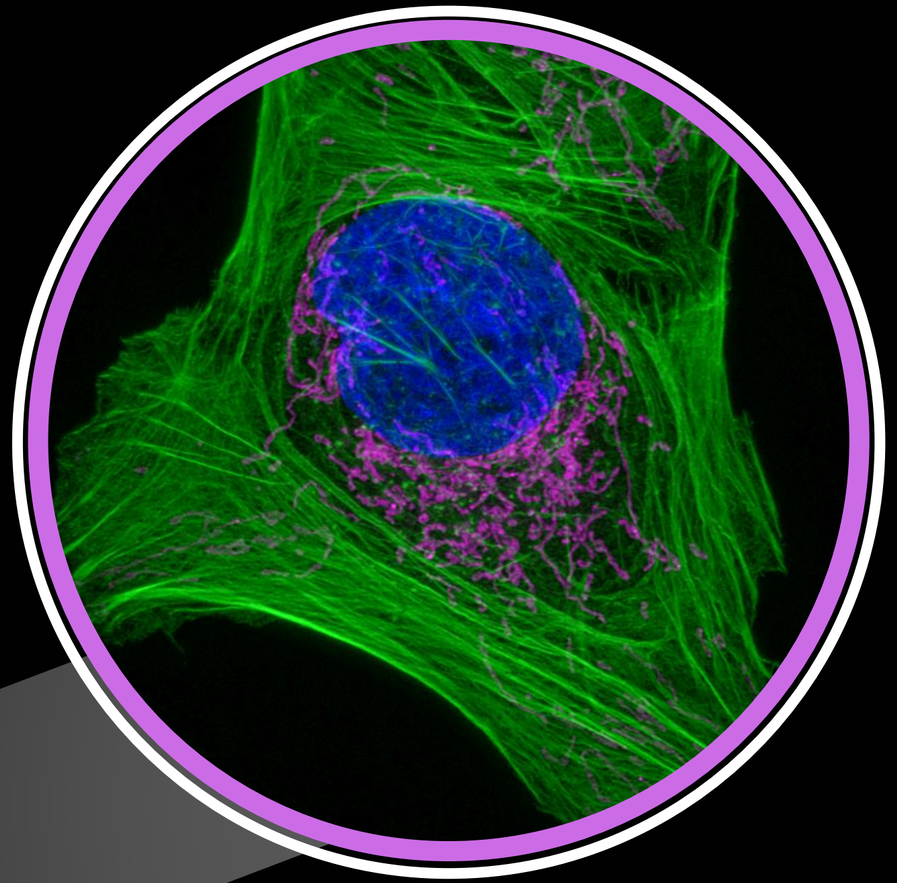
Sample preparation in PRINCIPLES



Ivan Novotny, LMCF IMG, Prague, CZ

IMG

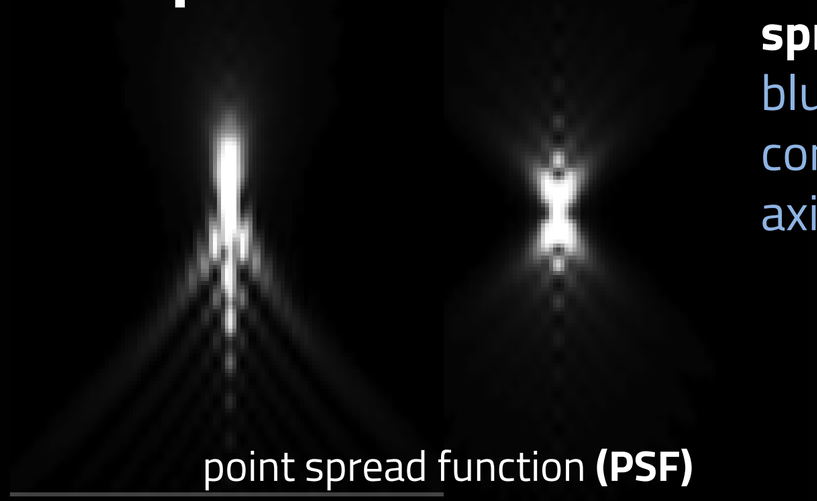
Rule 01

GARBAGE-IN
GARBAGE-OUT

Garbage

In microscopy, 'garbage' refers to an insufficient sample, primarily characterized by pronounced spherical aberration.

Spherical aberration in light microscopy **distorts the point spread function (PSF)** by elongating or blurring it, reducing image resolution and contrast, particularly along the optical axis.

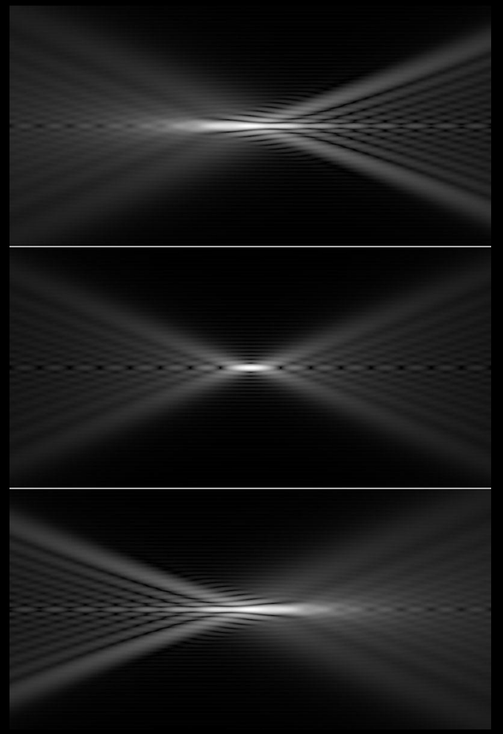


point spread function **(PSF)**

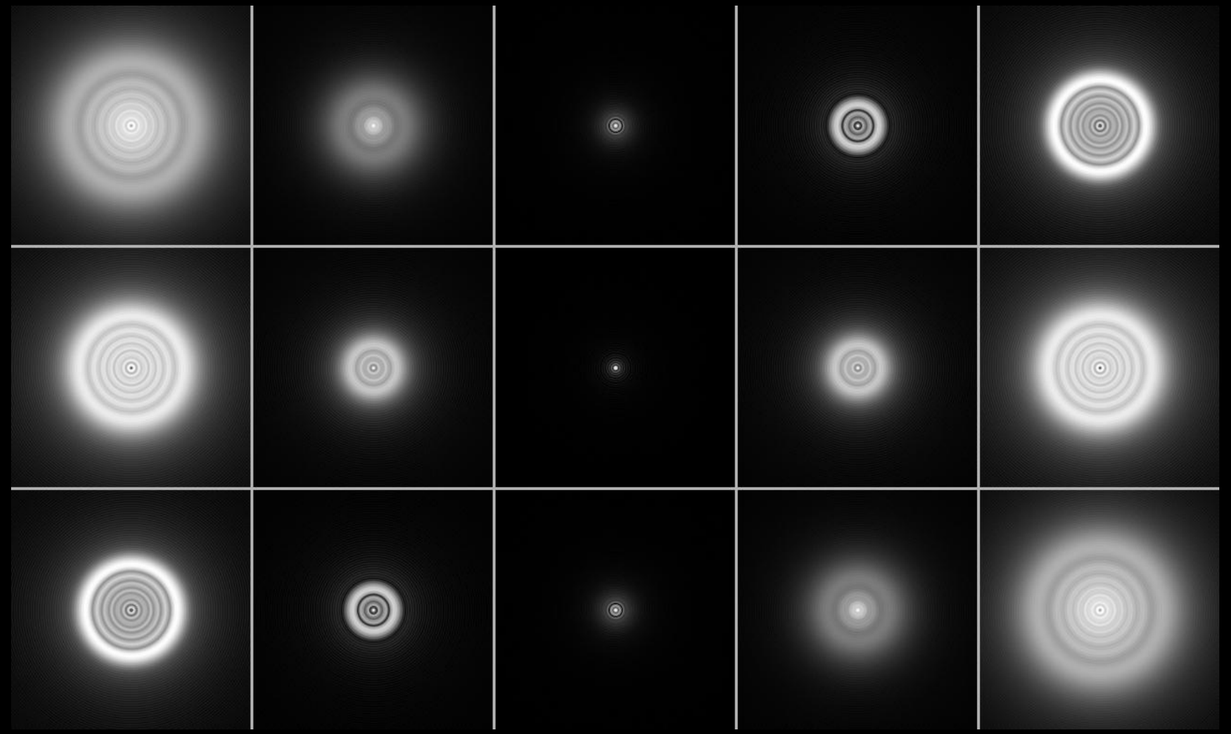
Spherical aberration

in light microscopy **distorts the point spread function (PSF)** by elongating or blurring it, reducing image resolution and contrast, particularly along the optical axis.

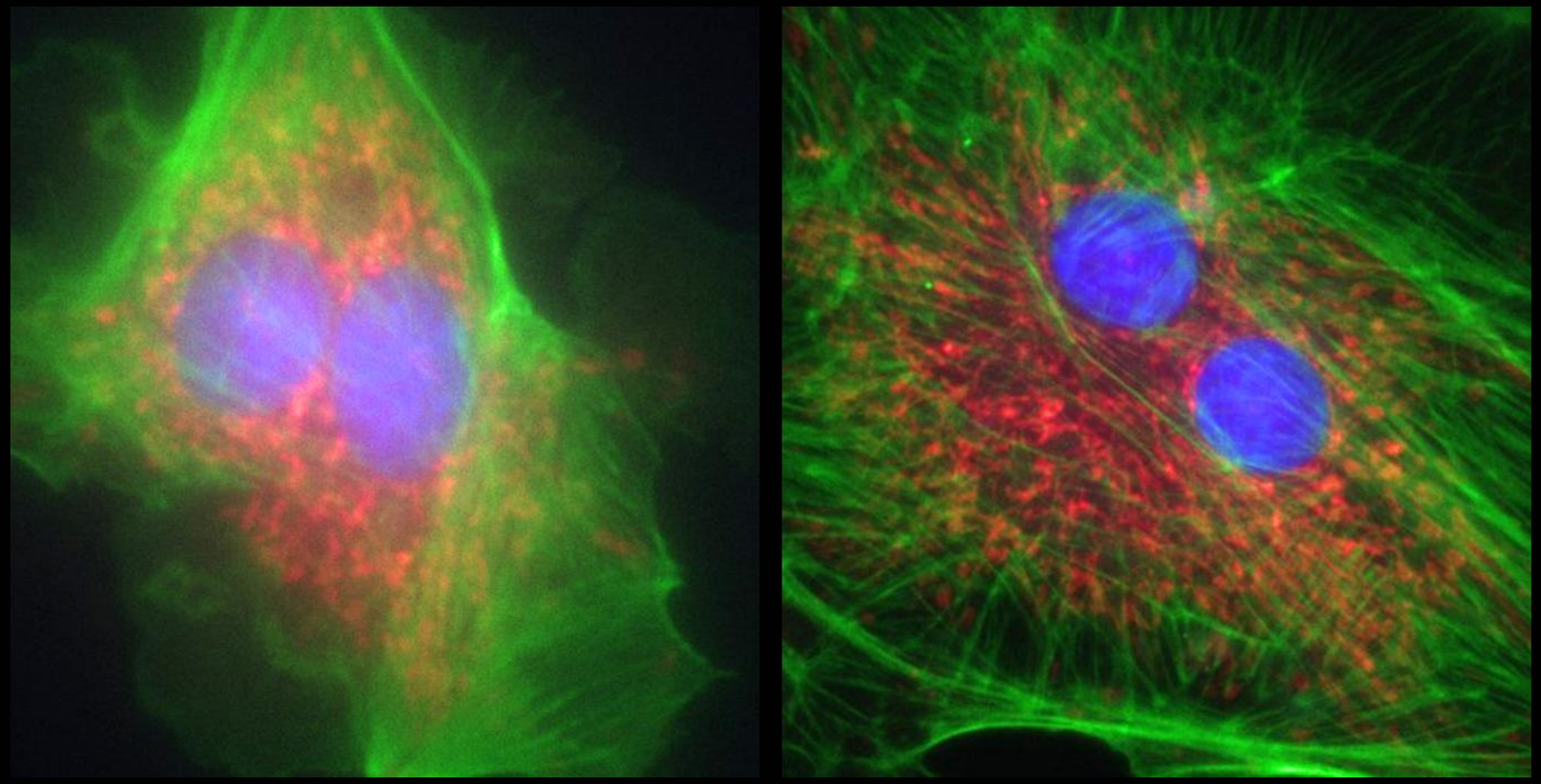
Axial section of PSF



Lateral section of PSF



The effect on an image

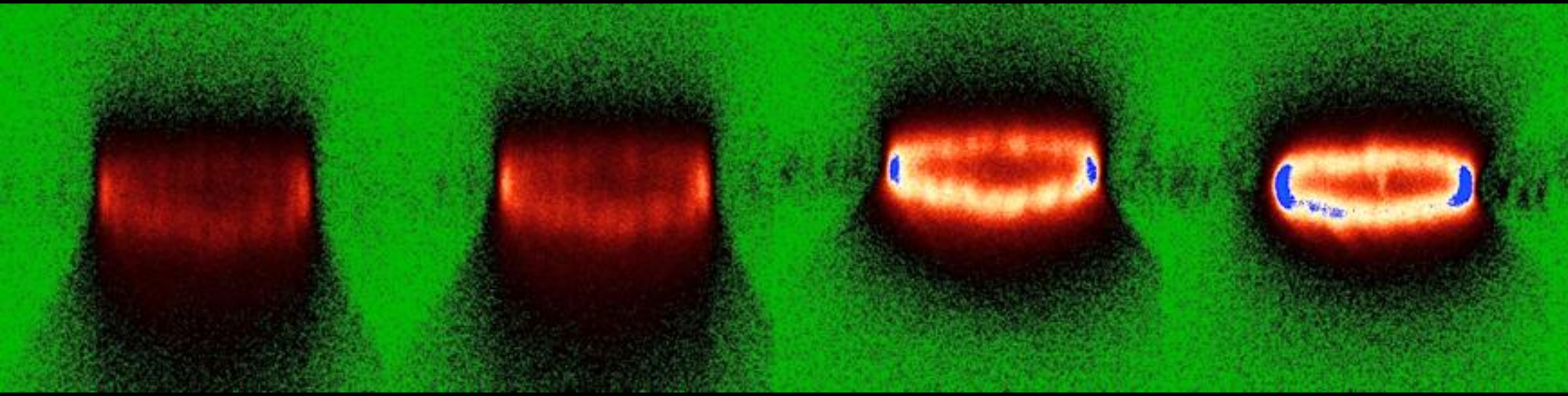


Images acquired using identical setup on WF fluorescent microscope

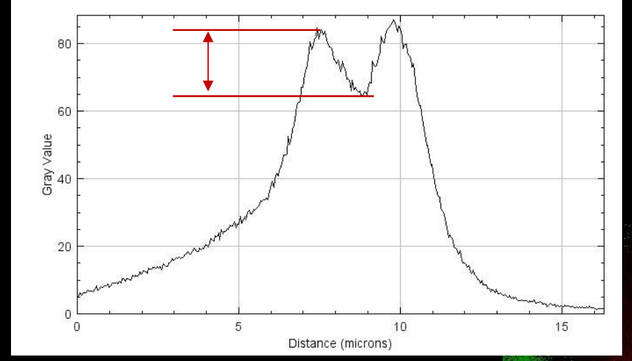
The effect of spherical aberration on an image causes **blurring, loss of signal intensity, and distortion of shapes, compromising image clarity and accuracy**.

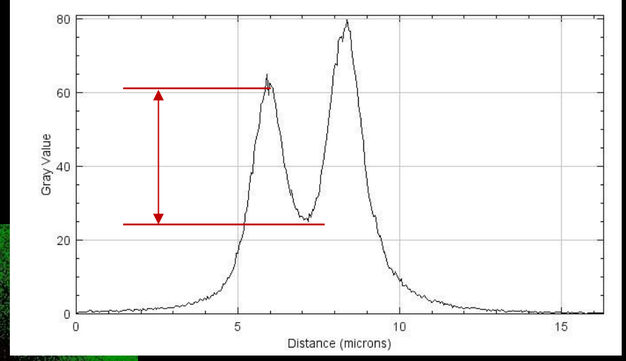
The same cell

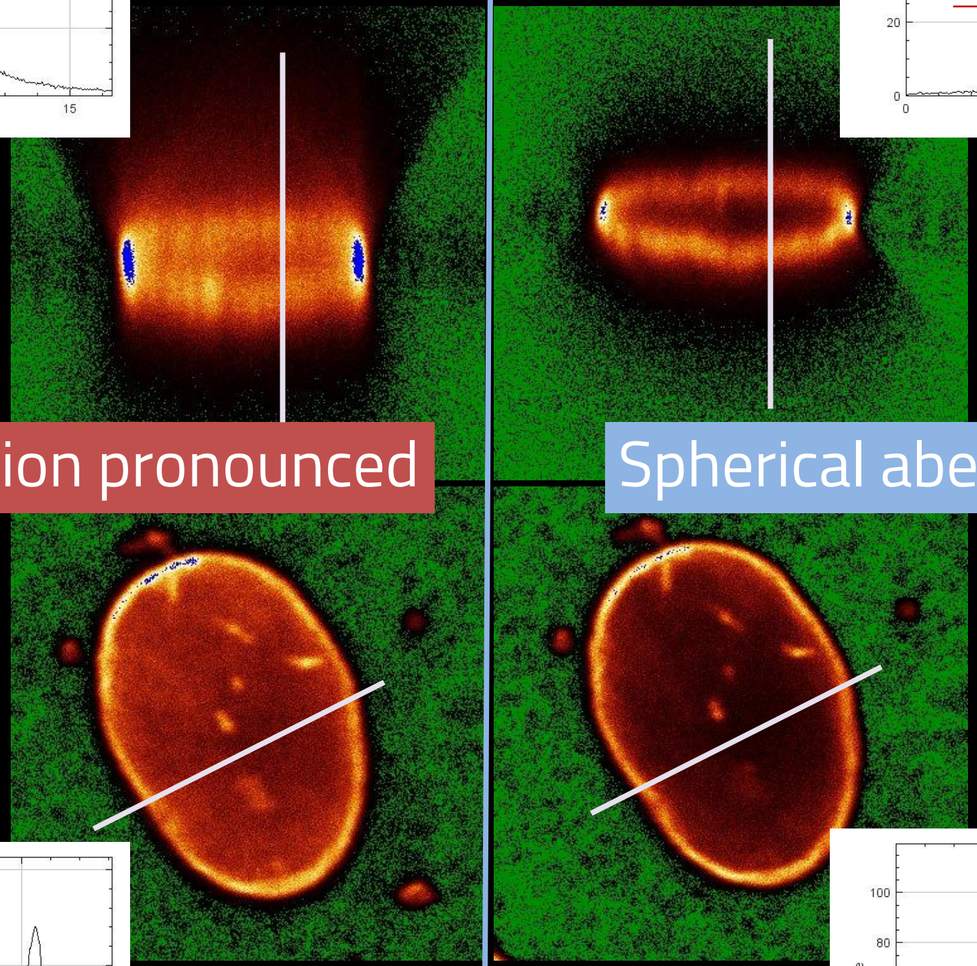
..different aberration.



The confocal microscope image, acquired with identical conditions. Only difference is the pronounced spherical aberration.

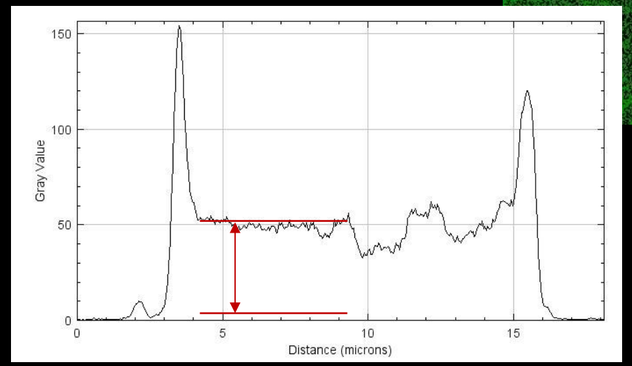


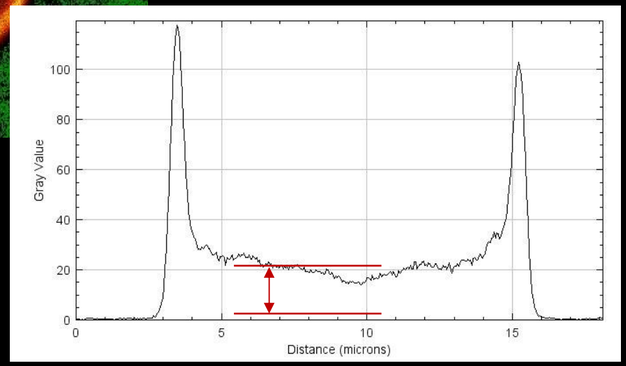




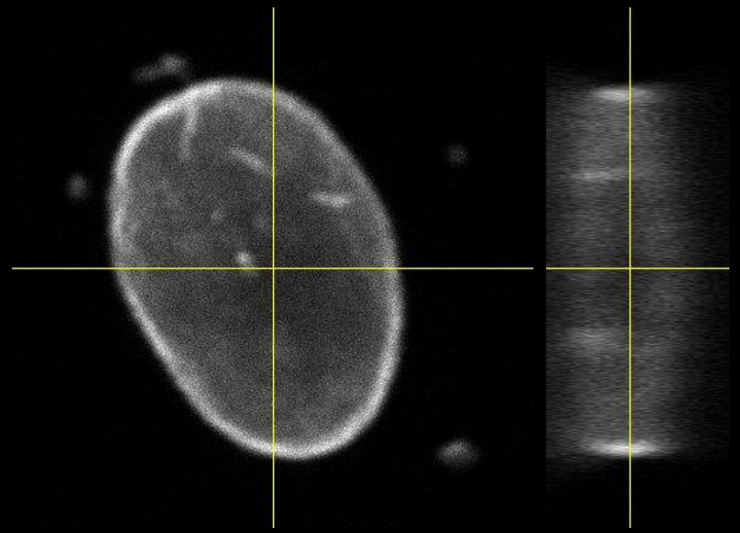
Spherical aberration pronounced

Spherical aberration corrected

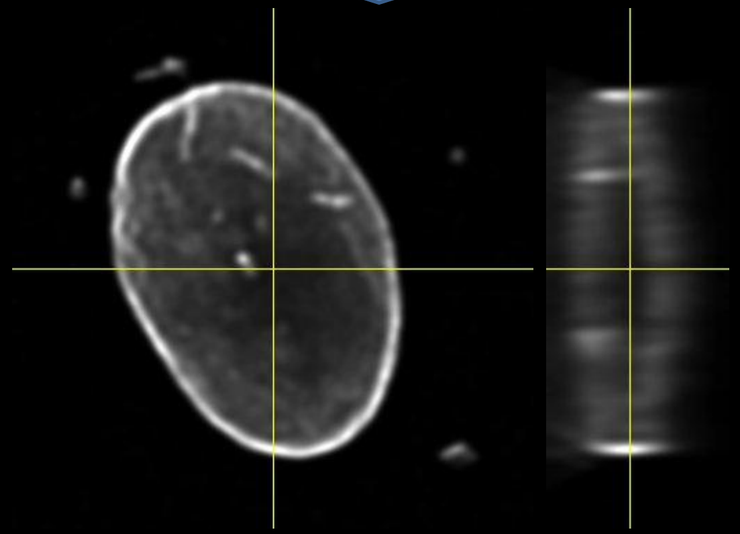




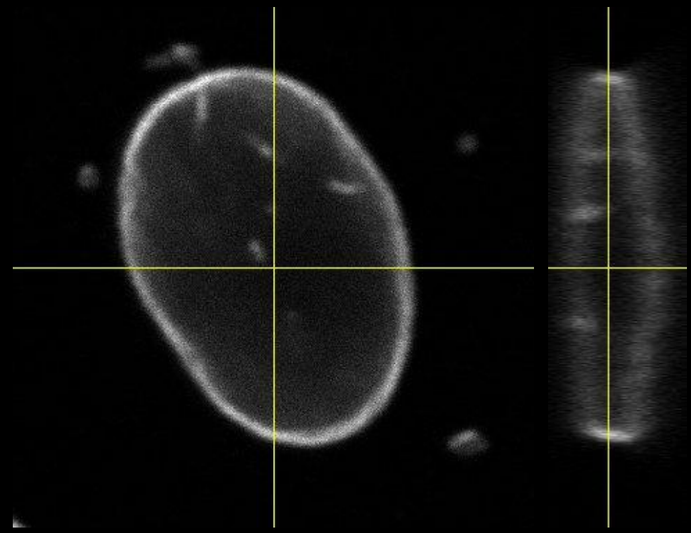
Spherical aberration pronounced



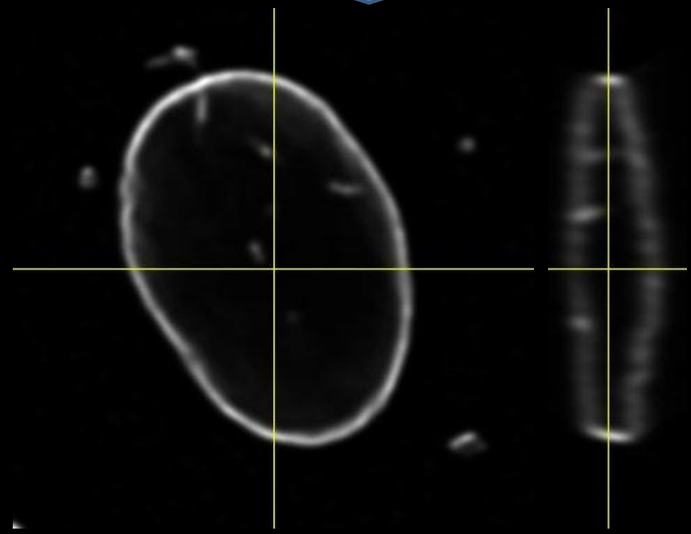
decon



Spherical aberration corrected

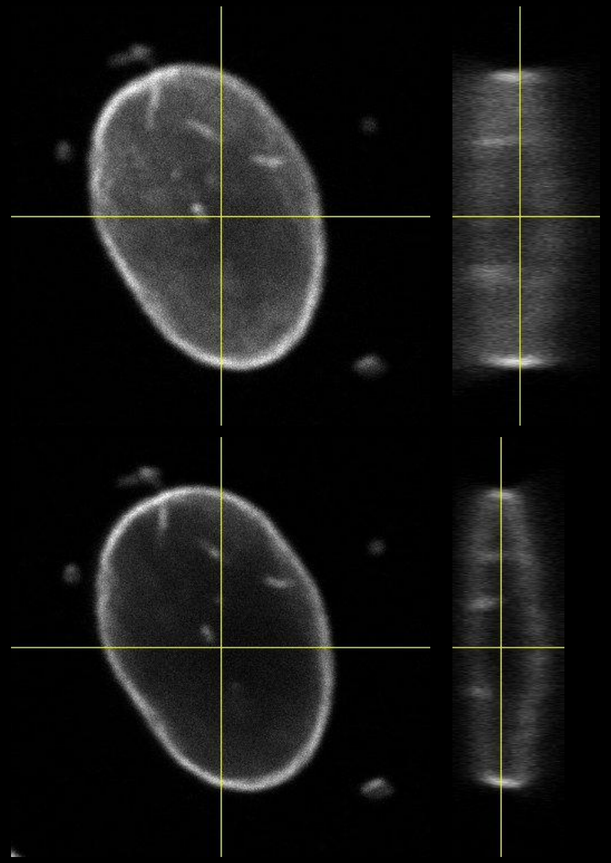


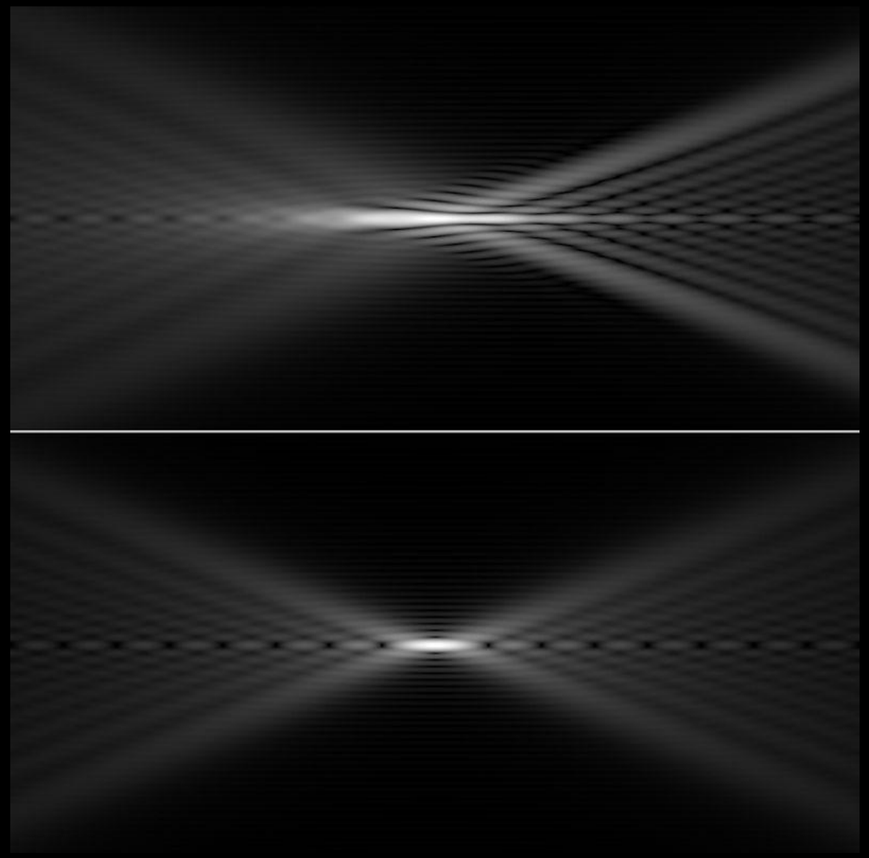
decon



Spherical aberration ...sorry, again

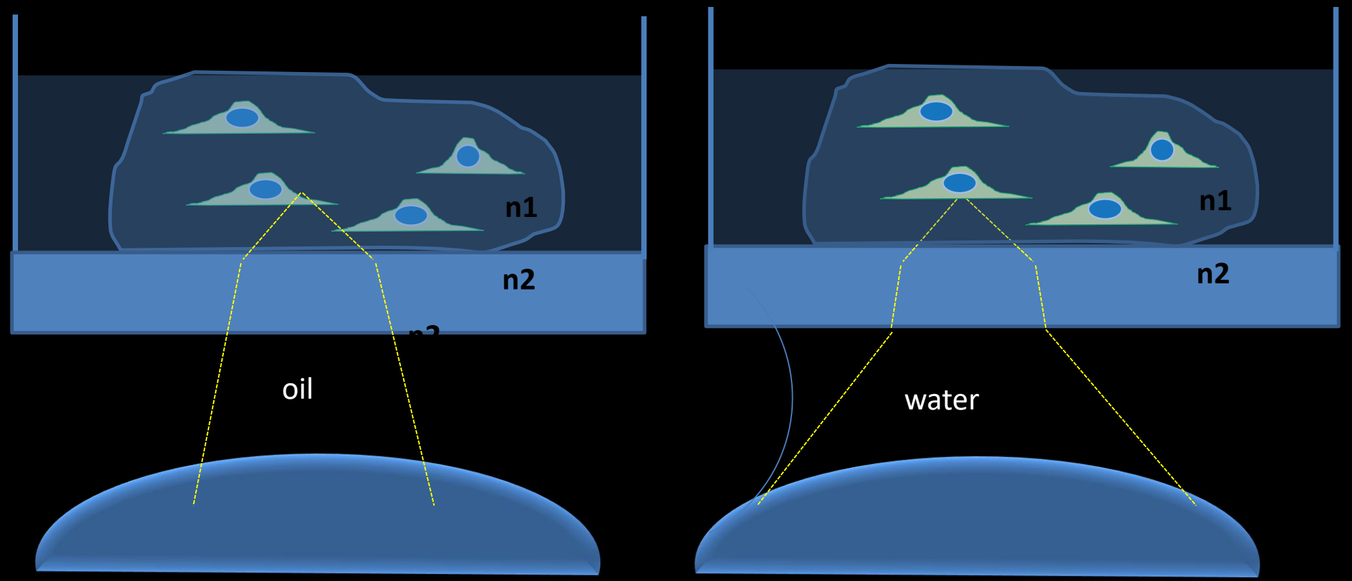
in light microscopy **distorts the point spread function (PSF)** by elongating or blurring it, reducing image resolution and contrast, particularly along the optical axis.





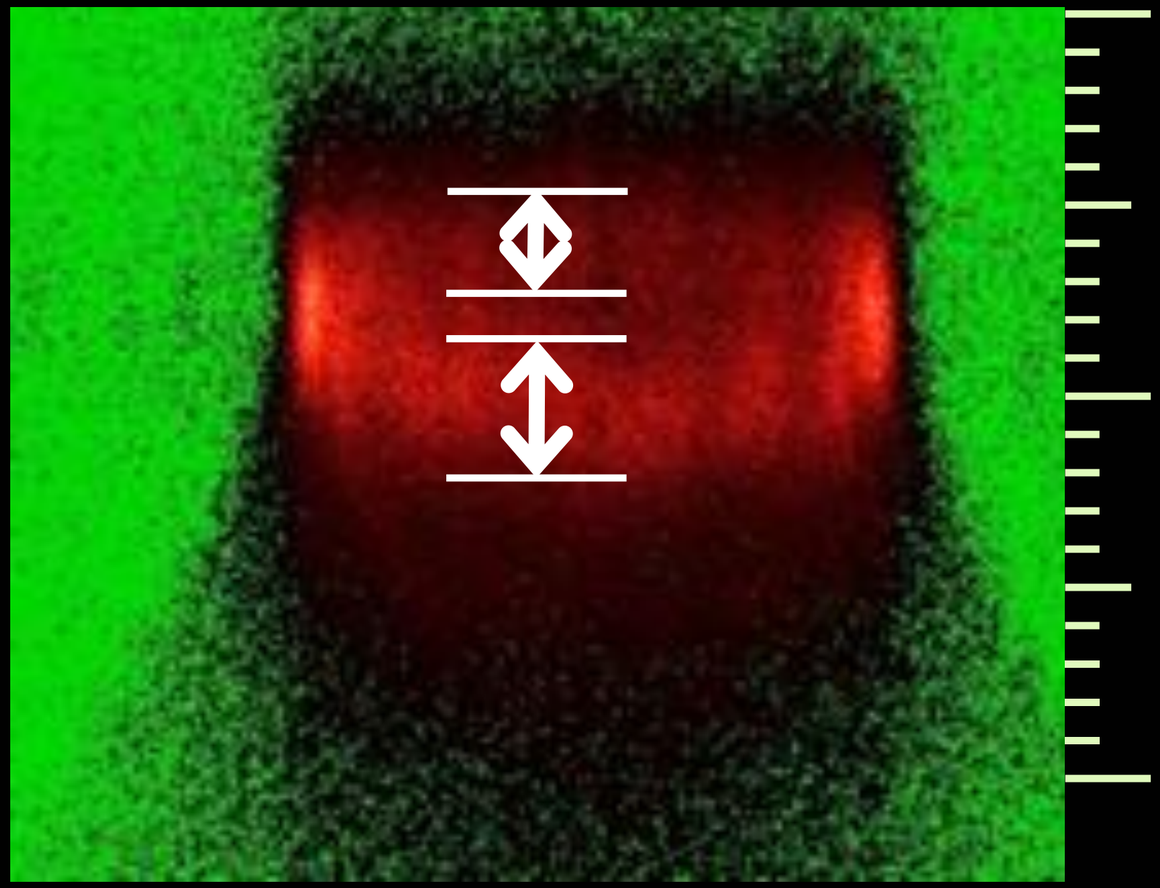
The refractive index mismatch

...causes spherical aberration



When the embedding media differs from the objective's immersion and correction, the refractive index mismatch causes spherical aberration.

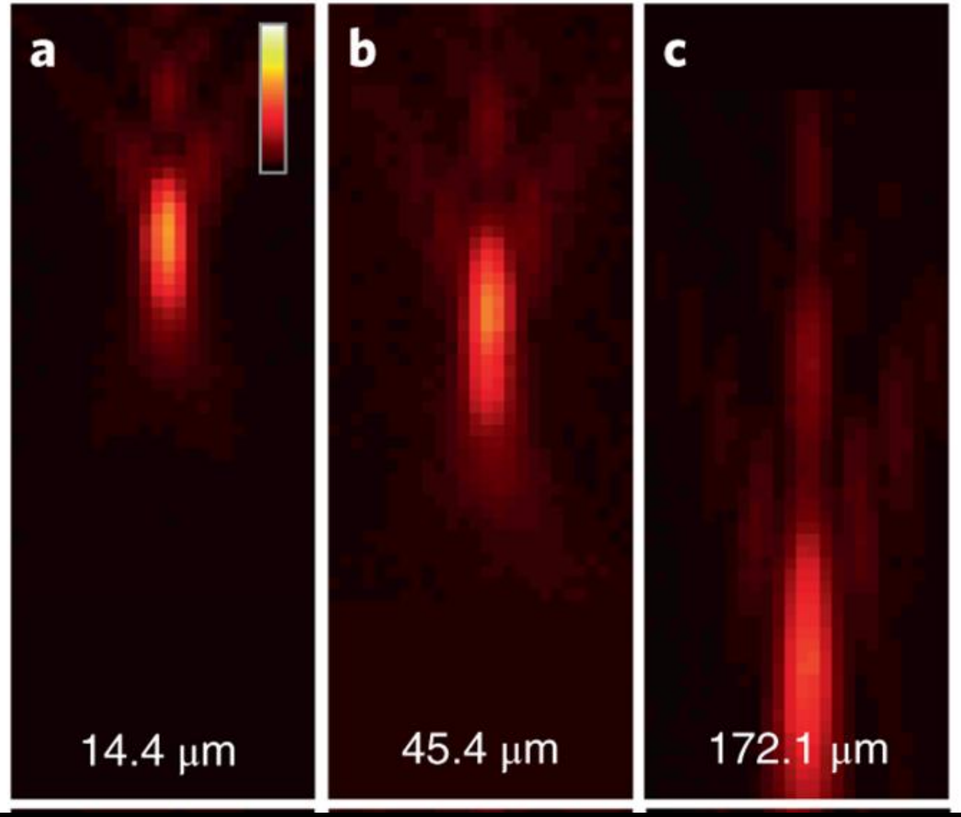
The effect of the depth



Spherical aberration becomes increasingly pronounced with greater sample depth due to the compounded effects of refractive index mismatch.

The effect of the depth

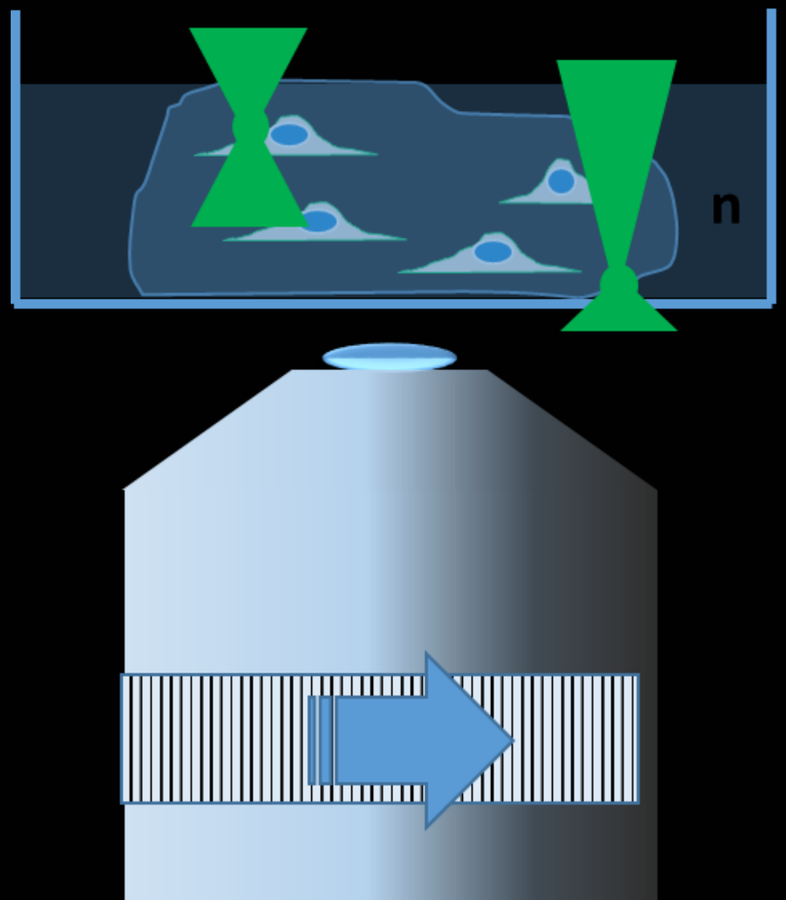
… in thick sample



Michael J. Mlodzianoski, Paul J. Cheng-Hathaway, Shane M. Bemiller, Tyler J. McCray, Sheng Liu, David A. Miller, Bruce T. Lamb, Gary E. Landreth & Fang Huang

Nature Methods **15**, 583–586(2018) | Cite this article

The trick of correction

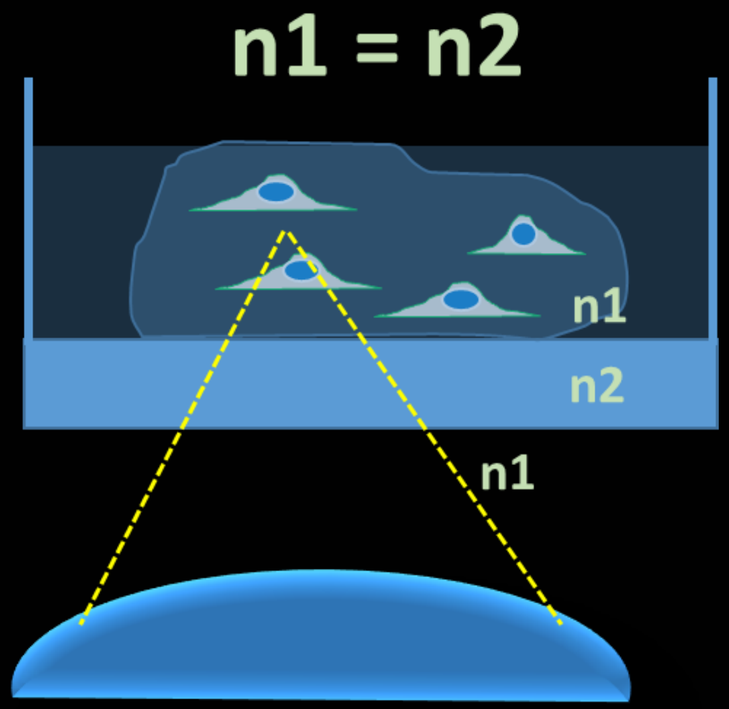


… works, but…

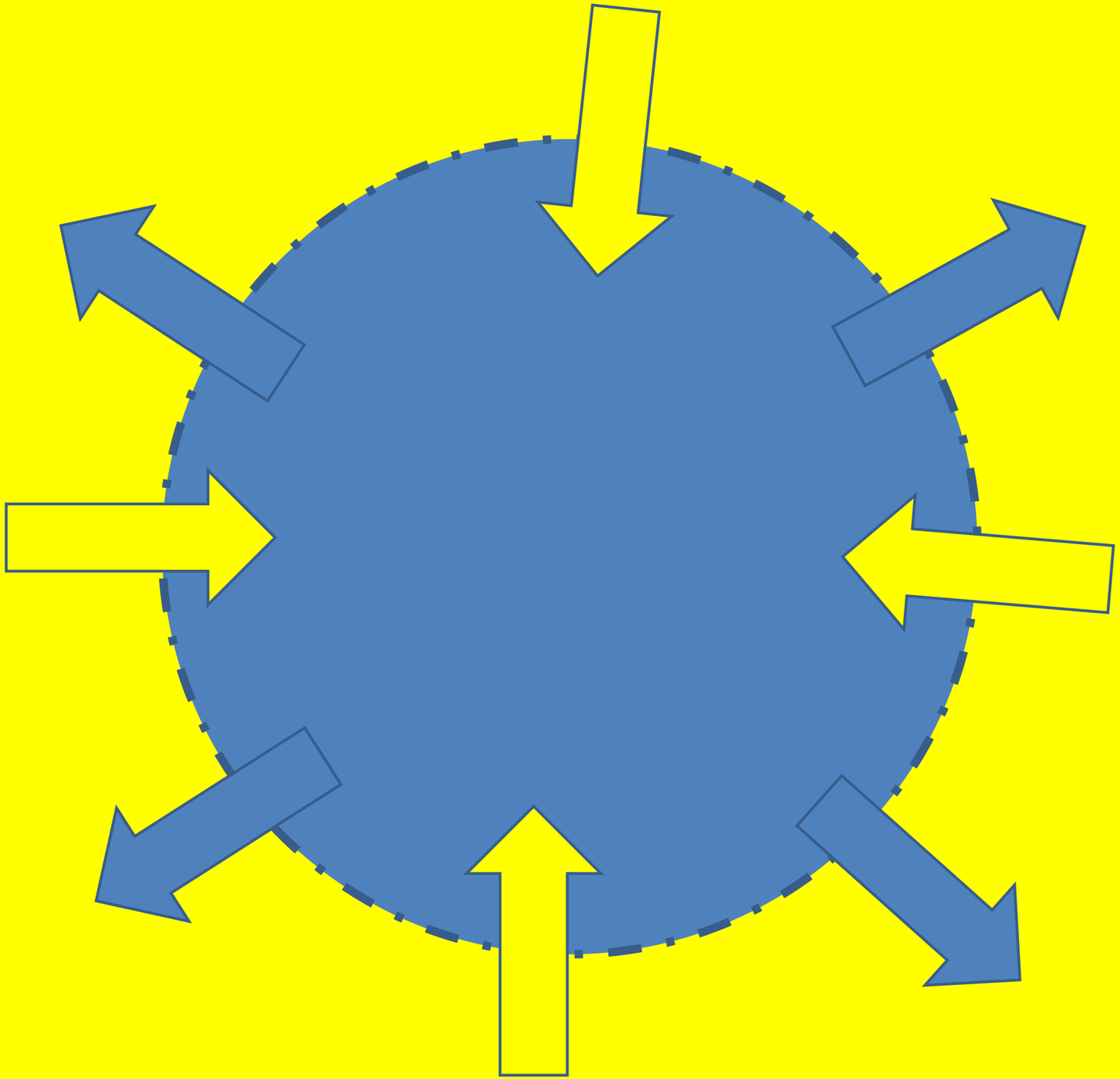
Using an objective with a correction collar, spherical aberration can be corrected, but only for a specific, defined depth.

The concept

..of sample preparation

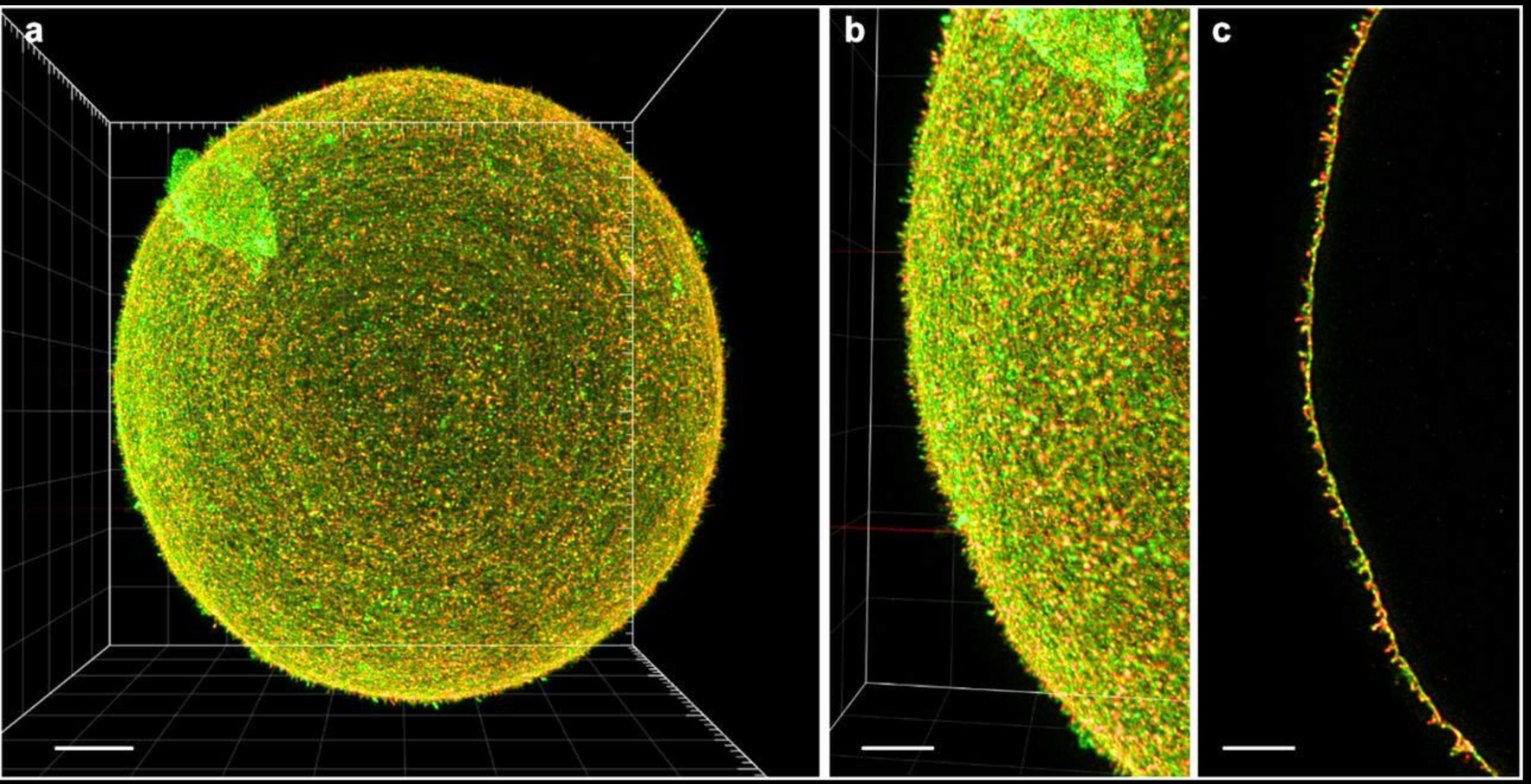


The golden rule is to minimize the refractive index mismatch throughout the entire sample to reduce spherical aberration and enhance image quality.



The long story short

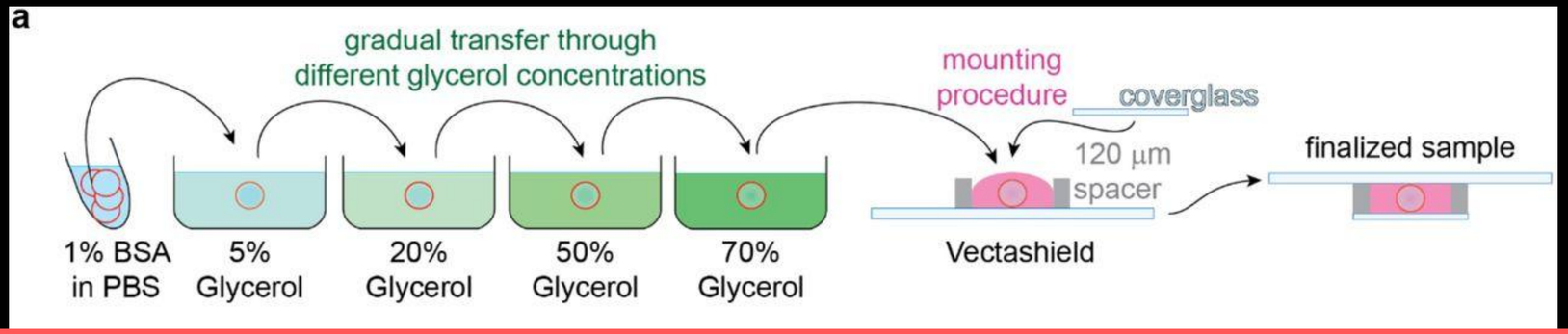
..of oocyte preparation

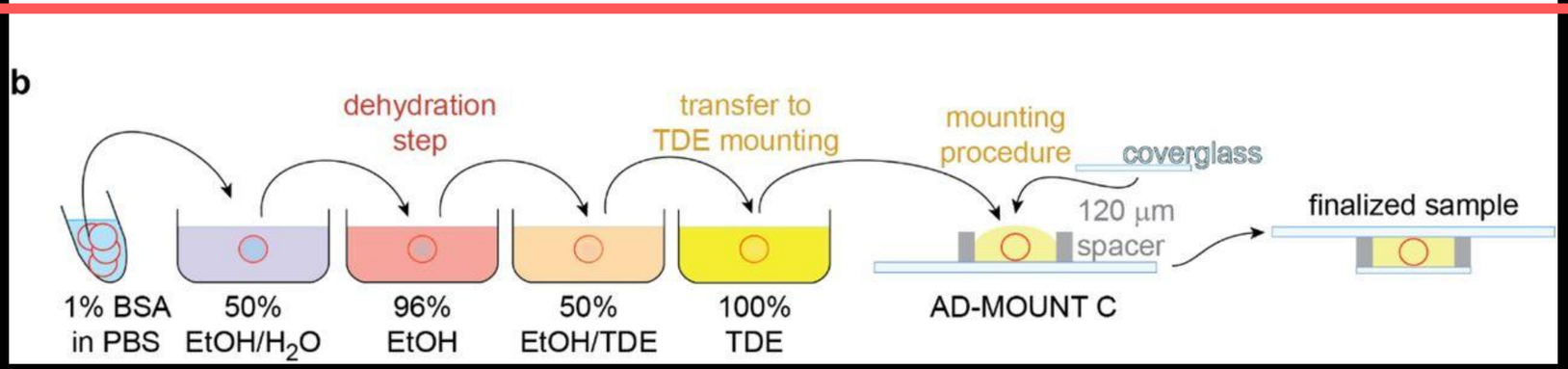


- Big
- Fragile
- Hard to handle

The key step in the procedure

… replace water with a mounting





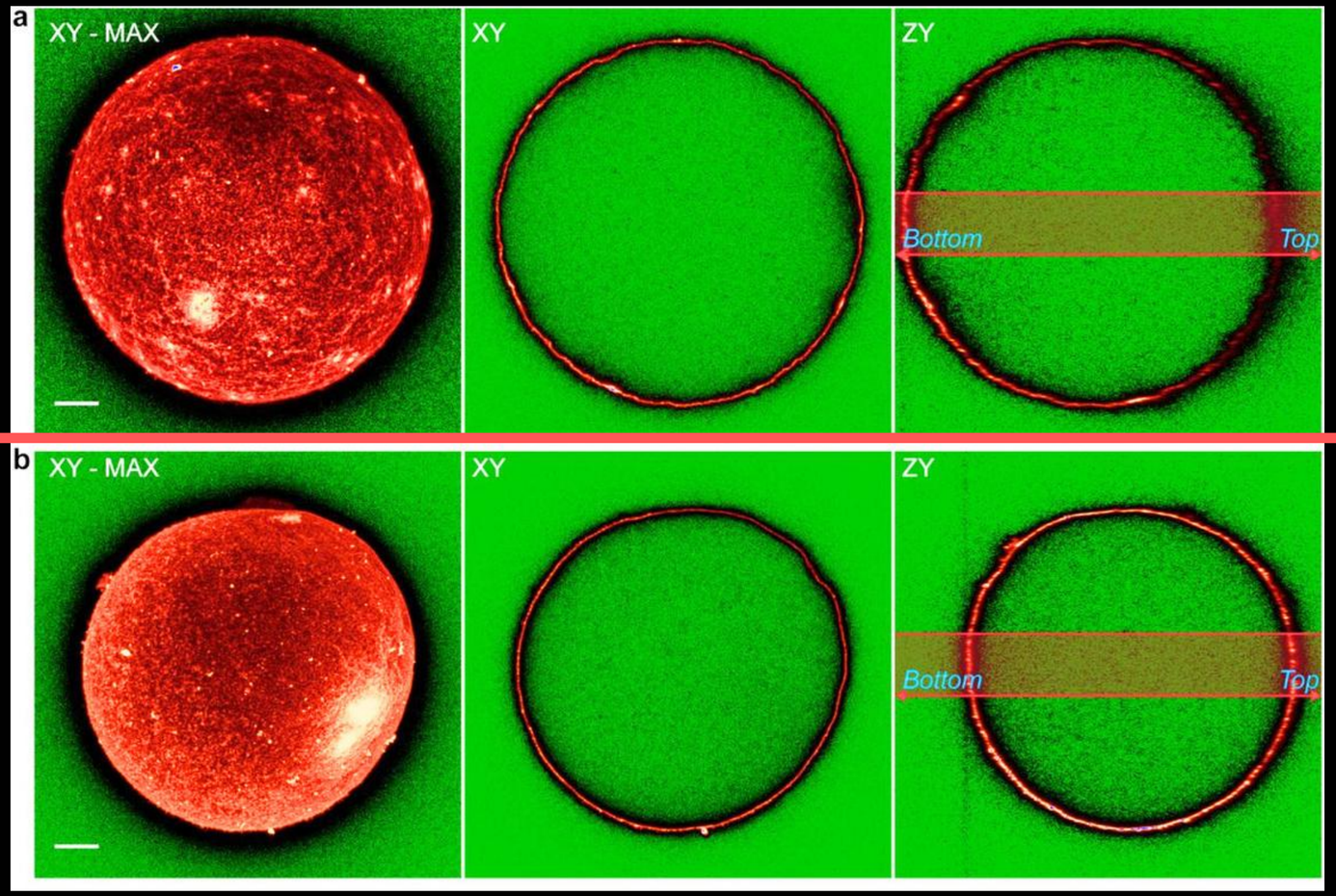
Gradual transfer to mounting media.

or

Gentle but thorough dehydration of the specimen.

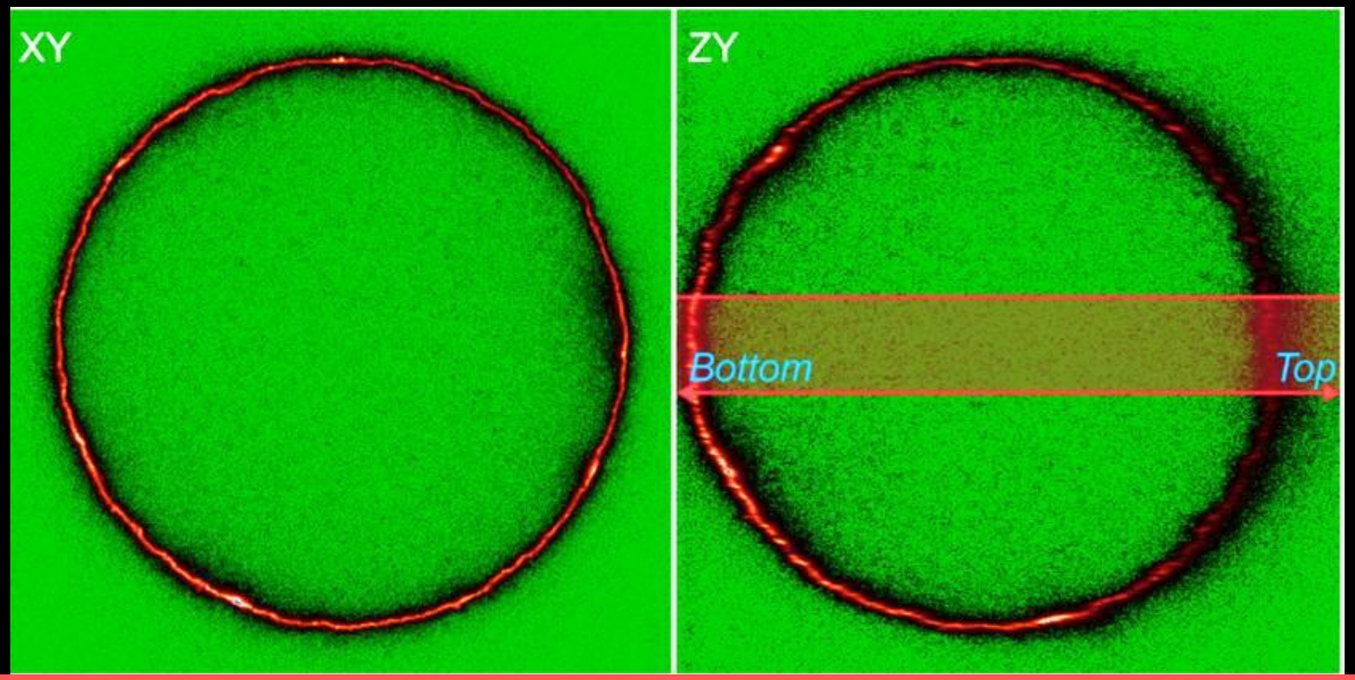
The extended procedure

… the dehydration

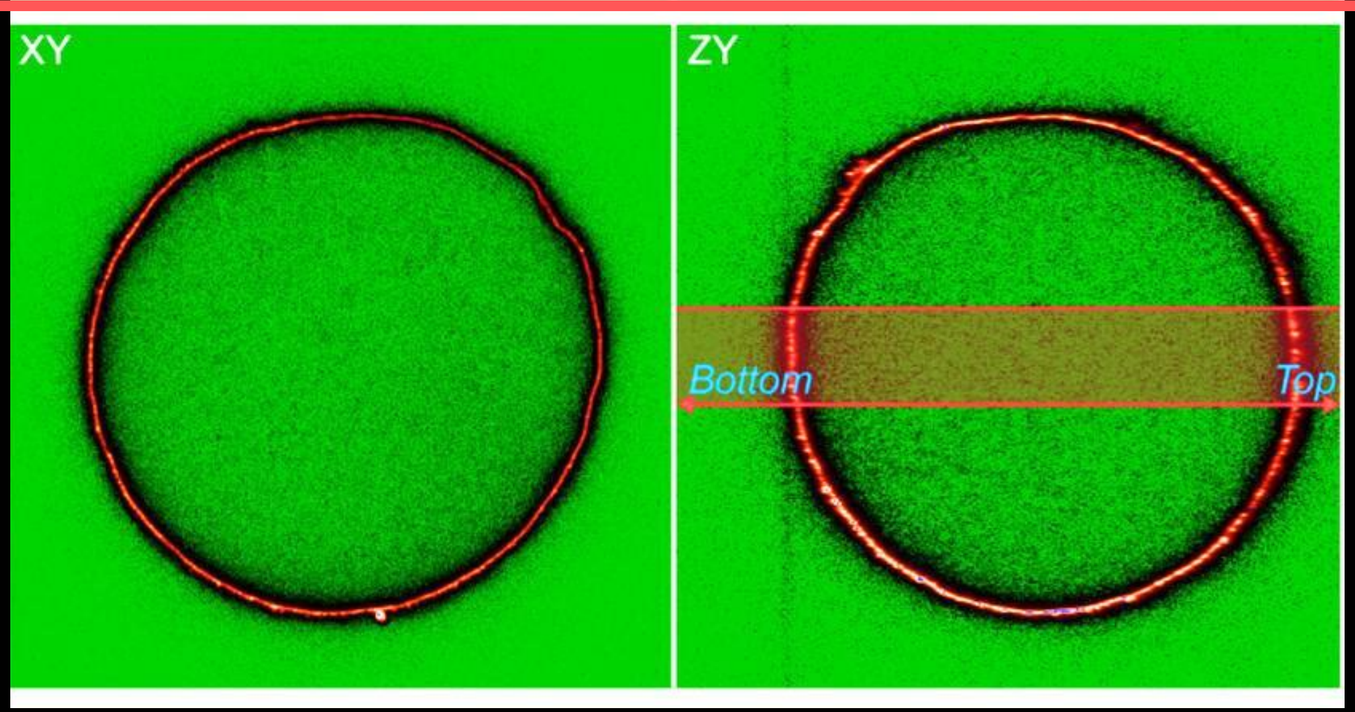


Standard smearing caused by spherical aberration

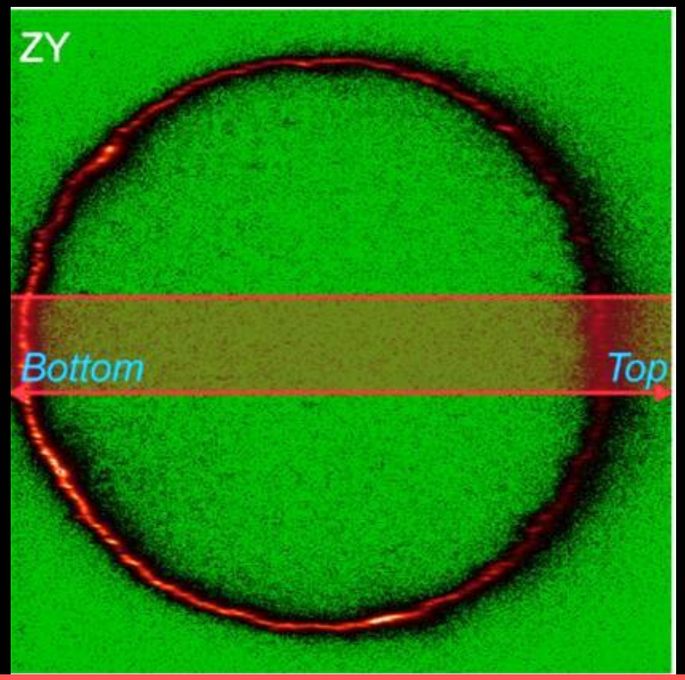
Minimized spherical aberration.

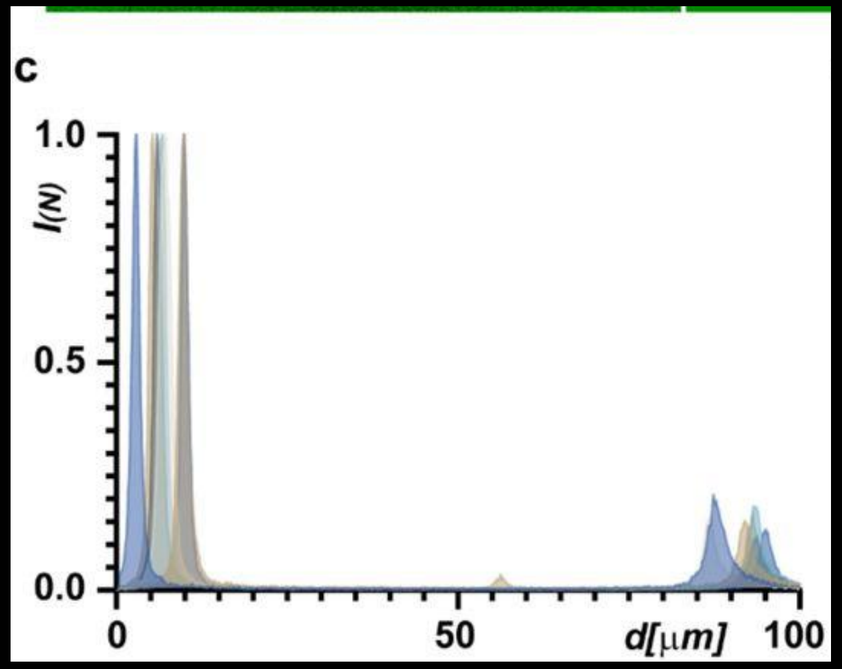


Standard smearing caused by spherical aberration

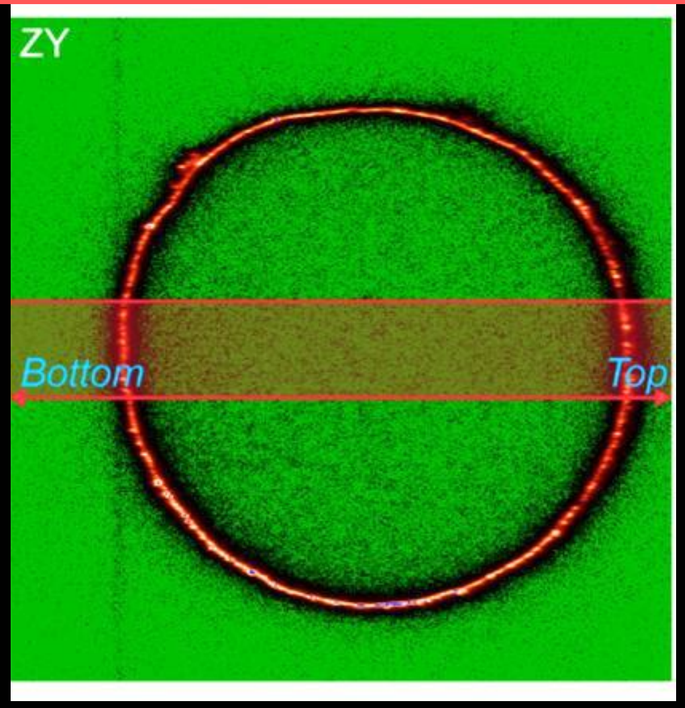


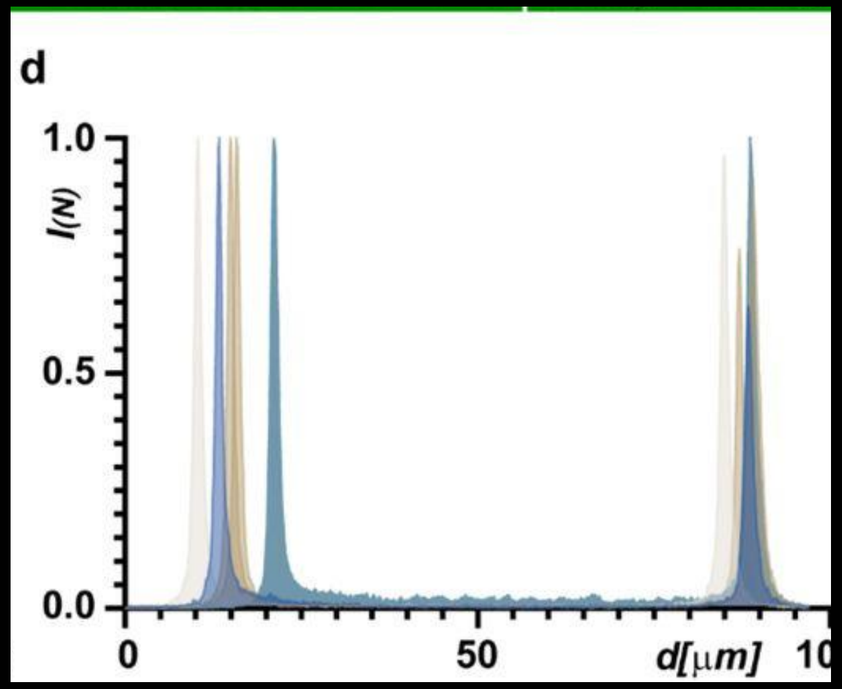
Minimized spherical aberration.





~85 % of signal intensity is lost; PSF is smeared on the other side of the oocyte.

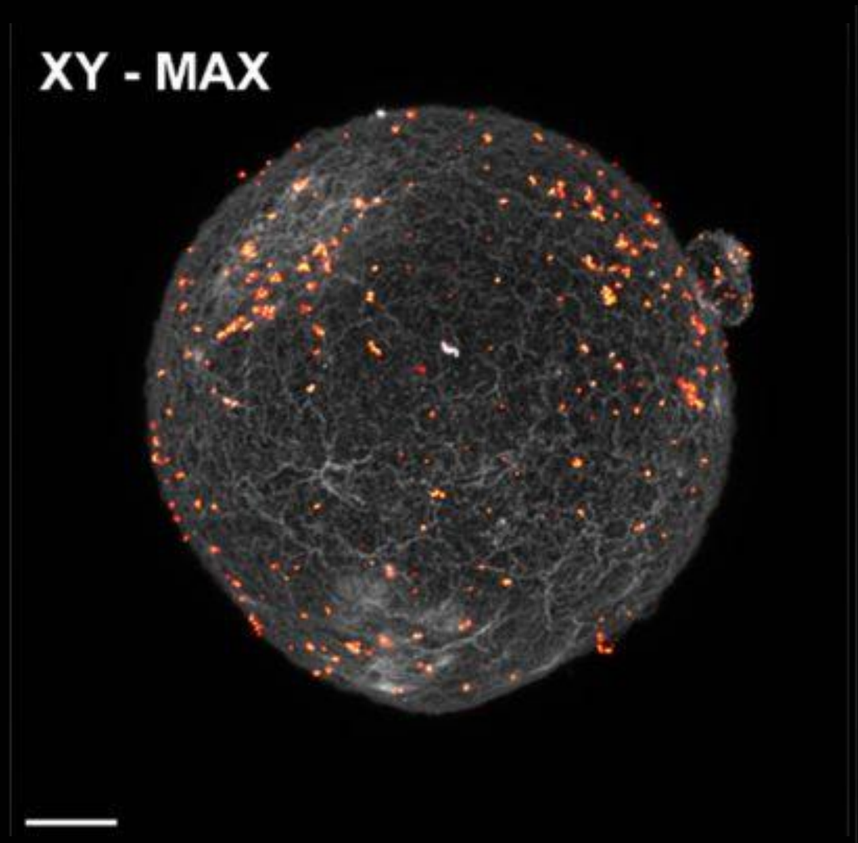


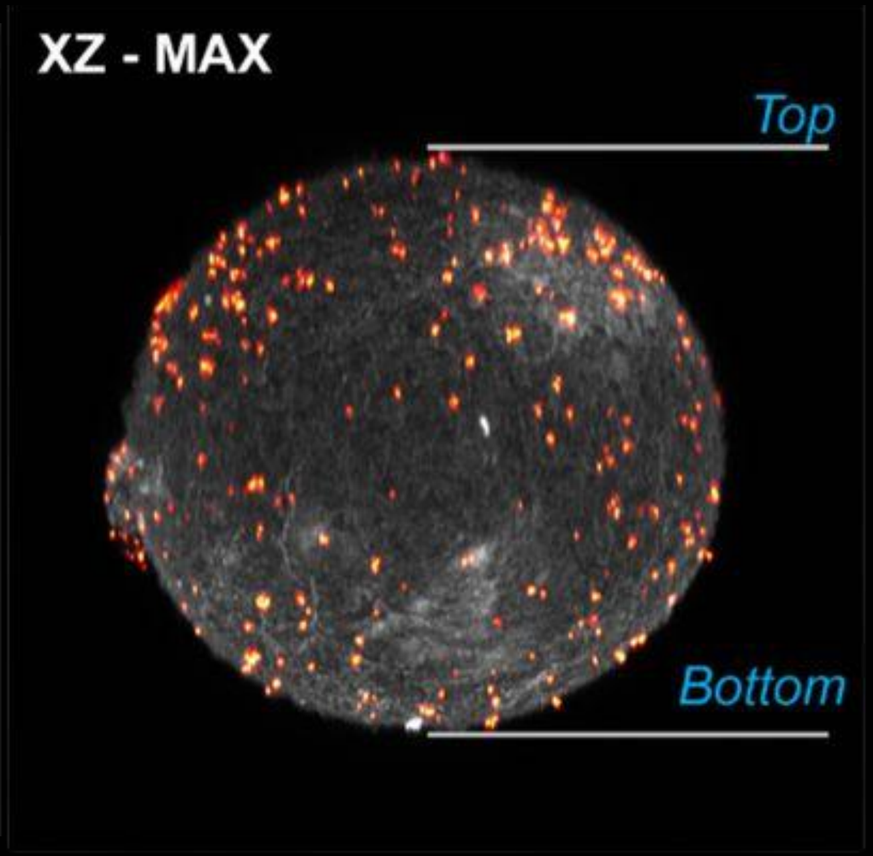


~10 % of signal intensity is lost; PSF shape is preserved through the entire oocyte.

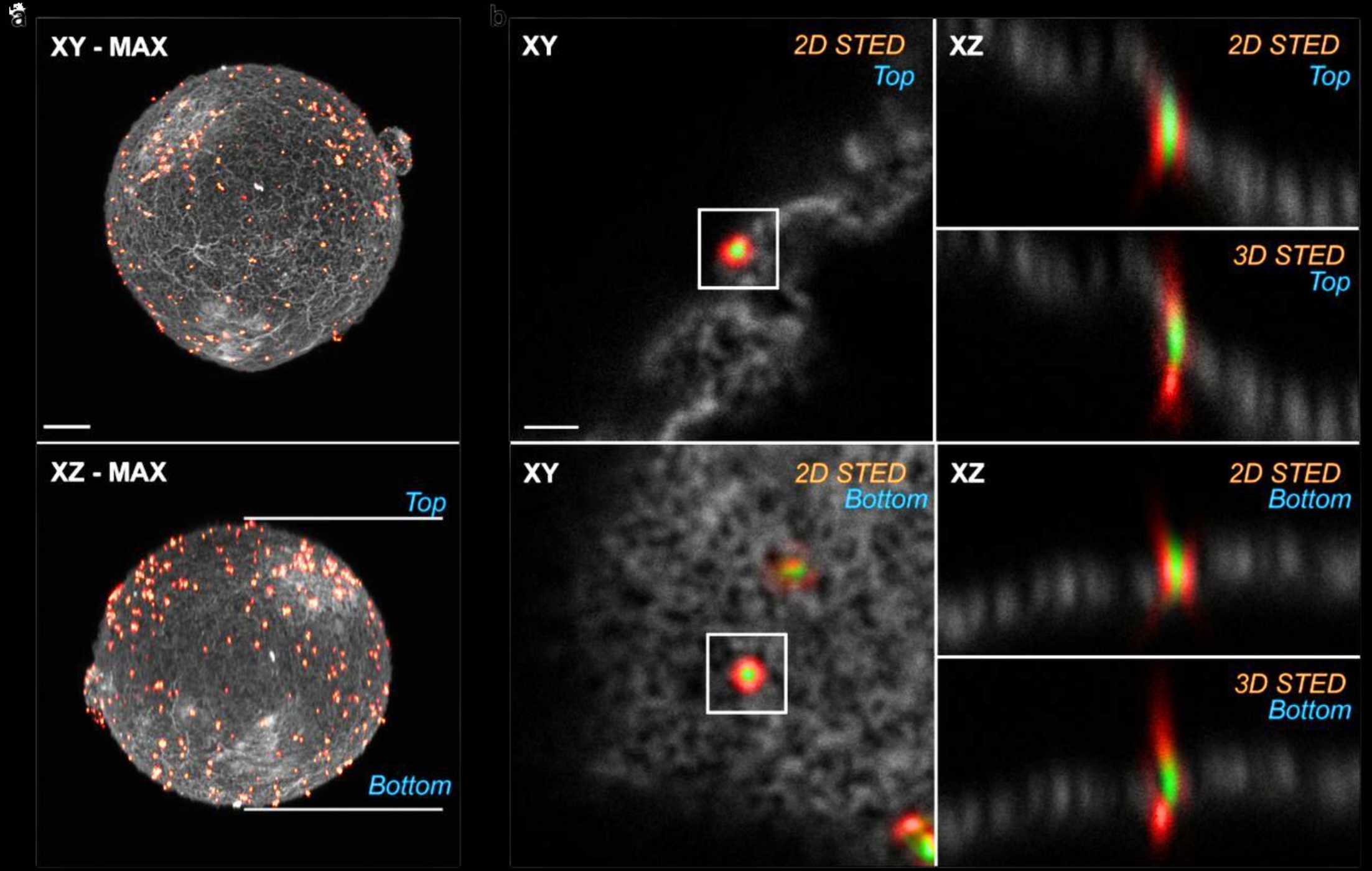
The proof of STED alignment

… within the sample





Gold beads adhered to the oocyte surface are used for visualizing the point spread function (PSF) and their co-alignment throughout the specimen.

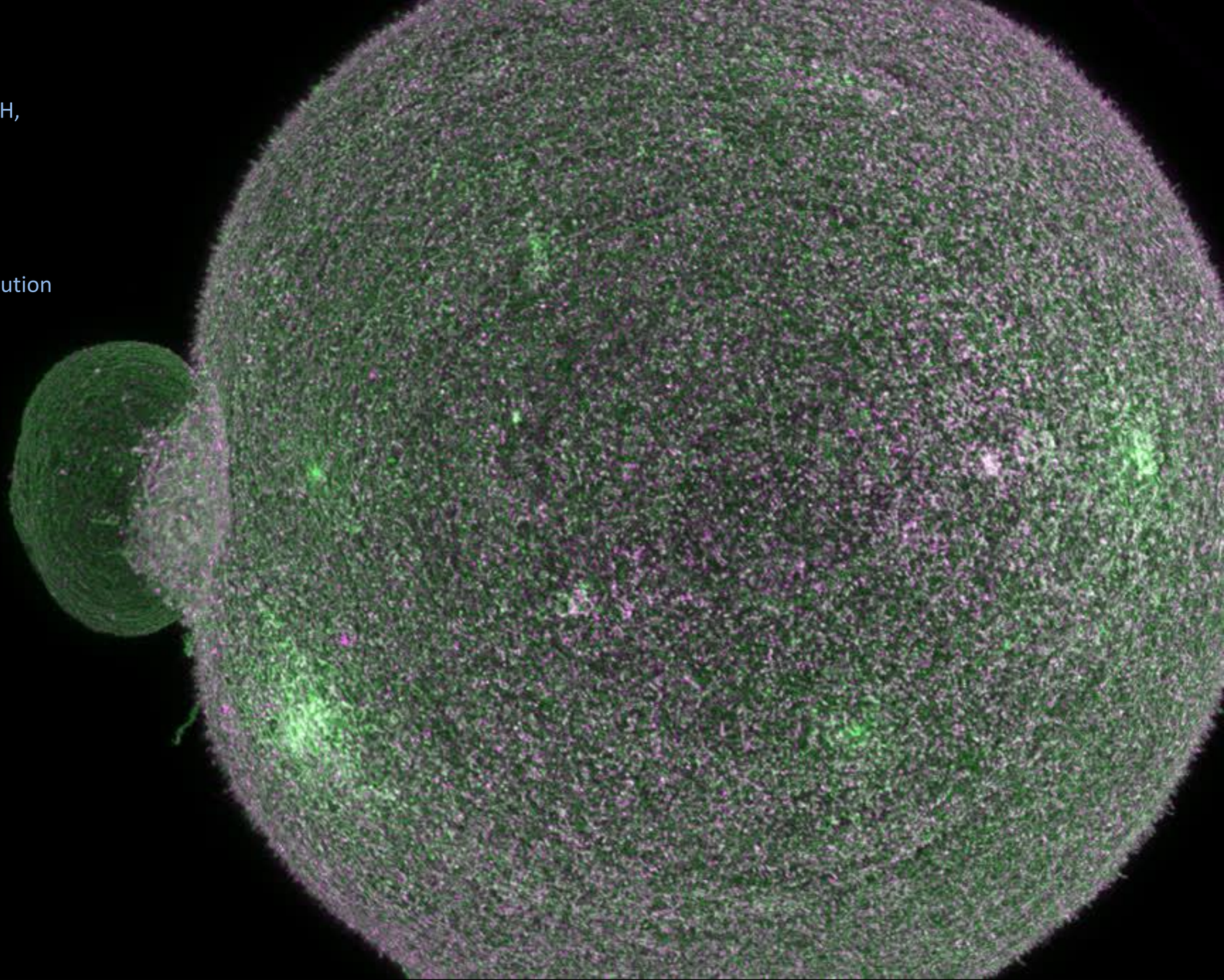

a
XY - MAX
XZ - MAX
Top
Bottom
b
XY
2D STED
Top
XZ
2D STED
Top
3D STED
Top
XY
2D STED
Bottom
XZ
2D STED
Bottom
3D STED
Bottom

Frolikova M, Blazikova M, Capek M, Chmelova H, Valecka J, Kolackova V, Valaskova E, Gregor M, Komrskova K, Horvath O, Novotny I.

Innovative sample preparation using alcohol dehydration and high refractive index medium enables acquisition of two-channel super-resolution 3D STED image of an entire oocyte.

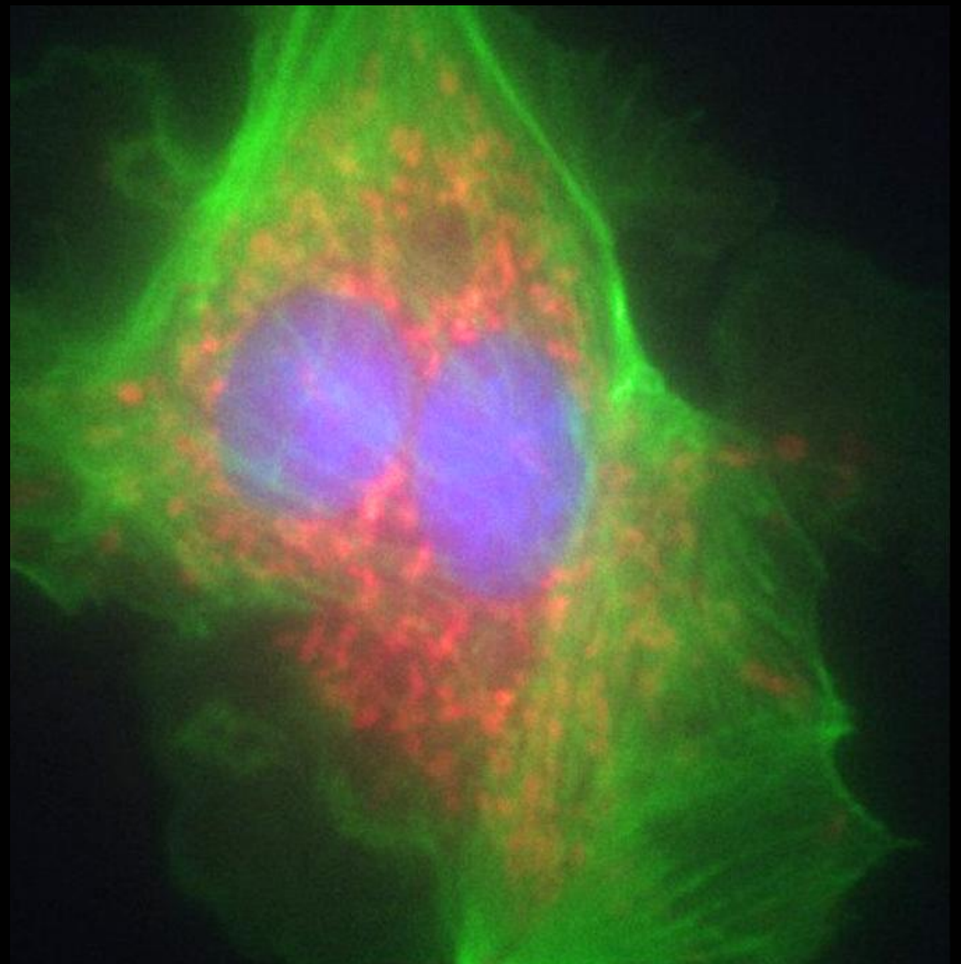
J Microsc. 2024 Oct 11.

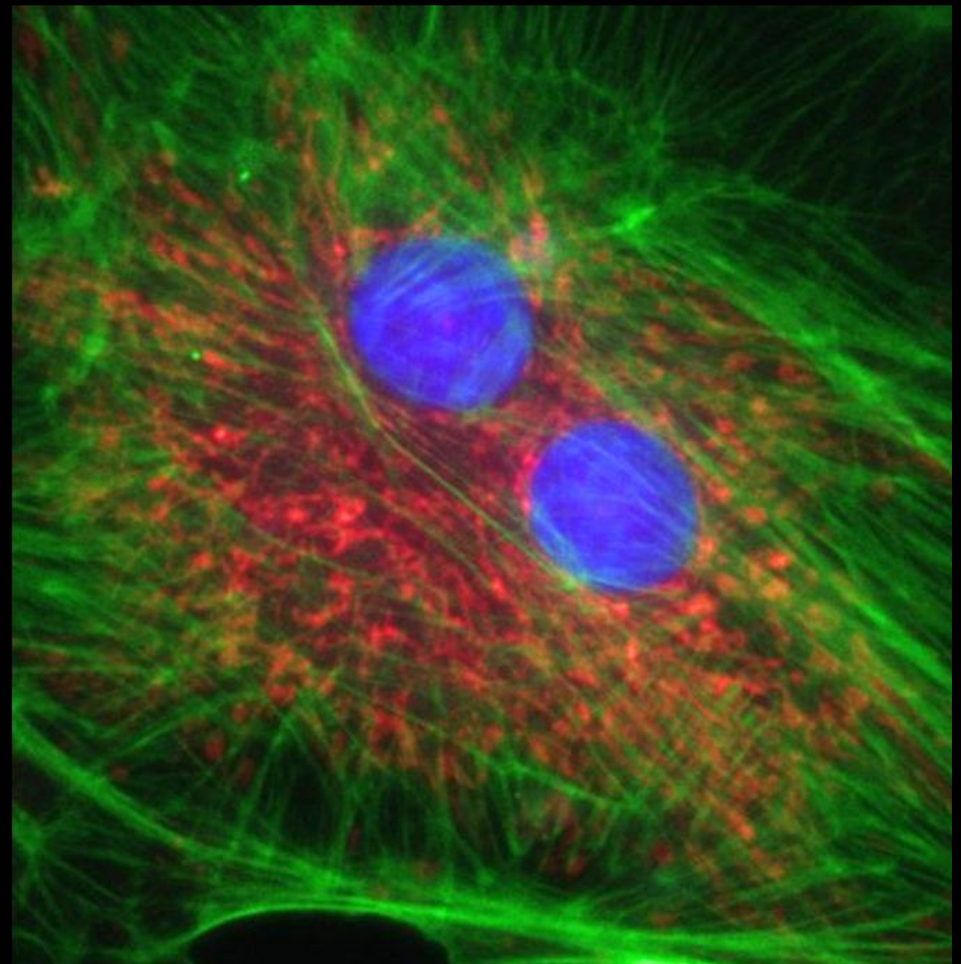
doi: 10.1111/jmi.13363
PMID: 39392013.



The concept

..minimize effect of spherical aberration





AD MOUNT - Mounting media evolution

IMG

CZECH BIOIMAGING
Imaging principles of life

MŠMT

ADVI

2011

IMG

Initial mixing and optimization of mounting media for super-resolution microscopy. Stability and anti-fade properties identified as key requirements.

2012

IMG

Mounting media successfully used in routine super-resolution microscopy experiments on the DeltaVision OMX SIM at MPI-CBG, Dresden, as part of a project conducted under the **Euro-BioImaging** Access Framework.

2015

IBT
IMG

Successfully applied mounting media for STED microscopy sample preparation and began providing it to LM IMG facility users, receiving positive feedback from users and collaborators.

2016

IPHYS
IBT
IMG

Successful application of the **Czech BioImaging** project prompted further work on optimizing and developing mounting media for super-resolution microscopy.Optimizing of TDE-based mounting medium.

2019

IEB
IMIC
IPHYS
IBT
IMG

Optimized pH and salt concentration for improved properties and introduced a second type of mounting media for wider usage. Optimizing of TDE-based mounting medium for STED experiments.

2020

CB SAS
IEB
IMIC
IPHYS
IBT
IMG

Optimized sample preparation protocol for super-resolution microscopy, including mounting with our optimized mounting media. Successfully acquired 3D STED images of an entire mouse oocyte mounted in TDE-based medium.

2024

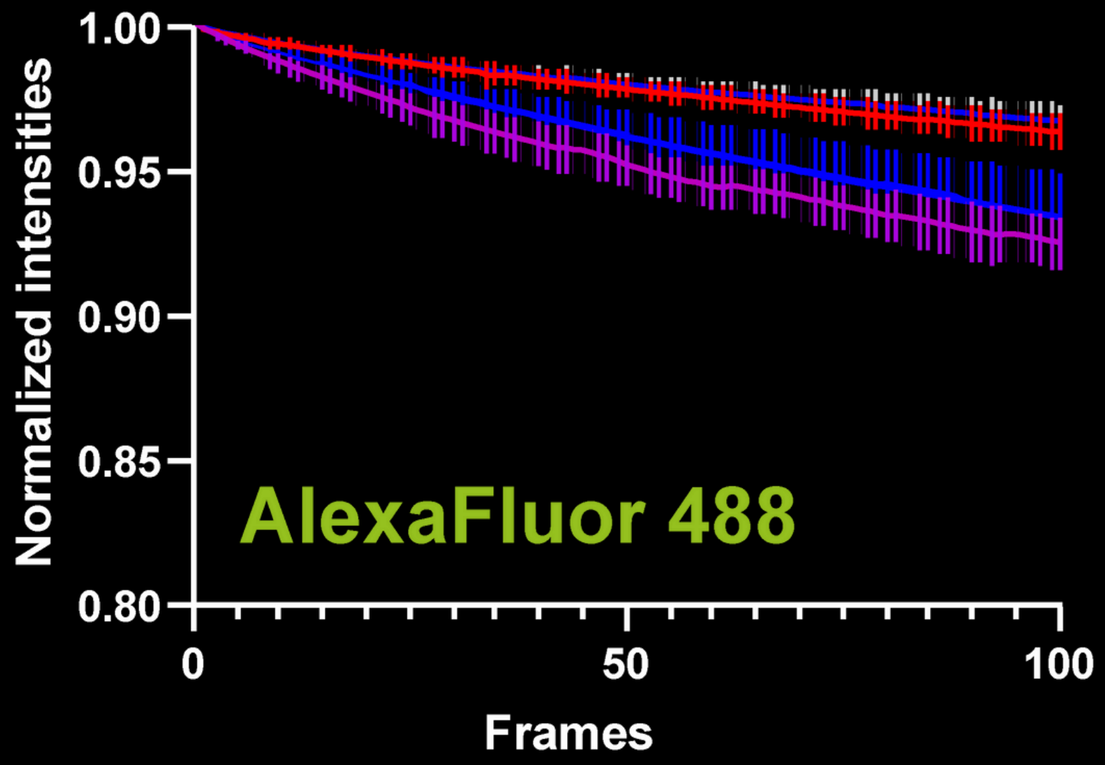
MANY OTHERS

CB SAS
IEB
IMIC
IPHYS
IBT
IMG

Since 2022, commercial licensing of our three types of mounting media to **ADVI**, a startup company specializing in advanced microscopy consumables and solutions. 2024 - newly optimized hardenning mounting medium.

AD MOUNT - Fluorescence stability assay

Non-hardening



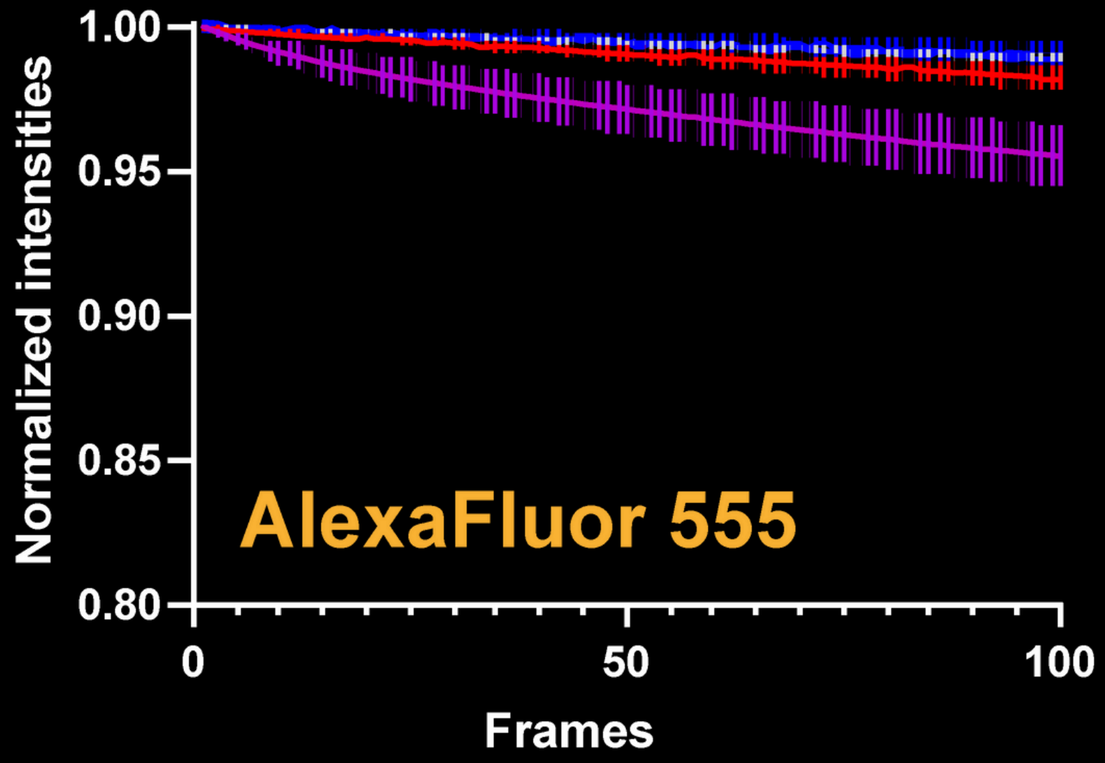
AD MOUNT | F universal
Universal Application
Refractive index: 1.45
Non-hardening
All fluorophores

AD MOUNT | S superior
Superior Stability
Refractive index: 1.47
Non-hardening
Synthetic dyes only

AD MOUNT | C clear
Clear view
Refractive index: 1.518
Non-hardening
Synthetic dyes

AD MOUNT - Fluorescence stability assay

Non-hardening



AD MOUNT

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AD MOUNT

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Synthetic dyes only

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C clear

Clear view
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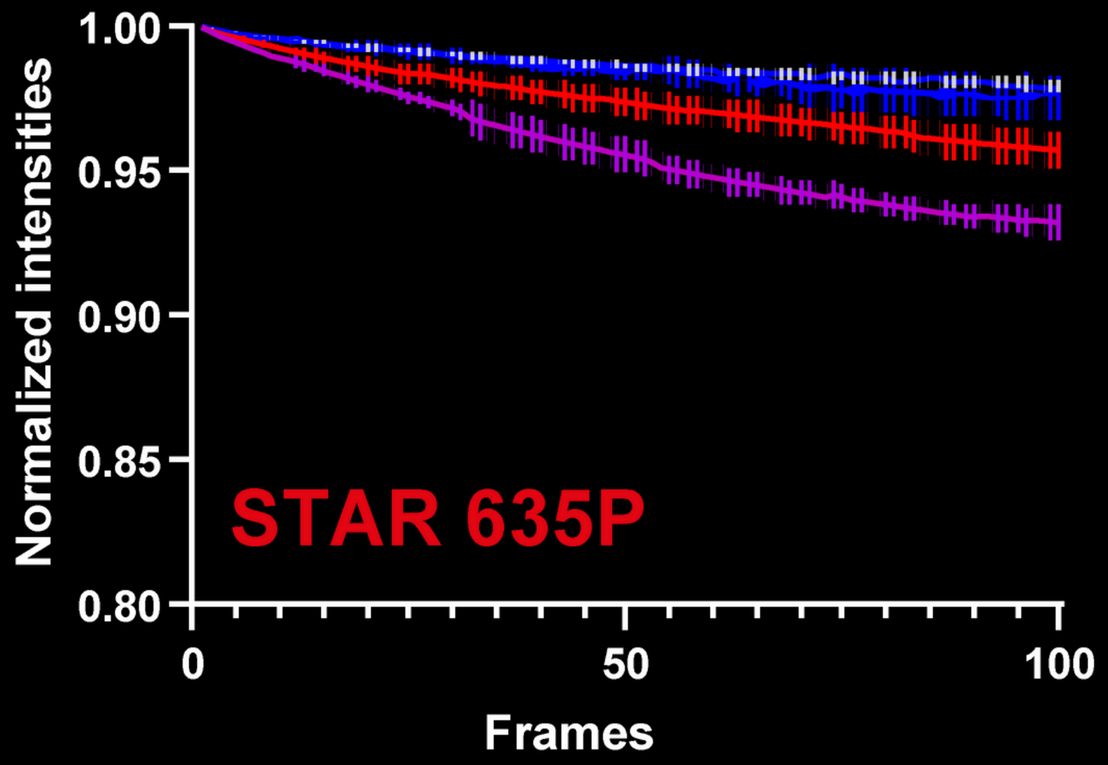
Synthetic dyes

AD MOUNT - Fluorescence stability assay

Non-hardening



commercial 01
ADF
ADS
ADC

AD MOUNT
F universal
Universal Application
Refractive index: 1.45
Non-hardening
All fluorophores

AD MOUNT
S superior
Superior Stability
Refractive index: 1.47
Non-hardening
Synthetic dyes only

AD MOUNT
C clear
Clear view
Refractive index: 1.518
Non-hardening
Synthetic dyes

AD MOUNT - the best achievement

Juno and CD9 protein network organization in oolemma of mouse oocyte

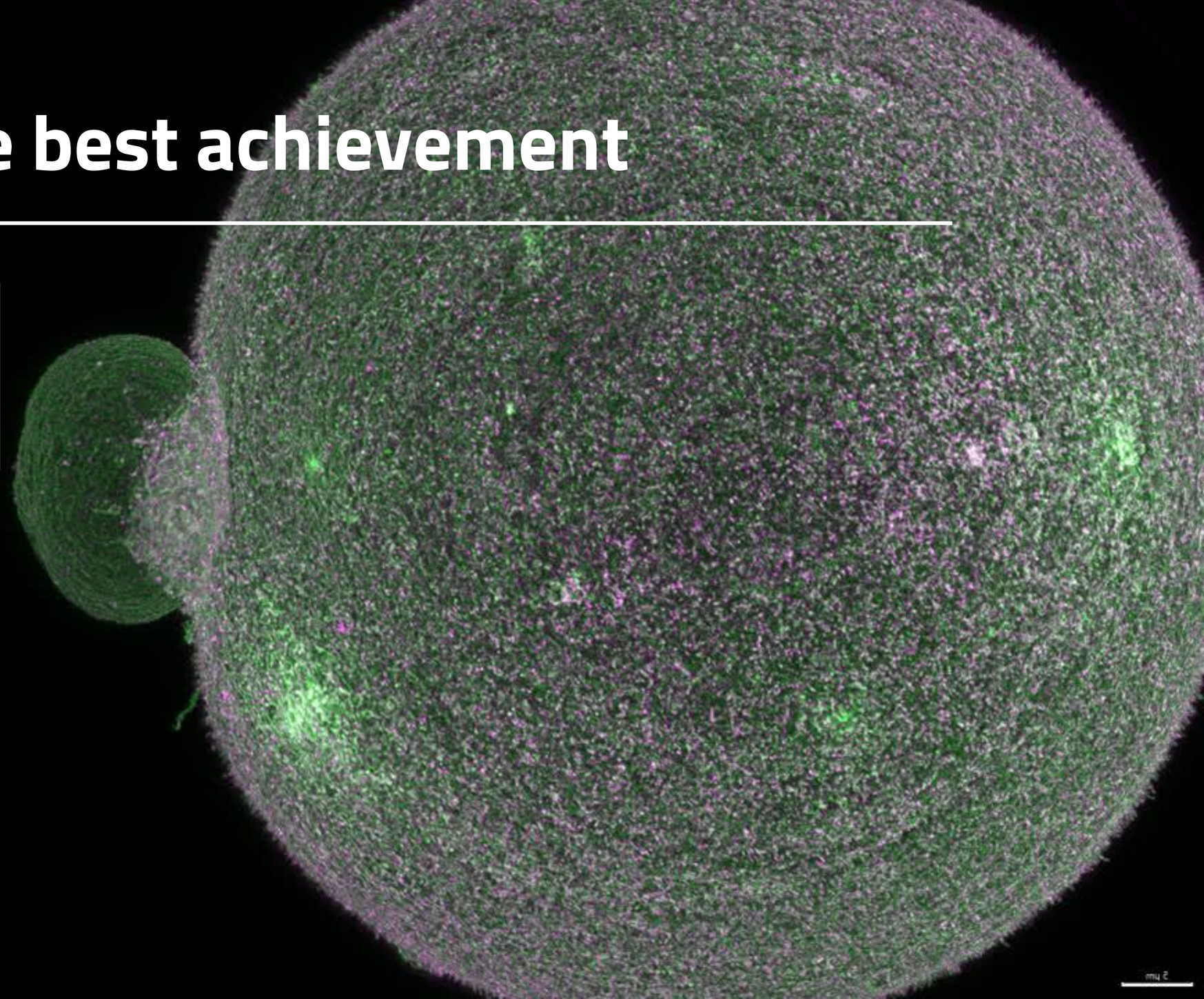
Juno (green) and CD9 (magenta) in 3D STED super-resolution image of entire mouse oocyte.

Frolikova et al. 2023, doi: 10.3389/fcell.2023.1110681

AD MOUNT

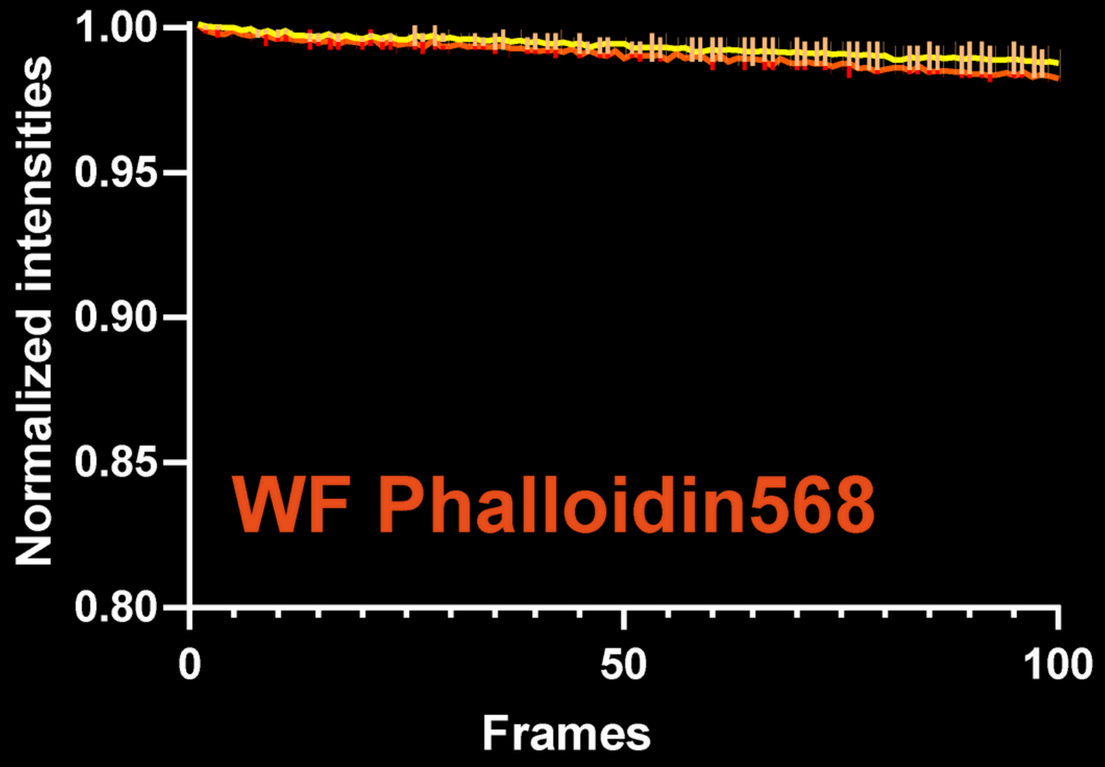
clear

Clear view
Refractive index: 1.518
Non-hardening
Synthetic dyes



AD MOUNT - Fluorescence stability assay

Hardening



commercial 02

ADHD

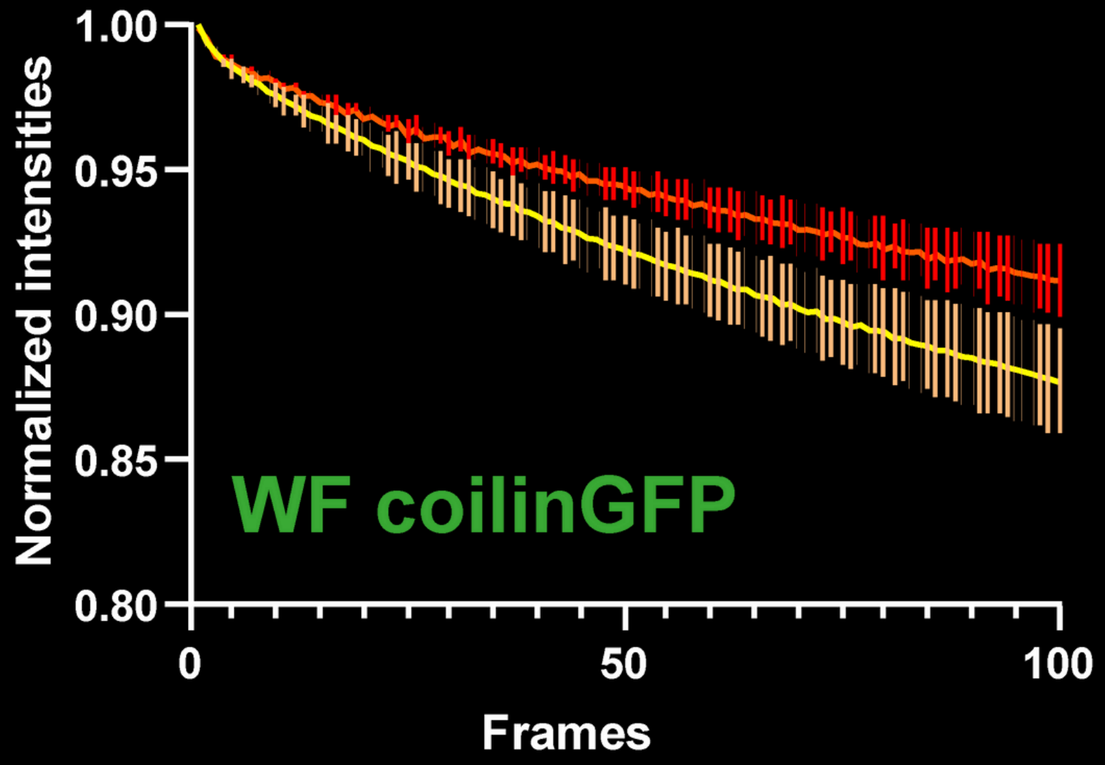
AD MOUNT

H hard

Universal Application
Refractive index: 1.46
Hardening
All fluorophores

AD MOUNT - Fluorescence stability assay

Hardening



commercial 02

ADHD

AD MOUNT

H hard

Universal Application
Refractive index: 1.46
Hardening
All fluorophores

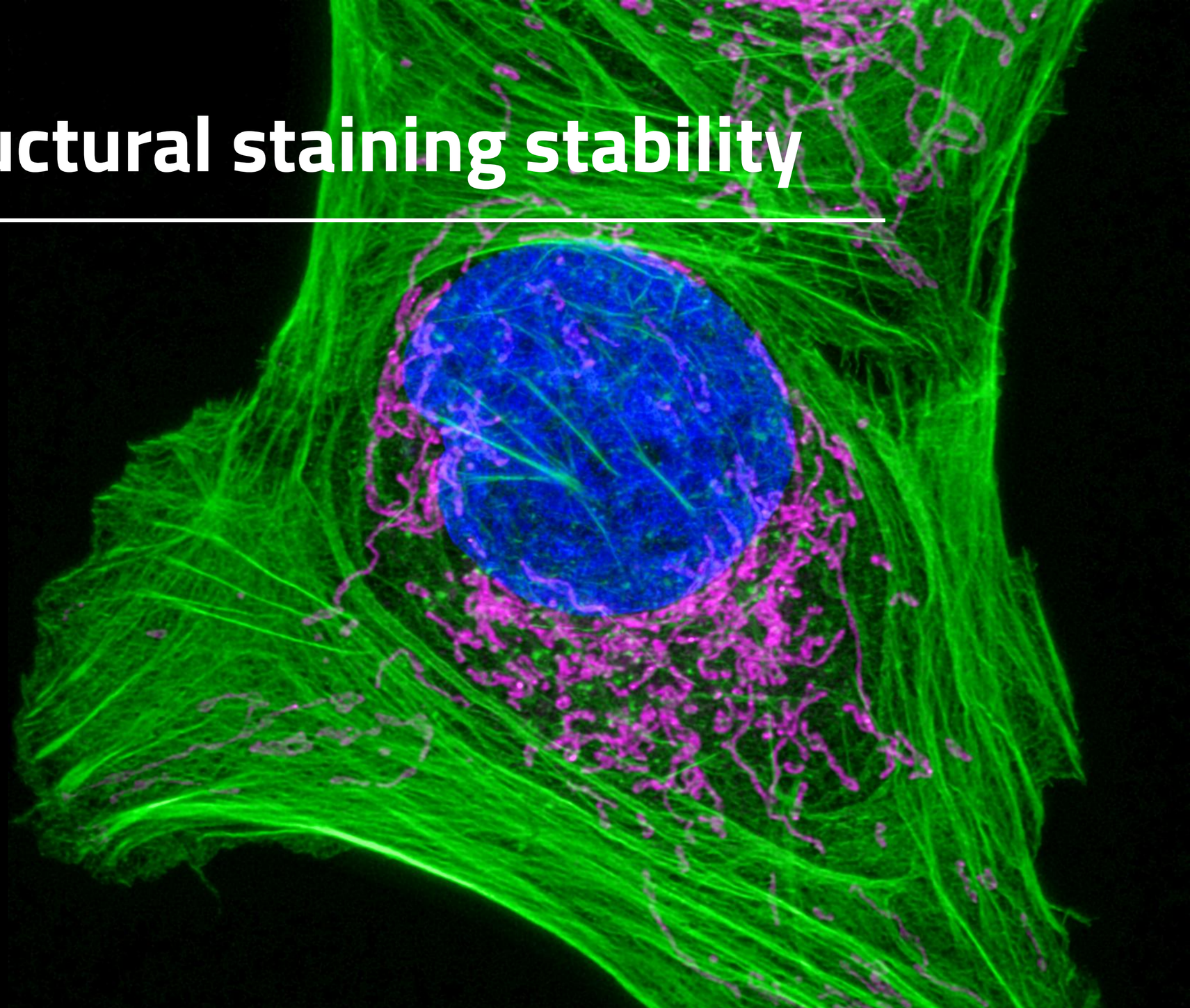
AD MOUNT - structural staining stability

Hardening

Phalloidin staining is non-covalent, hydrophobic interaction based visualization of actin structures.
AD-MOUNT H provides a long-lasting stabilizing effect, enhancing the visibility of these structures.

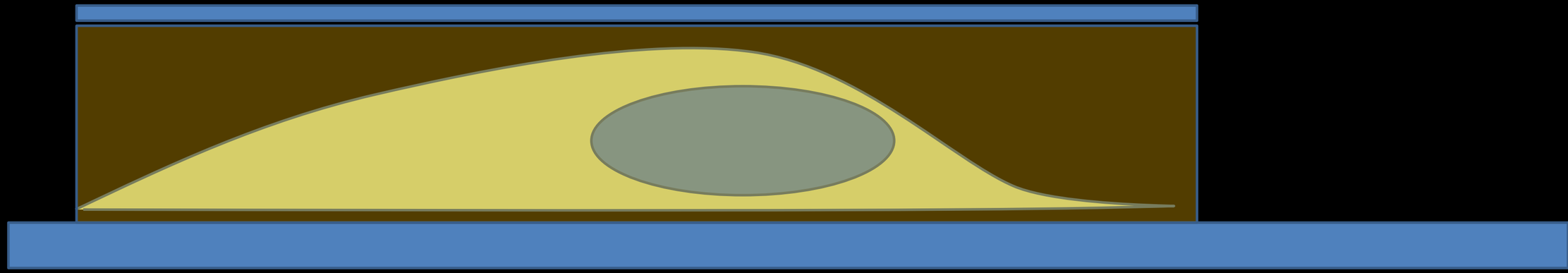
H hard

Universal Application
Refractive index: 1.46
Hardening
All fluorophores



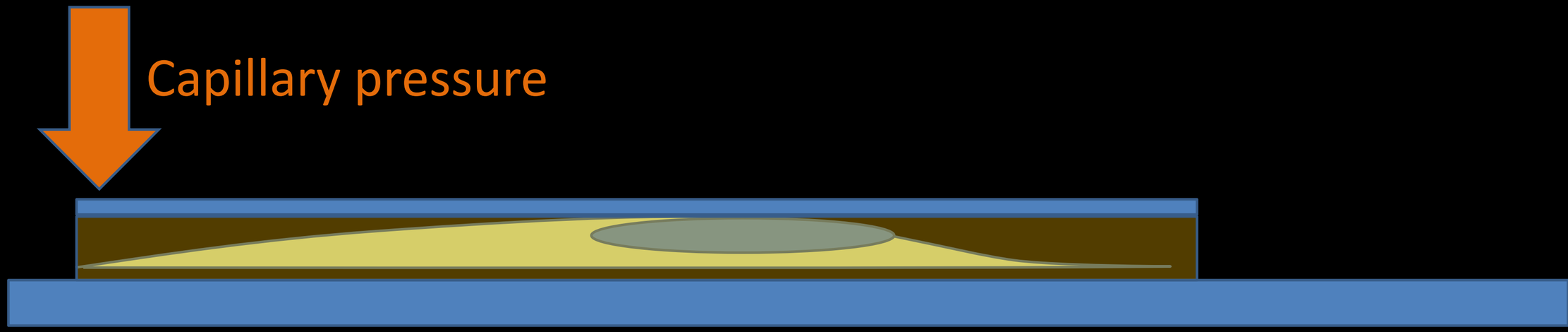
Important factor for sample flattening

and collapse of fragile structures.



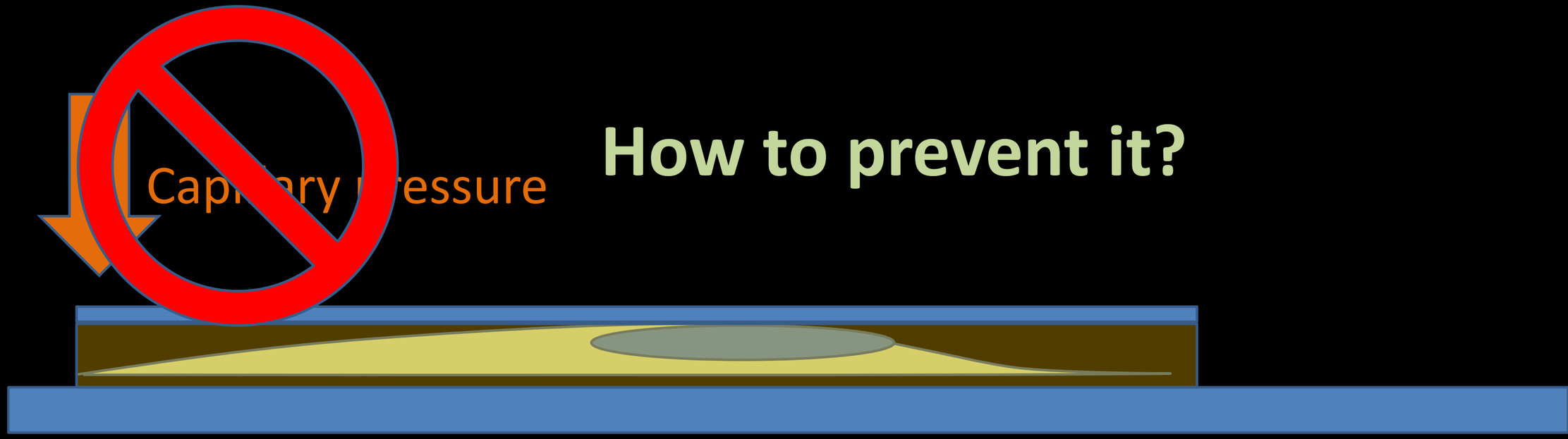
Important factor for sample flattening

and collapse of fragile structures.



Important factor for sample flattening

and collapse of fragile structures.

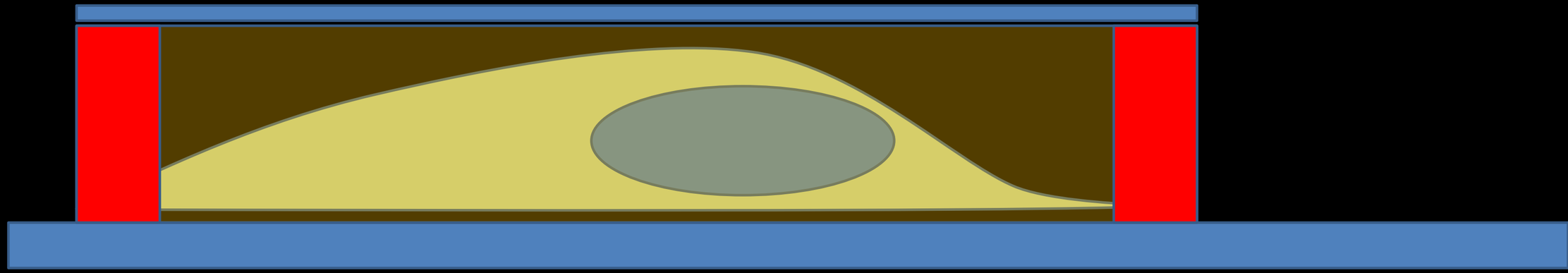


How to prevent it?

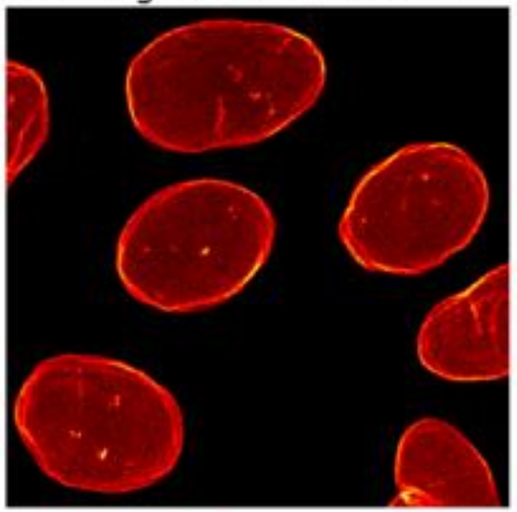
Important factor for sample flattening

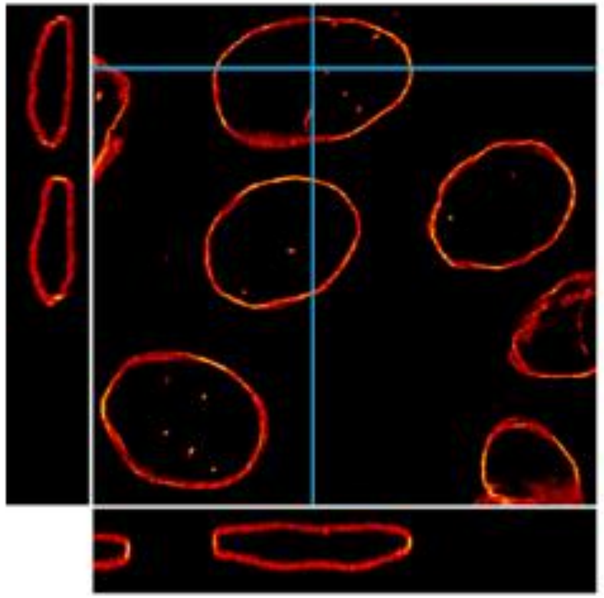
and collapse of fragile structures.

Use mounting spacers

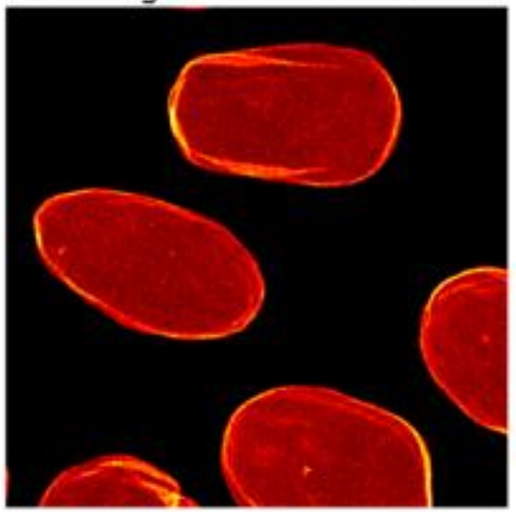


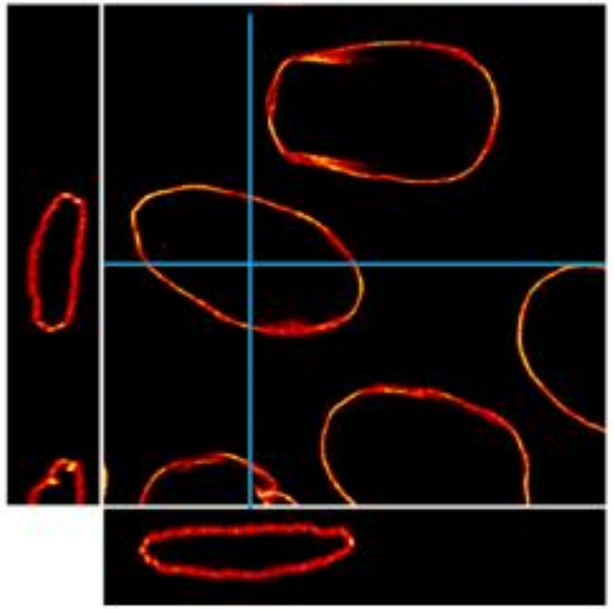
Standard drying
Mounting Spacers: AD-SEALS
Mounting medium: AD-MOUNT C



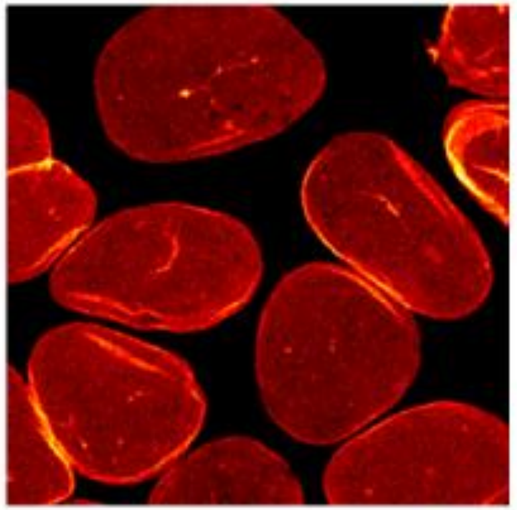


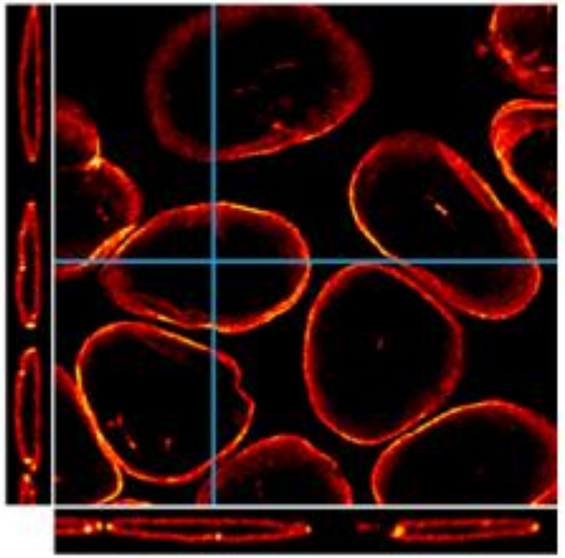
Standard drying
Mounting Spacers: AD-SEALS
Mounting medium: AD-MOUNT S



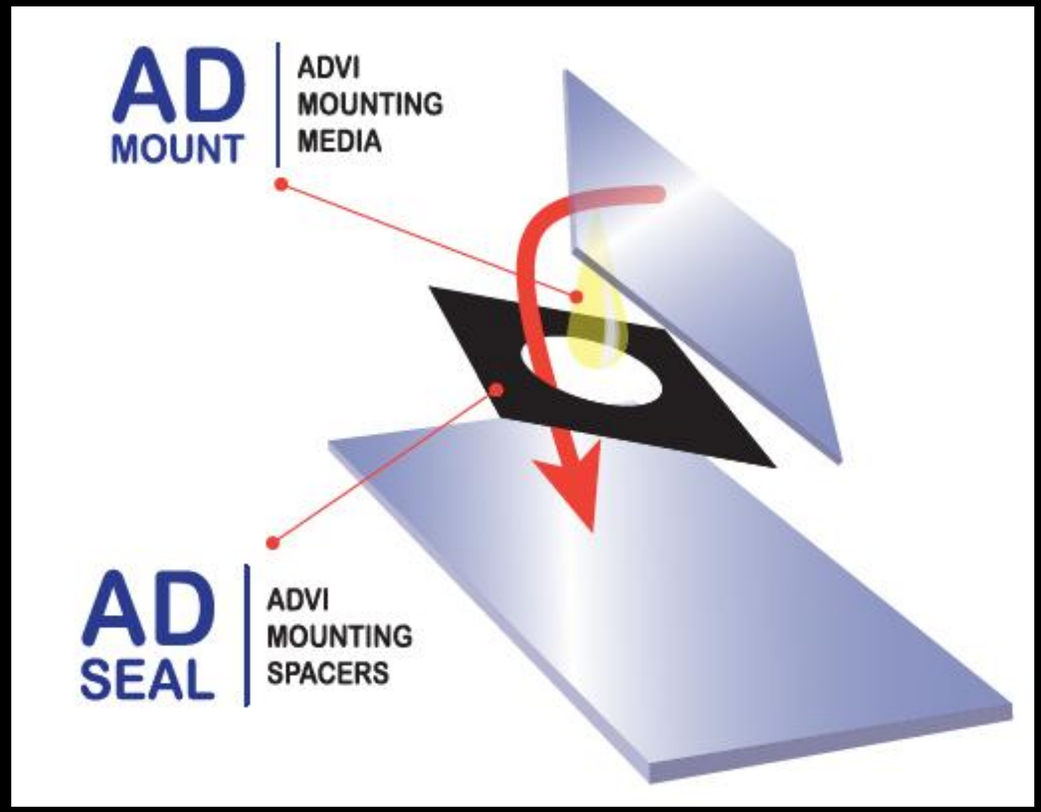


Standard drying
direct mounting to 90% Gly





How to Use Mounting Spacers: A Video Protocol



https://www.advi-web.com/en/mounting-spacers-how-to-easy-use/

Thank you

..see you