

Marqués G, Pengo T, Sanders MA (2020) eLife 9: e55133

In AGILE: each image gets an ID (DCx_001) and a row in the Images tab of the AGILE registry.
Stage 1: stored locally and securely; templates at msca-agile.eu.

Take-home: ten questions first

1. What exactly will I measure?
2. Which resolution and dimensions does that need?
3. Fixed or live, whole-mount or isolated nuclei?
4. Which label, and which controls?
5. Which fixation, clearing and mounting medium?
6. Which microscope and objective?
7. Which pixel size, z-step and Δt?
8. Which settings, frozen after the pilot?
9. How many biological replicates?
10. How will I name, register and store the images?

Fill the survey again – see what you've learned

https://www.surveymonkey.com/r/5JRVRB5



Hidden to fit 50 min: paper title only – cite on the next slides

Image acquisition – what you need to know (or at least be aware of)

https://doi.org/10.1038/s41592-021-01156-w

nature methods

FOCUS | PERSPECTIVE

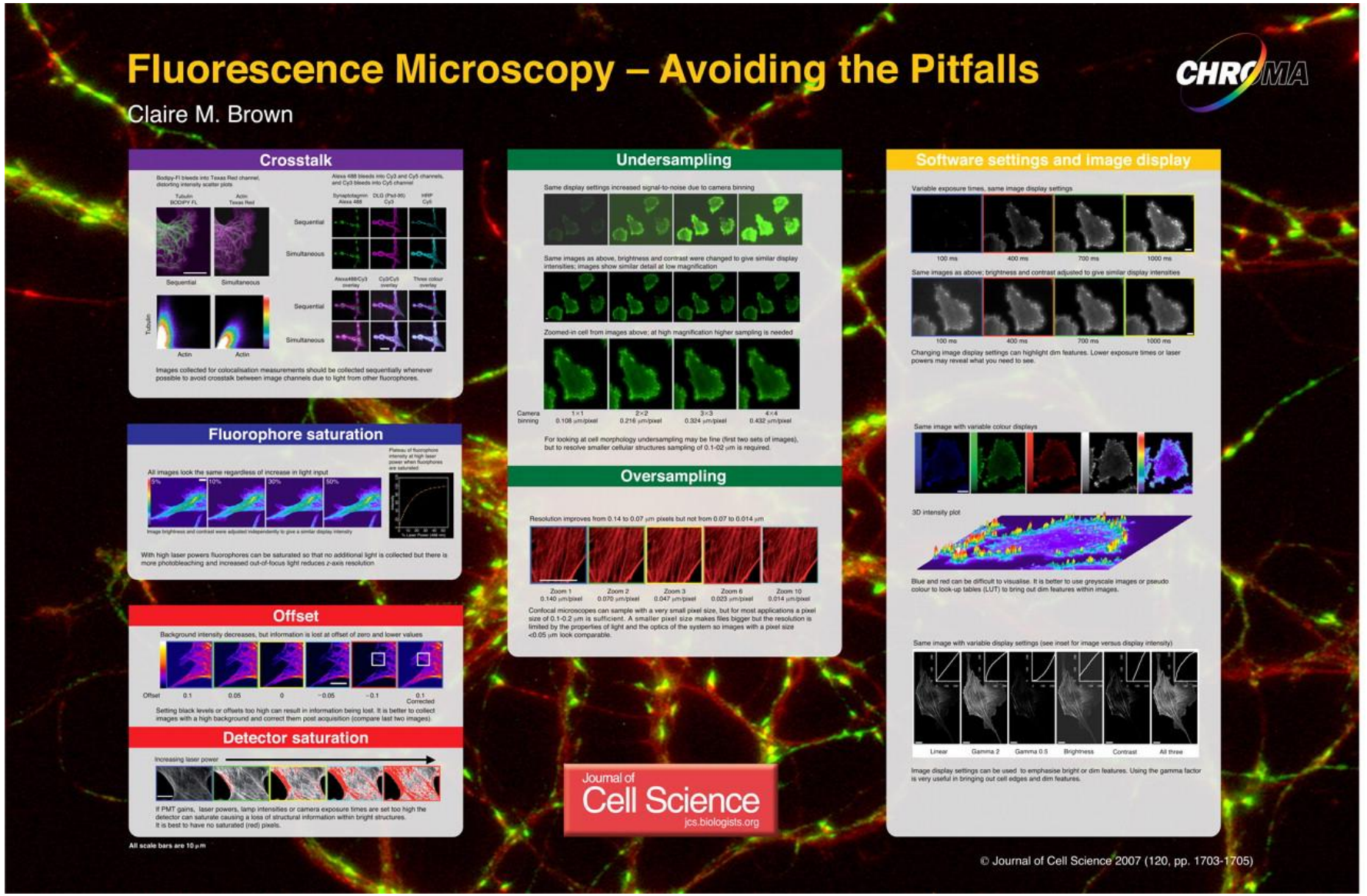
https://doi.org/10.1038/s41592-021-01156-w

Check for updates

Best practices and tools for reporting reproducible fluorescence microscopy methods

Paula Montero Llopis[1]✉, Rebecca A. Senft[2], Tim J. Ross-Elliott[1], Ryan Stephansky[1], Daniel P. Keeley[3], Preman Koshar[3], Guillermo Marqués[4], Ya-Sheng Gao[5], Benjamin R. Carlson[5], Thomas Pengo[6], Mark A. Sanders[4], Lisa A. Cameron[5] and Michelle S. Itano[3]

Although fluorescence microscopy is ubiquitous in biomedical research, microscopy methods reporting is inconsistent and perhaps undervalued. We emphasize the importance of appropriate microscopy methods reporting and seek to educate researchers about how microscopy metadata impact data interpretation. We provide comprehensive guidelines and resources to enable accurate reporting for the most common fluorescence light microscopy modalities. We aim to improve microscopy reporting, thus improving the quality, rigor and reproducibility of image-based science.



https://jcs.biologists.org/content/120/10/1703

Published Online: 3 January, 2006 | Supp Info: http://doi.org/10.1083/jcb.200507103
Downloaded from jcb.rupress.org on December 2, 2019

Seeing is believing? A beginners' guide to practical pitfalls in image acquisition

Imaging can be thought of as the most direct of experiments. You see something; you report what you see. If only things were truly this simple. Modern imaging technology has

word "confocal pinhole" or sigh as they watch their cells fade and die in front of their very eyes time and time again at the microscope. Take heart, beginners, if microscopes were actually so simple then many people (including myself) would suddenly be out of a job!

All data are subject to interpretation

Deliberate scientific fraud exists, but in modern microscopy a far greater number of errors are introduced in complete innocence. As an example of a common problem, take colocalization. Upstairs in the lab, a researcher collects a predominantly yellow merged image on a basic microscope, naturally interpreted as colocalization of green and red signals.

short article cannot be an exhaustive "how to" guide, I have also assembled a bibliography of a few highly recommended textbooks and microscopy web sites, which readers should consult for more extensive treatments of the critical issues introduced here.

Sample preparation

"Garbage in = garbage out" is the universal motto of all microscopists. A worrying tendency today is to assume that deconvolution software or confocal microscopes can somehow override the structural damage or suboptimal immunolabeling induced by poor sample preparation. The importance of appropriate fixation, permeabilization, and labeling methods for preserving cellular morphology or protein localization is well known to electron microscopists (Hayat, 2000),

Published Online: 29 June, 2009 | Supp Info: http://doi.org/10.1083/jcb.200903097
Downloaded from jcb.rupress.org on January 14, 2019

Accuracy and precision in quantitative fluorescence microscopy

Jennifer C. Waters

Harvard Medical School, Department of Cell Biology, Boston, MA 02115

The light microscope has long been used to document the localization of fluorescent molecules in cell biology research. With advances in digital cameras and the discovery and development of genetically encoded fluorophores, there has been a huge increase in the use of fluorescence microscopy to quantify spatial and temporal measurements of fluorescent molecules in biological specimens. Whether simply comparing the relative intensities of two fluorescent specimens, or using advanced techniques like Förster resonance energy transfer (FRET) or fluorescence recovery after photobleaching (FRAP), quantitation of fluorescence re-

During acquisition of the digital image, the photons that are detected at each pixel are converted to an intensity value that is correlated to, but not equal to, the number of detected photons (Pawley, 2006c). In fluorescence microscopy, the intensity value of a pixel is related to the number of fluorophores present at the corresponding area in the specimen. We can therefore use digital images to extract two types of information from fluorescence microscopy images: (1) spatial, which can be used to calculate such properties as distances, areas, and velocities; and (2) intensity, which can be used

accuracy is obvious. Precision is equally important in quantitative fluorescence microscopy because we are often forced to make only one measurement (for example, one time-point in a live-cell time-lapse experiment). In addition, we are usually measuring biological specimens that have some level of natural variability, so variance seen in measurements made on different cells will be caused by both biological variability and that which is introduced when making the measurement. To use a fluorescence microscope and digital detector to quantitate spatial and intensity informa-

NUCLEUS
2019, VOL. 10, NO. 1, 181–212
https://doi.org/10.1080/19491034.2019.1644592

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REVIEW

OPEN ACCESS Check for updates

Probing the 3D architecture of the plant nucleus with microscopy approaches: challenges and solutions

Tao Dumur[a], Susan Duncan [b], Katja Graumann [c], Sophie Desset [d], Ricardo S Randall [e], Ortrun Mittelsten Scheid [a], Hank W Bass [f], Dimiter Prodanov [g], Christophe Tatout [d], and Célia Baroux [e]

[a]Gregor Mendel Institute (GMI) of Molecular Plant Biology, Austrian Academy of Sciences, Vienna Biocenter (VBC), Vienna, Austria; [b]Norwich Research Park, Earlham Institute, Norwich, UK; [c]Department of Biological and Medical Sciences, Oxford Brookes University, Oxford, UK; [d]GReD, Université Clermont Auvergne, CNRS, INSERM, Clermont–Ferrand, France; [e]Department of Plant and Microbial Biology, Zürich-Basel Plant Science Center, University of Zürich, Zürich, Switzerland; [f]Department of Biological Science, Florida State University, Tallahassee, FL, USA; [g]Environment, Health and Safety, Neuroscience Research Flanders, Leuven, Belgium

ABSTRACT

The eukaryotic cell nucleus is a central organelle whose architecture determines genome function at multiple levels. Deciphering nuclear organizing principles influencing cellular responses and identity is a timely challenge. Despite many similarities between plant and animal nuclei, plant nuclei present intriguing specificities. Complementary to molecular and biochemical approaches, 3D microscopy is indispensable for resolving nuclear architecture. However, novel solutions are required for capturing cell-specific, sub-nuclear and dynamic processes. We provide a pointer for utilising high-to-super-resolution microscopy and image processing to probe plant nuclear architecture in 3D at the best possible spatial and temporal resolution and at quantitative and cell-specific levels. High-end imaging and image-processing solutions allow the community now to transcend conventional practices and benefit from continuously improving approaches. These promise to deliver a comprehensive, 3D view of plant nuclear architecture and to capture spatial dynamics of the nuclear compartment in relation to cellular states and responses.

Abbreviations: 3D and 4D: Three and Four dimensional; AI: Artificial Intelligence; ant: antipodal nuclei (ant); CLSM: Confocal Laser Scanning Microscopy; CTs: Chromosome Territories; DL: Deep Learning; DLIm: Dynamic Live Imaging; ecn: egg nucleus; FACS: Fluorescence-Activated Cell Sorting; FISH: Fluorescent In Situ Hybridization; FP: Fluorescent Proteins (GFP, RFP, CFP, YFP, mCherry); FRAP: Fluorescence Recovery After Photobleaching; GPU: Graphics Processing Unit; KEEs: KNOT Engaged Elements; INTACT: Isolation of Nuclei TAgged in specific Cell Types; LADs: Lamin-Associated Domains; ML: Machine Learning; NA: Numerical Aperture; NADs: Nucleolar Associated Domains; PALM: Photo-Activated Localization Microscopy; Pixel: Picture element; pn: polar nuclei; PSF: Point Spread Function; RHF: Relative Heterochromatin Fraction; SIM: Structured Illumination Microscopy; SLIm: Static Live Imaging; SMC: Spore Mother Cell; SNR: Signal to Noise Ratio; SRM: Super-Resolution Microscopy; STED: STimulated Emission Depletion; STORM: STochastic Optical Reconstruction Microscopy; syn: synergid nuclei; TADs: Topologically Associating Domains; Voxel: Volumetric pixel

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Three-dimensional microscopy imaging; image processing and analysis; tracking; plant nucleus; live imaging; whole-mount; nuclear organization

in effective resolution [38]. This particularly affects the sub-micrometric scale and thereby impedes resolution of closely-related and complex subnuclear structures. An alternative to imaging in fresh tissue consists in applying optical clearance methods on fixed tissue preparations. Imaging isolated nuclei can also circumvent these problems. The benefits and limitations of each approach are discussed in the following sections.

Checklist for a good imaging design

At the start of each imaging experiment, it is important to careful evaluate (i) the choice of fluorophore, (ii) sample preparation, (iii) the optical/microscopy setup and (iv) the image acquisition parameters. We refer to this collectively as *imaging design*. A good imaging design allows to resolve fine-scale details in the plant nucleus, as shown by several illustrations in this review. Conventional microscopy, accessible to the majority of plant cell biologists, allows to resolve, for instance, large chromatin domains; genomic regions; protein complexes forming speckles or located in nuclear bodies in the 200 nm range or below and detected by immunostaining, *in situ* hybridization or tagged fluorescent proteins.

Expedited routine practices tend towards a simplified imaging design at the detriment of signal quality and signal contrast, hence poor spatial resolution. General considerations of image resolution [38,39], and more generally of microscopy imaging of plant tissues, are published elsewhere. This reading material is strongly recommended prior to starting an experiment [37,40,41], to avoid classical pitfalls and to prepare well for image analysis and quantification [39,42,43]. More directly related to imaging the plant cell nucleus, we recently reviewed practical considerations for plant chromatin imaging, including a comparison of imaging instruments and their resolution [44]. The motivation for a good imaging design is to achieve the best-possible resolution within a constrained imaging procedure, including sample viability and accessibly, available fluorophores and imaging systems.

be made wherever possible to avoid low quantum efficiency and poor photostability (for live reporters, see reference [45] for a comparison of several FPs). In addition, specific fluorophores are required for super-resolution imaging instruments [46,47]. As mentioned, multiple fluorophores should offer well-separated spectral properties among each other and potential background fluorescence [34].

(ii) *sample preparation* will greatly influence the Signal-to-Noise Ratio (SNR, which measures the amount of informative signal relative to undesired, background noise) and thus merits serious consideration. In addition to endogenous fluorescence and the scattering properties detailed above, the mounting of thick samples or the presence of air bubbles trapped in the preparation will reduce excitation efficiency and signal collection considerably due to low light penetration and light scattering at each interface. Mounting and lens immersion media should be matched with identical (or as similar as possible) refractive indices [35,48].

(iii) *microscopy setup*. While appearing a basic recommendation, it is important to select an objective lens with suitable Numerical Aperture (NA), immersion medium, working distance and imaging depth and corrections for chromatic and axial aberrations.

(iv) *image acquisition*. We do not discuss here manufacturer-specific recommendations, but recall that image resolution and the possibility to perform downstream quantification depend on signal contrast, signal quality and intensity range [38,39]. Hence resolving fine-scale nuclear structures requires a well-thought setup for signal acquisition. A conservative practice includes avoiding near-saturation intensity levels and averaging modes as these

Imaging design checklist: full version

Design

- ❑ Biological question → measurable feature
- ❑ Dimensions: 2D, 3D, 4D
- ❑ Biological replicates and n per condition
- ❑ Positive, negative and unlabelled controls
- ❑ Blinding and randomised imaging order
- ❑ Pilot run to fix settings and n

Sample preparation

- ❑ Fixed or live; whole-mount or isolated nuclei
- ❑ Cell-type targeting (reporter, FACS, INTACT)
- ❑ Label and fluorophore vs lasers, filters, autofluorescence
- ❑ Fixation and permeabilisation; known artefacts
- ❑ Clearing; mounting medium and RI
- ❑ Labelling controls: unlabelled, single, secondary only, biological negative

Microscope and acquisition

- ❑ Modality: resolution, speed, depth, light dose
- ❑ Objective: NA, immersion, WD, correction collar
- ❑ Nyquist: pixel size, z-step, Δt
- ❑ Detector, gain, offset, bit depth; no saturation
- ❑ Sequential channels; chromatic shift measured
- ❑ Lowest light dose; bleaching checked; settings frozen

Documentation

- ❑ Image ID and registry row (AGILE)
- ❑ Sample metadata: genotype, age, treatment, labelling, mounting
- ❑ Microscope metadata: objective, lasers, filters, detector, voxel size
- ❑ Raw files kept; processing recorded
- ❑ Controls stored with the data