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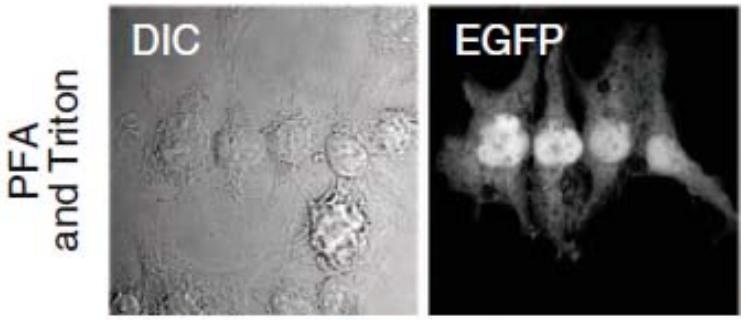
Martin Spitaler, FILM

Immunolabelling artifacts

"Immunolabeling artifacts and the need for live-cell imaging"

Ulrike Schnell, Freark Dijk, Klaas A Sjollema &
Ben N G Giepmans (Groningen, NL)

Nature Methods 9/2: 152-158



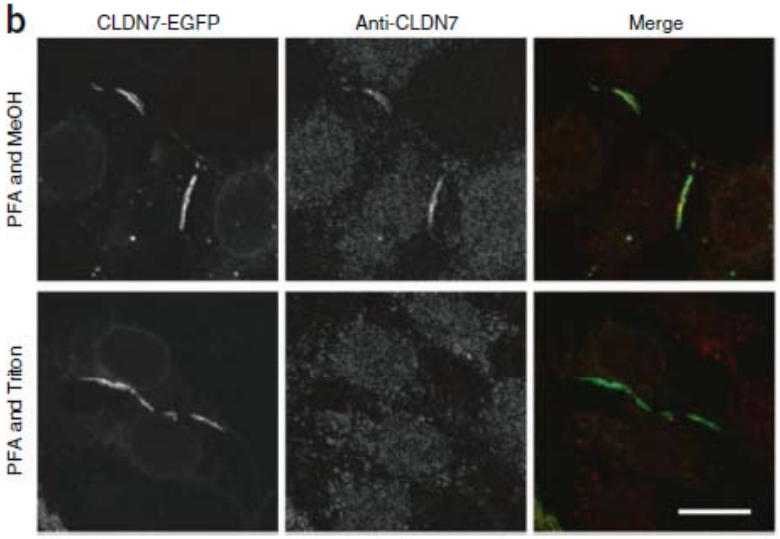
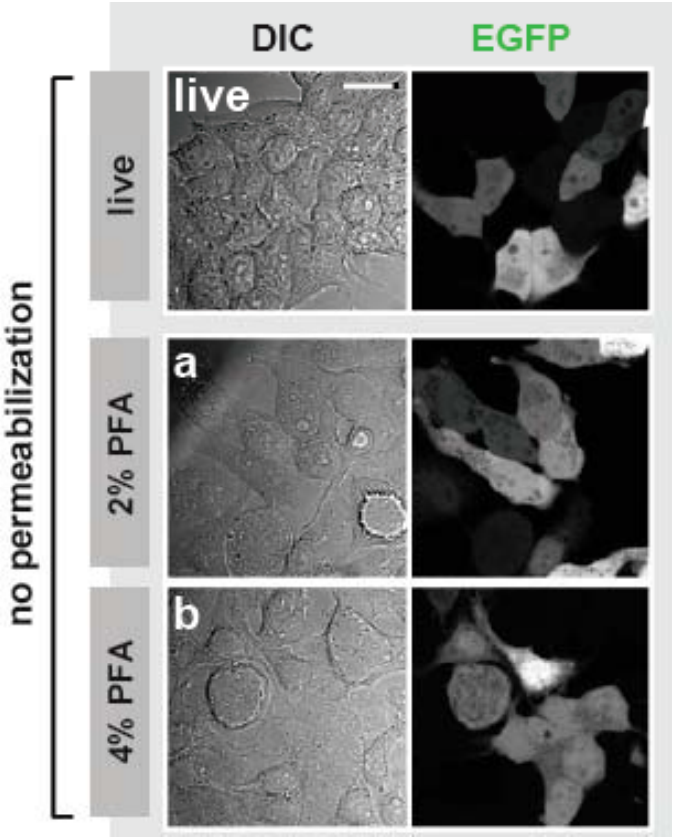


Figure 1 | Fixation and permeabilization can affect epitope



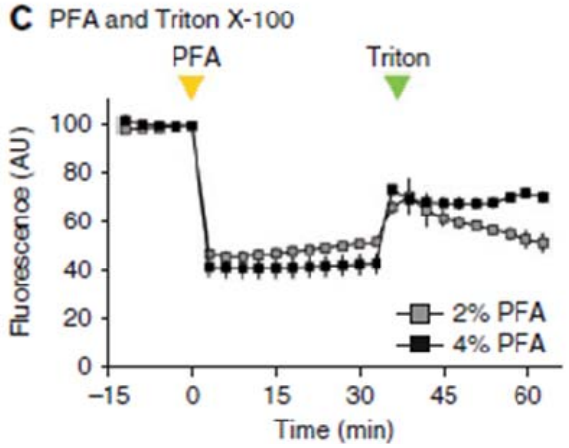
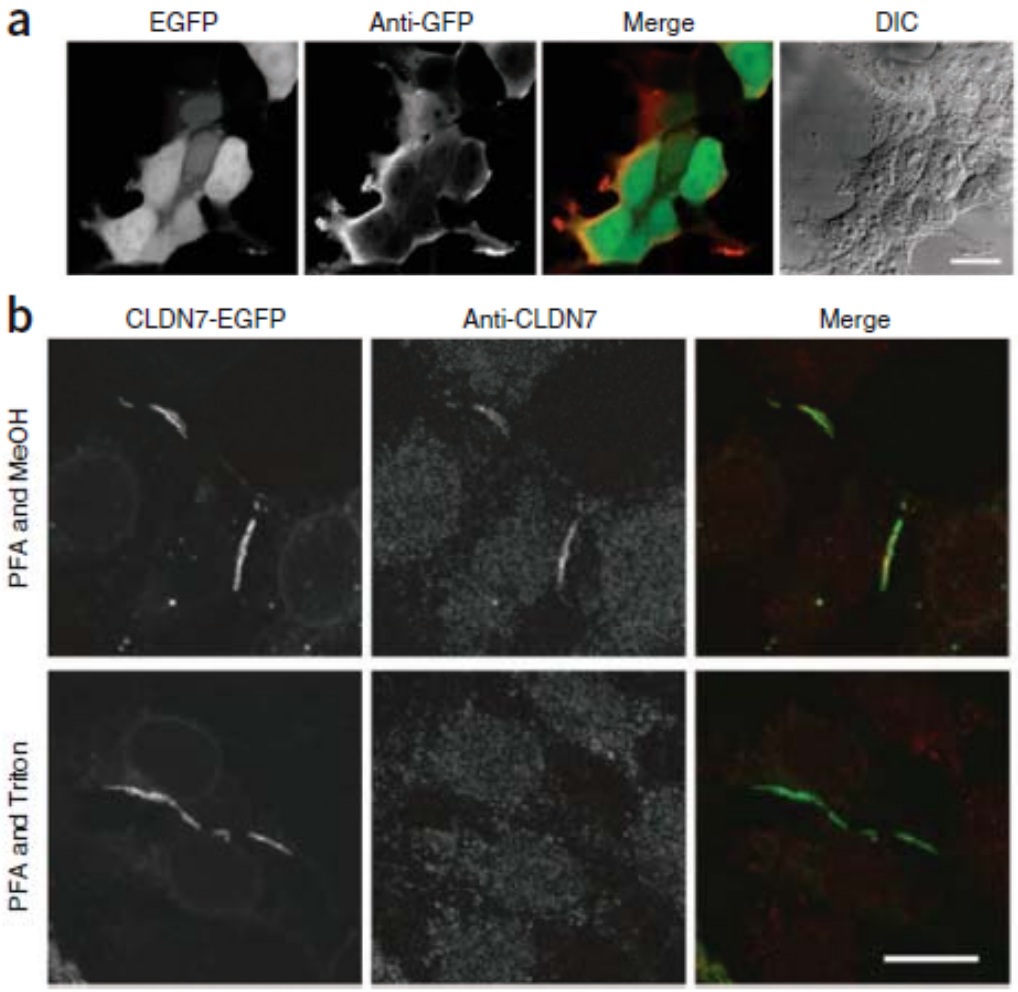


Figure 3



2% PFA +
0.05% glutaraldehyde

2% PFA +
MetOH (-20C)
1min

2% PFA +
0.1% triton
15min

Figure 1 | Fixation and permeabilization can affect epitope

Why fixation artifacts?
What happens during fixation and permabilisation?

Fixation:
- arrests the sample in a live-like state
- ideally should fix everything (proteins, lipids, nucleic acids, …)
- must not block access for labels (antibodies, lipid dyes, DNA dyes, …)

Common fixatives:
- dehydrating:
  - methanol (precipitates proteins)
- aldehydes (crosslinking):
  - formaldehyde
  - glutaraldehyde

Permeabilisation:
- opens cell boundaries (plasma membrane, organelles) to antibodies and other labels
- should not affect the shape of the cell or organelles
- should leave localisation of proteins in membranes intact

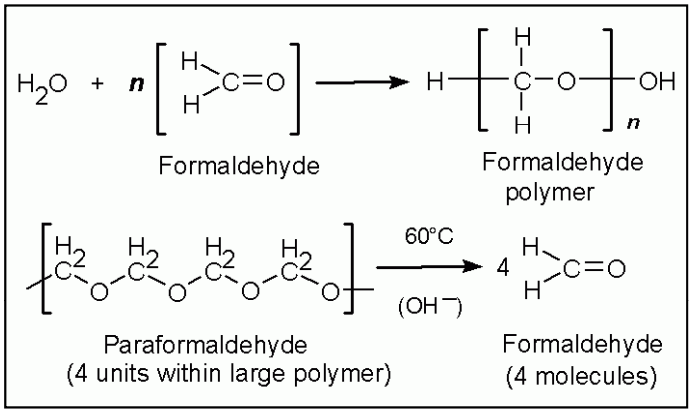
Permeabilising agents:
- saponin
- non-ionic detergents
- methanol

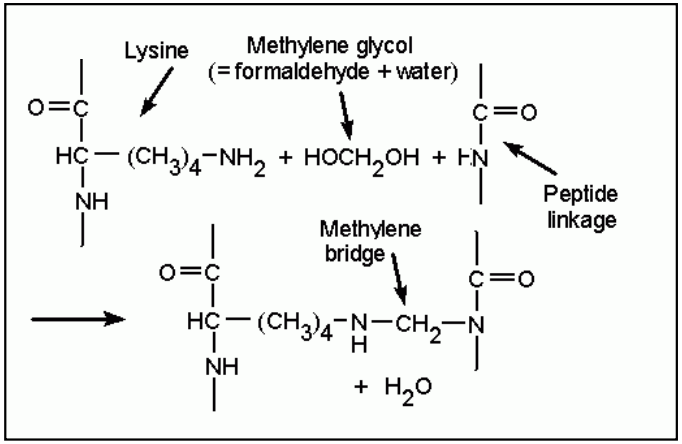
dehydrating fixatives:
methanol, ethanol, acetone

- for microscopy, usually methanol is used
- replaces water from the proteins surface, thereby inducing precipitation
- low temperature (usually -20C) and short incubation avoid denaturation of proteins (preserves antigens for staining)
- → *also solubilises membrane lipids (permeabilisation)*

aldehyde-based fixatives: formaldehyde, glutaraldehyde

formalin = formaldehyde gas dissolved in water
(100% = 37% w/v or 40% v/v in solution; contains 10-15% MetOH to prevent polymerisation)
paraformaldehyde (PFA) = formaldehyde polymer, powder

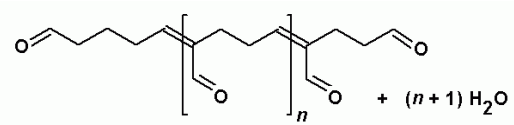


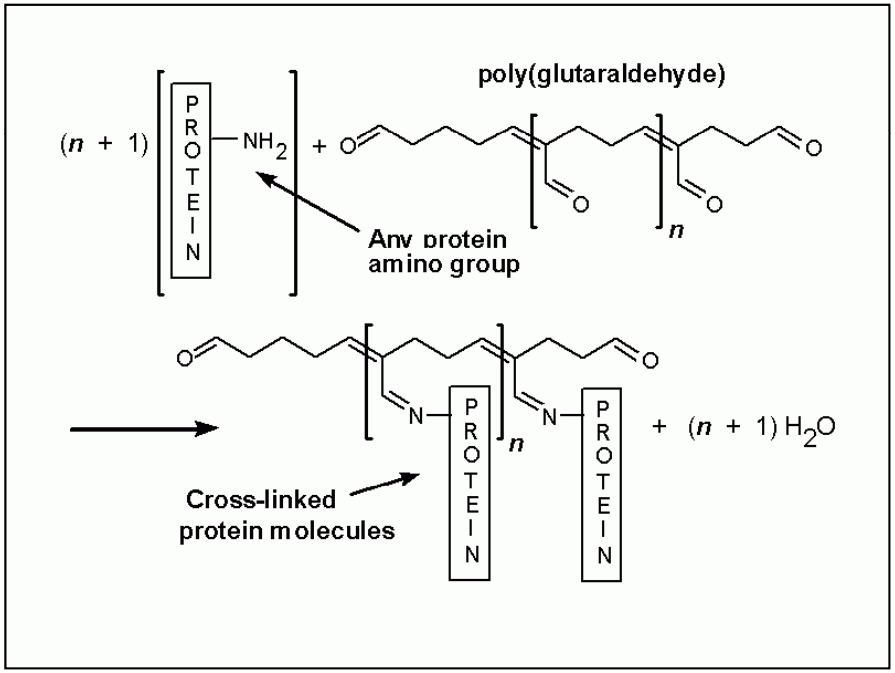


- The fixative action of formaldehyde is due to its reactions with proteins
- Substances such as carbohydrates, lipids and nucleic acids are trapped in a matrix of insolubilized and cross-linked protein molecules but are not chemically changed by formaldehyde

glutaraldehyde

poly (glutaraldehyde)





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<http://publish.uwo.ca/~jkiernan/formglut.htm>

aldehyde-based fixatives:
formaldehyde, glutaraldehyde

Advantages:

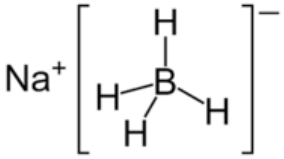
- strong crosslinking and fixation of proteins
- also fixes DNA (crosslinking histones to DNA)
- largely irreversible

Possible side effects and considerations

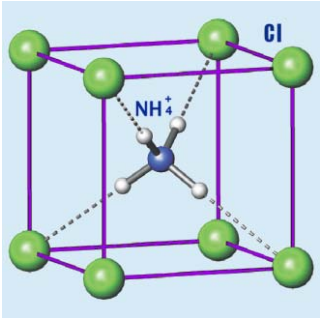
- destruction or shielding of epitopes for antibody staining *(worse with glutaraldehyde)*
- most proteins are fixed within seconds, but some very dense or loose complexes can take hours
- changed protein conformation (loss of fluorescence of fluorescent proteins)
- left-over aldehyde groups react with any amine group, i.e. also the lysins of the antibodies → high background staining *(worse with glutaraldehyde)*
- fluorescent side products (aldehyde reactions) *(worse with glutaraldehyde)*
- lipids are <u>not</u> crosslinked

Remedies

- blocking of free aldehyde groups after fixation with sodium borohydride or ammonium chloride
- good blocking with protein-rich solutions (fish skin gelatine, skimmed milk, BSA)



sodium borohydride
$NaBH_4$

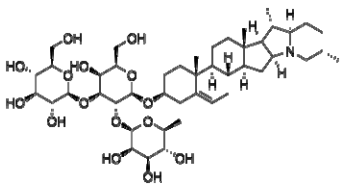


ammonium chloride
NH_4Cl

permeabilisation

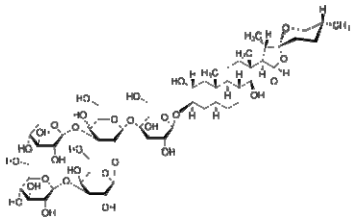
- Saponins (saponin, digitonin):
  - dissolves cholesterol from cholesterol-rich membranes (mainly plasma membrane)
  - leaves nucleus and Golgi largely intact

saponin



Saponaria officinalis (soapwort)

digitonin



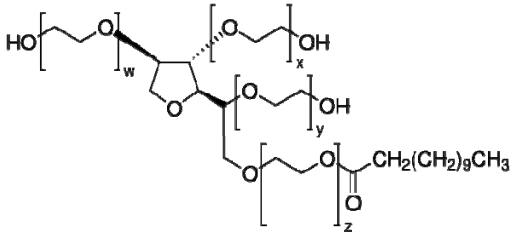
Digitalis purpurea (foxglove)

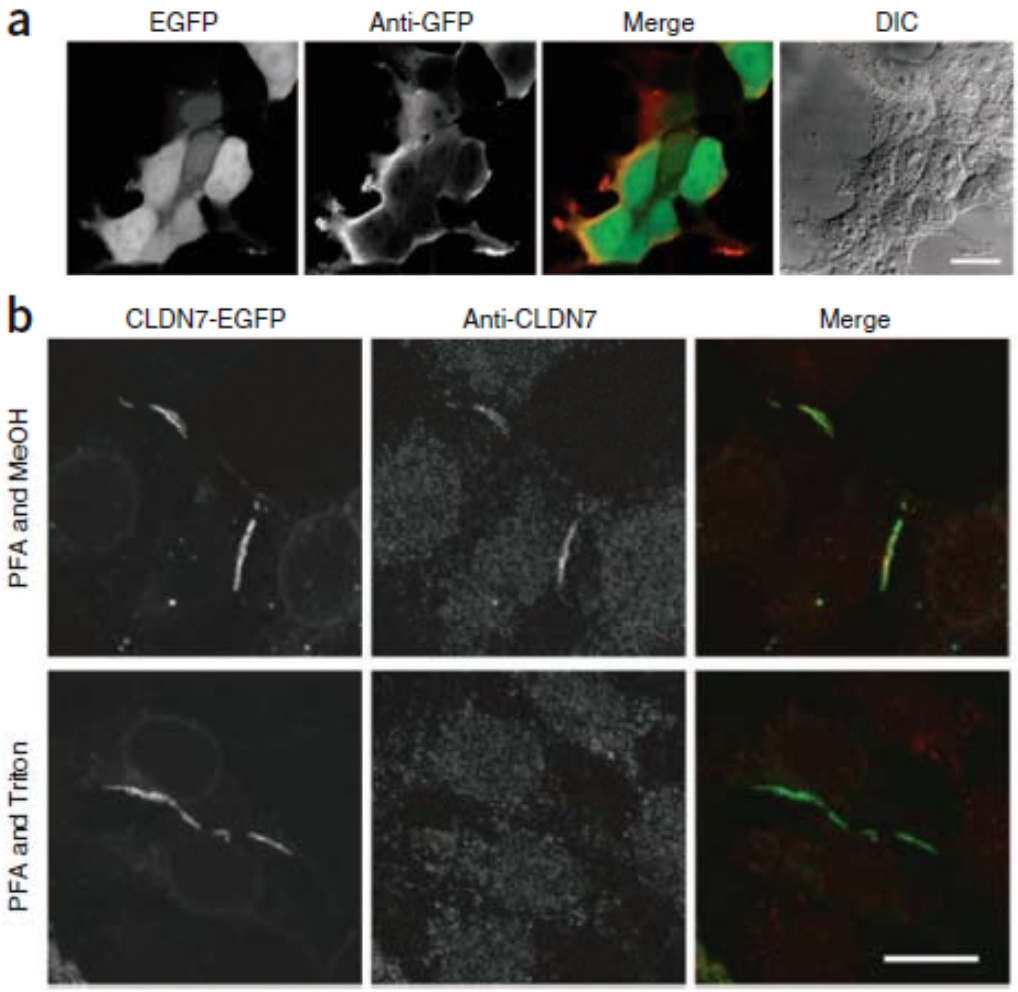
- non-ionic detergents (Triton-X100, NP40):
  - dissolve all lipids

triton X-100

- methanol:
  - dissolves all lipids
  - → *precipitates proteins at the same time*

tween-20





2% PFA +
0.05% glutaraldehyde

2% PFA +
MetOH (-20C)
1min

2% PFA +
0.1% triton
15min

Figure 1 | Fixation and permeabilization can affect epitope

Effect of fixation and permeabilisation on the cell structure and content

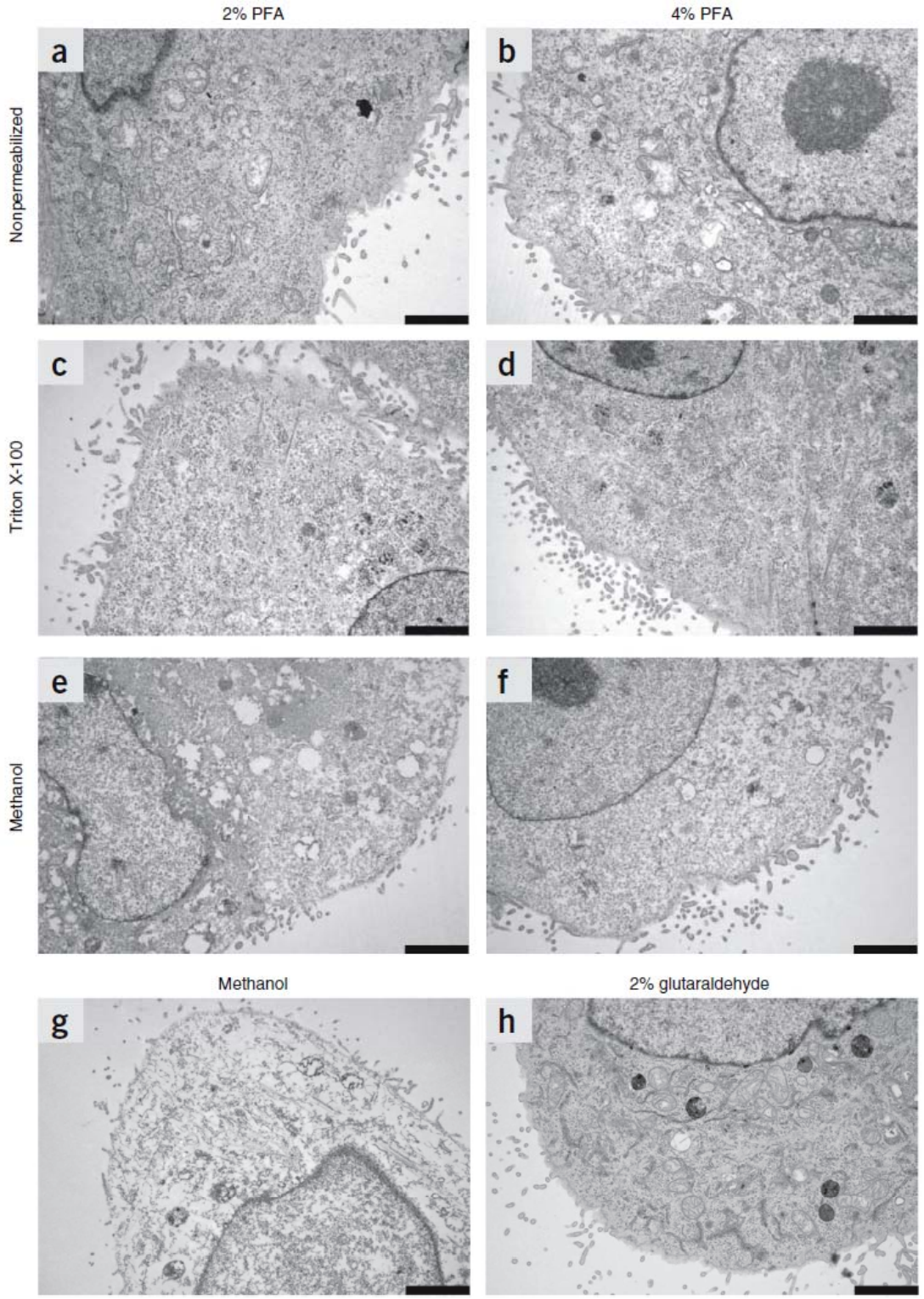


Figure 4

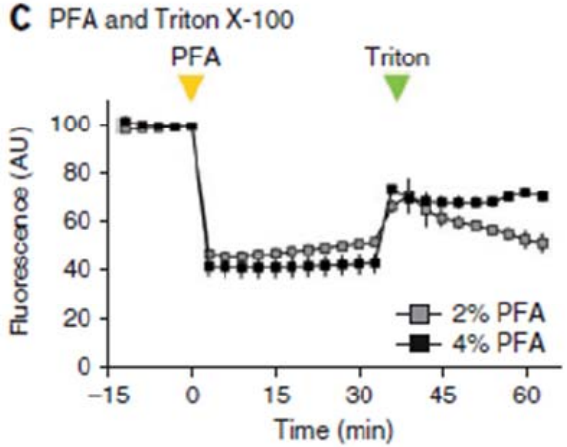
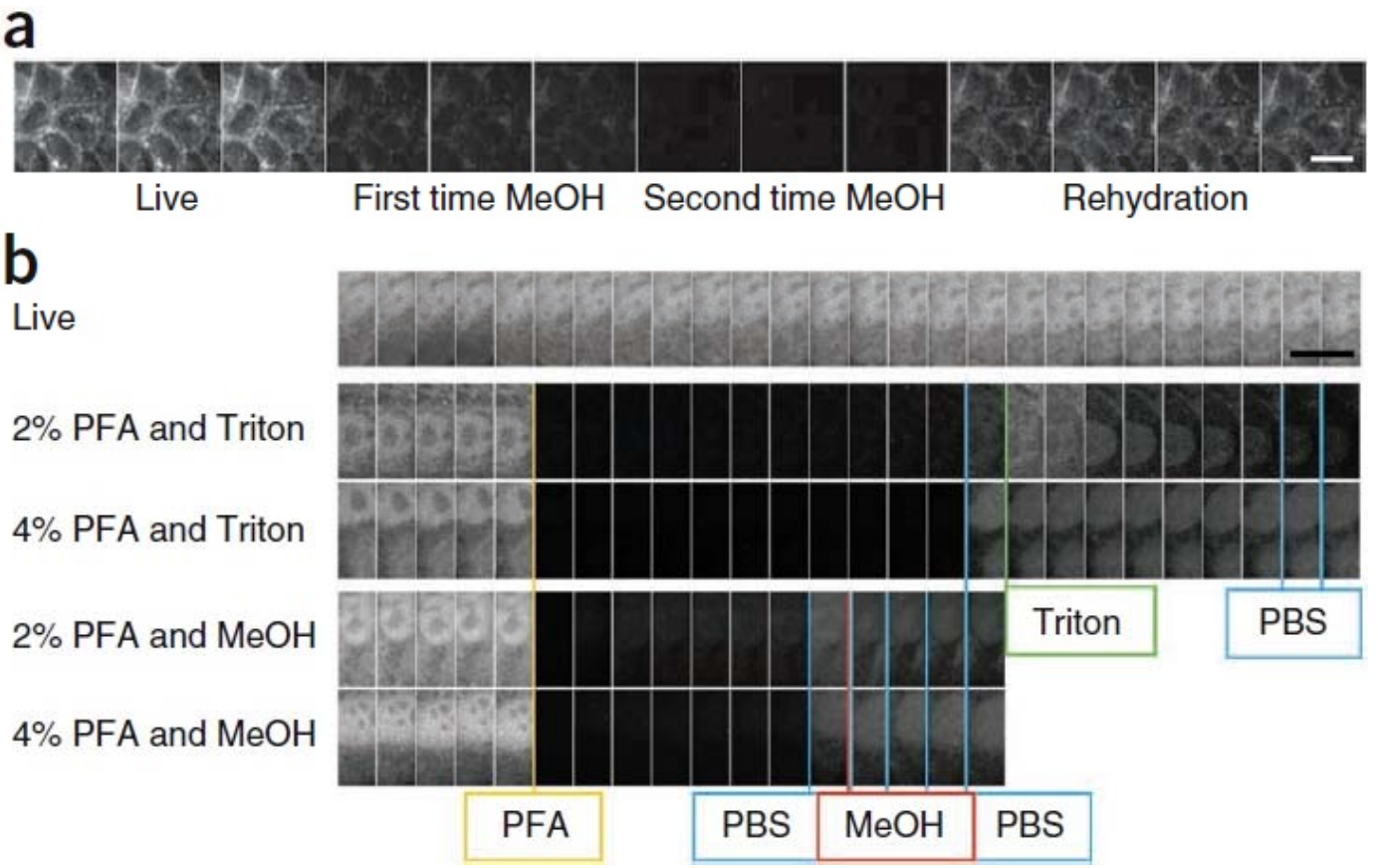

c PFA and Triton X-100
PFA
Triton
Fluorescence (AU)
100
80
60
40
20
0
−15
0
15
30
45
60
Time (min)
2% PFA
4% PFA

Fig 3


a
Live
First time MeOH
Second time MeOH
Rehydration
b
Live
2% PFA and Triton
4% PFA and Triton
2% PFA and MeOH
4% PFA and MeOH
Triton
PBS
PFA
PBS
MeOH
PBS

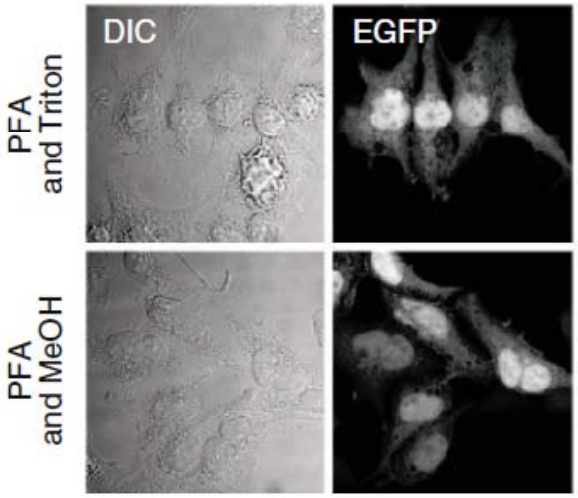

DIC
EGFP
PFA and Triton
PFA and MeOH

Fig 2b

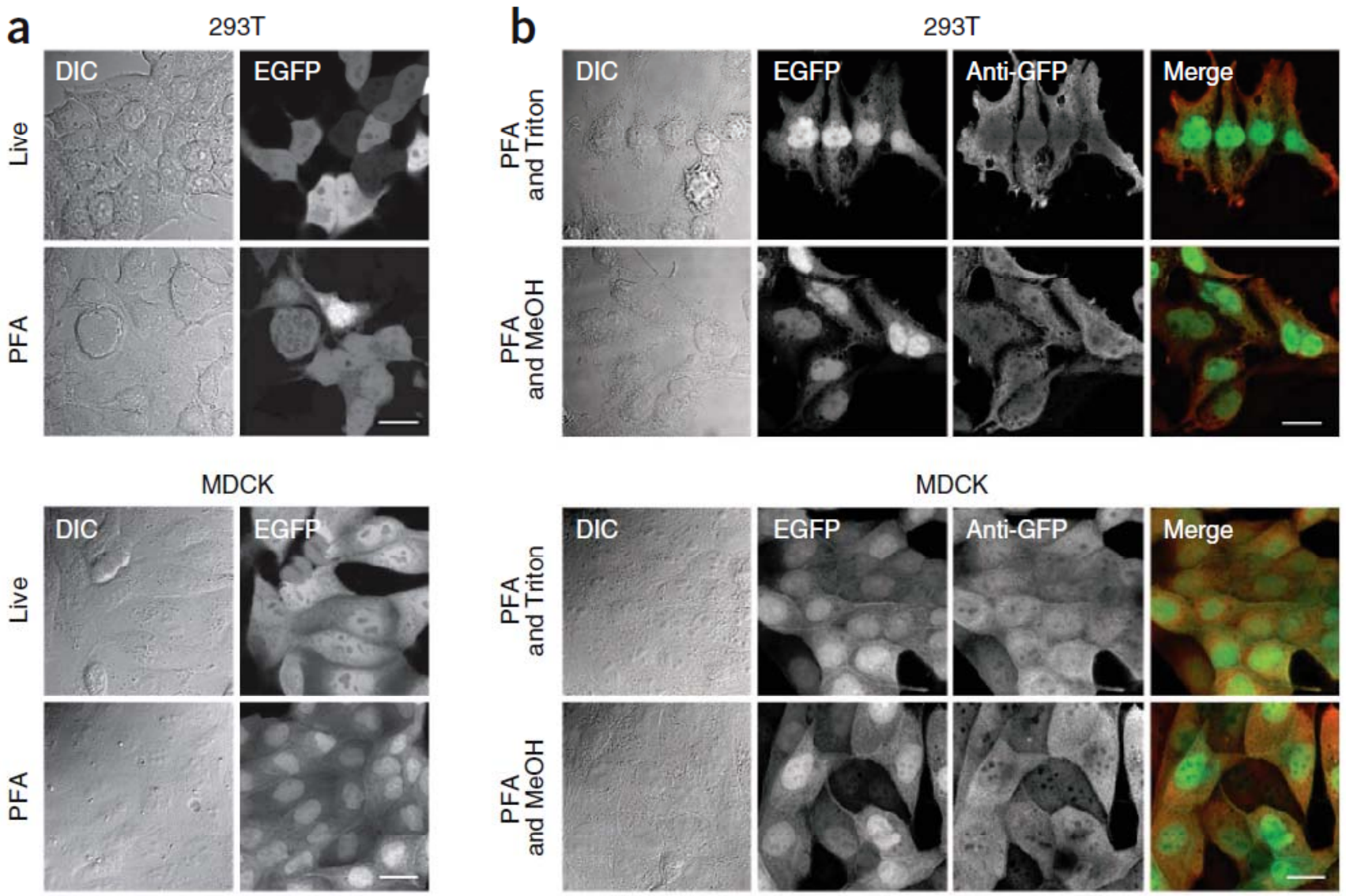

a
293T
DIC
EGFP
Live
PFA
MDCK
DIC
EGFP
Live
PFA
b
293T
DIC
EGFP
Anti-GFP
Merge
PFA and Triton
PFA and MeOH
MDCK
DIC
EGFP
Anti-GFP
Merge
PFA and Triton
PFA and MeOH

Figure 2

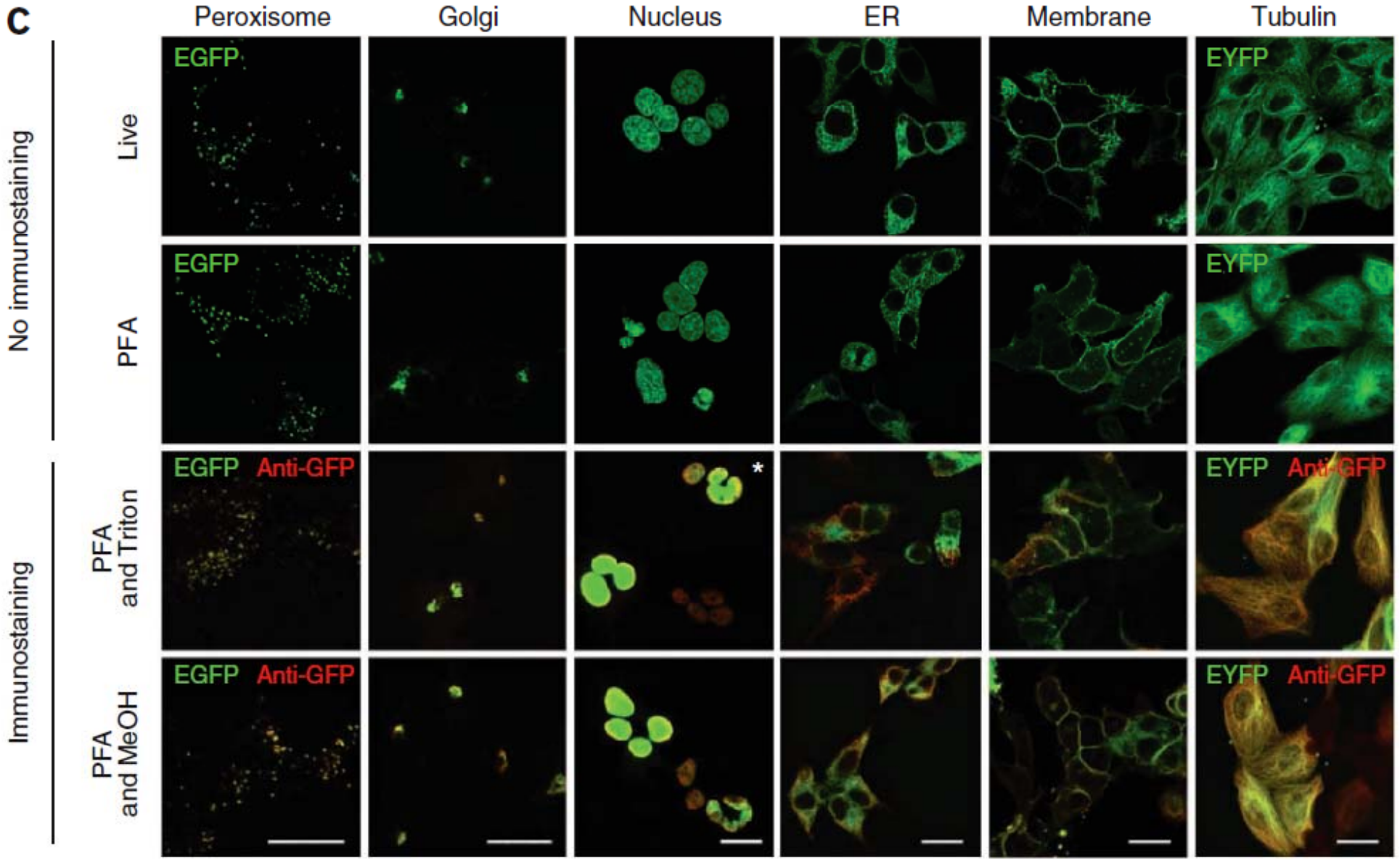
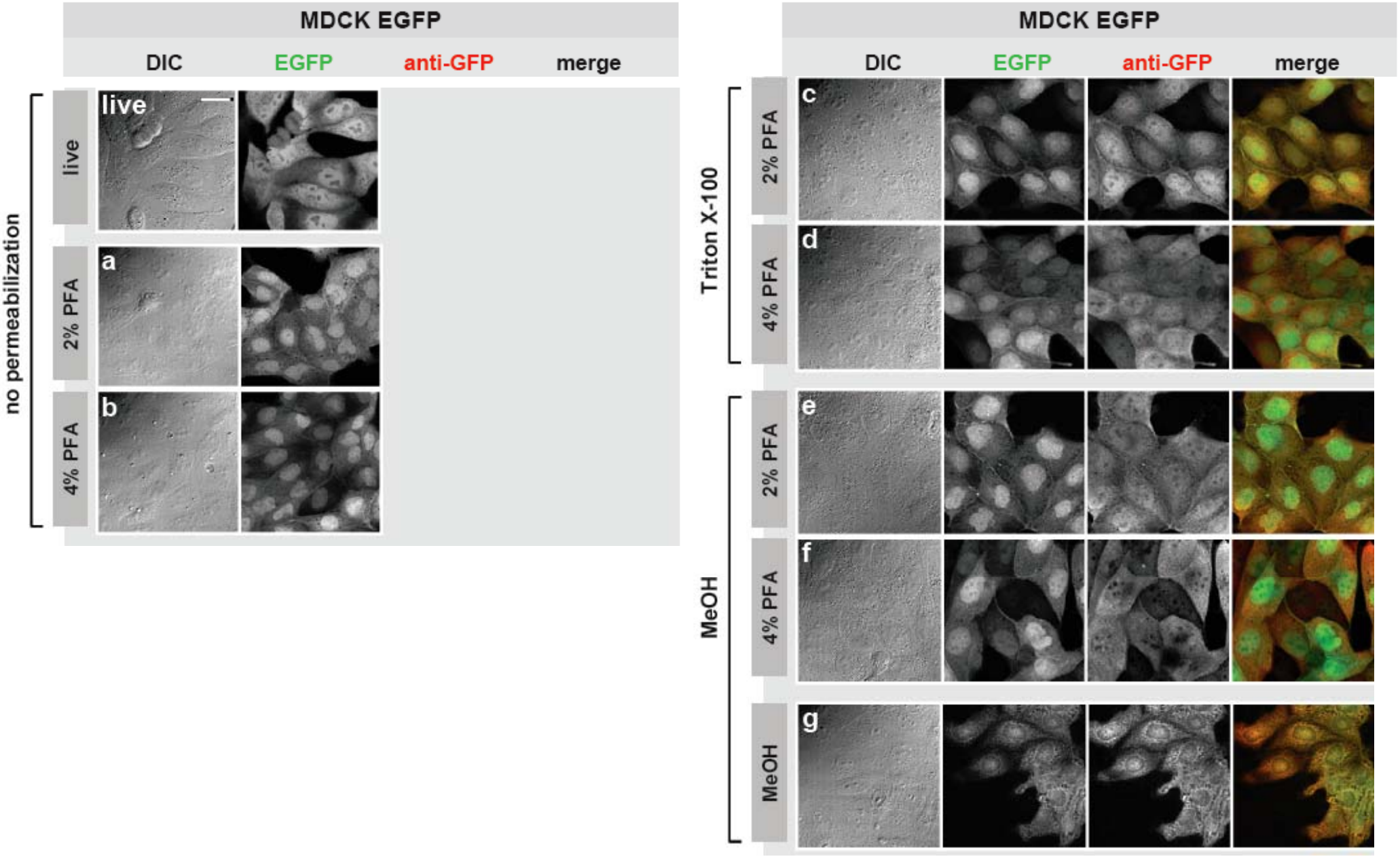
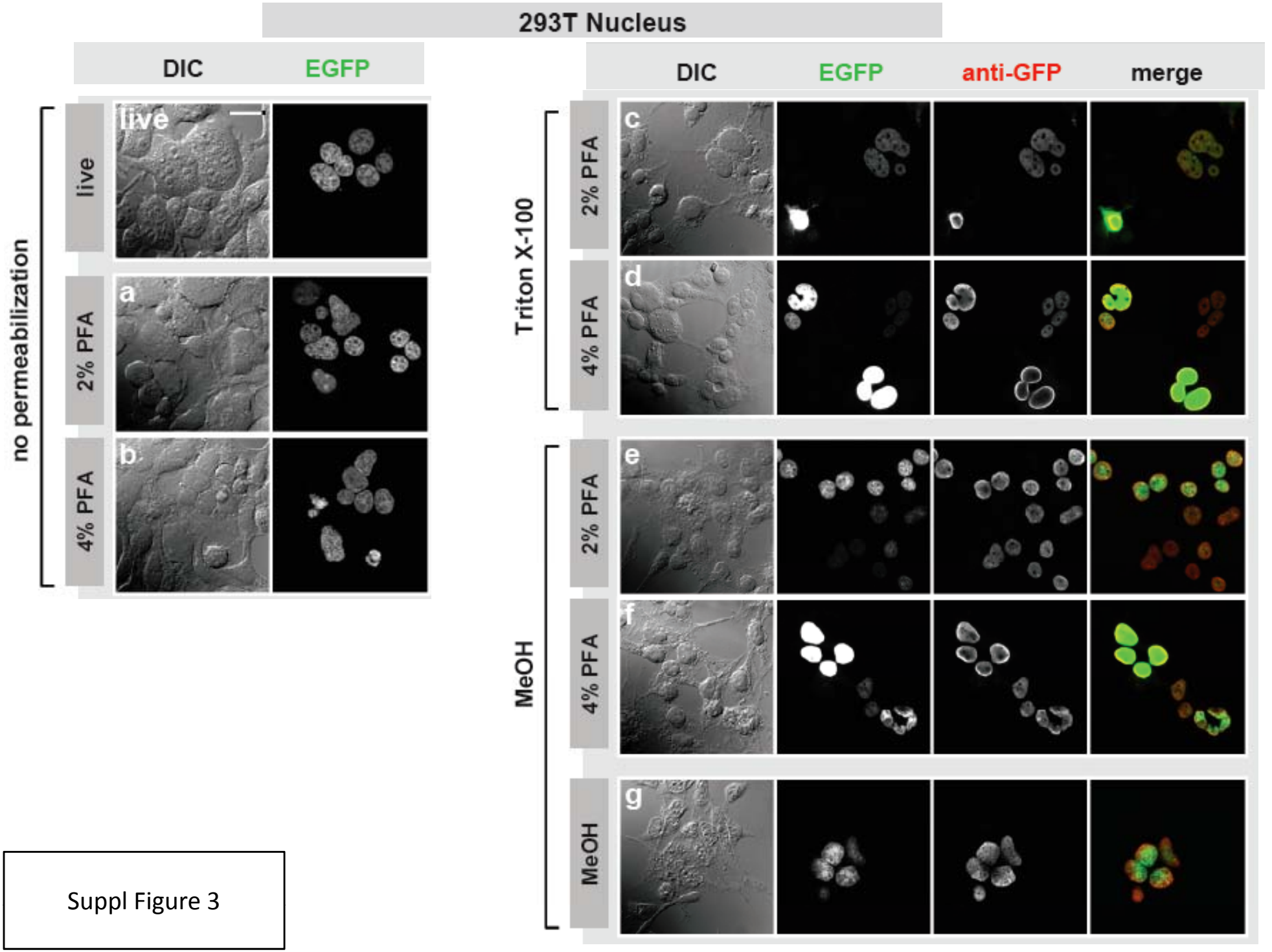

c
Peroxisome
Golgi
Nucleus
ER
Membrane
Tubulin
No immunostaining
Live
PFA
Immunostaining
PFA and Triton
PFA and MeOH
EGFP
EYFP
Anti-GFP
*

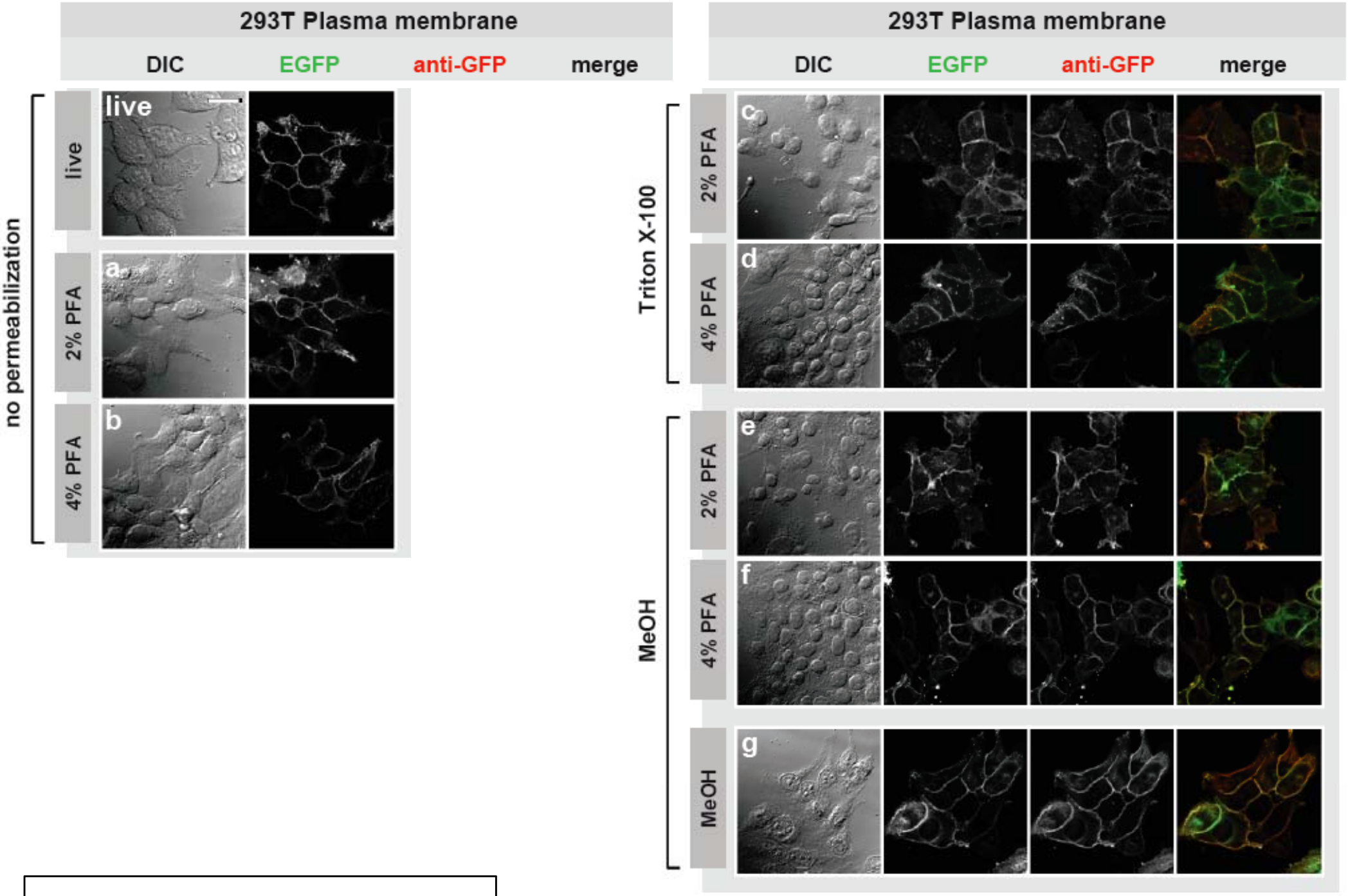
Figure 2



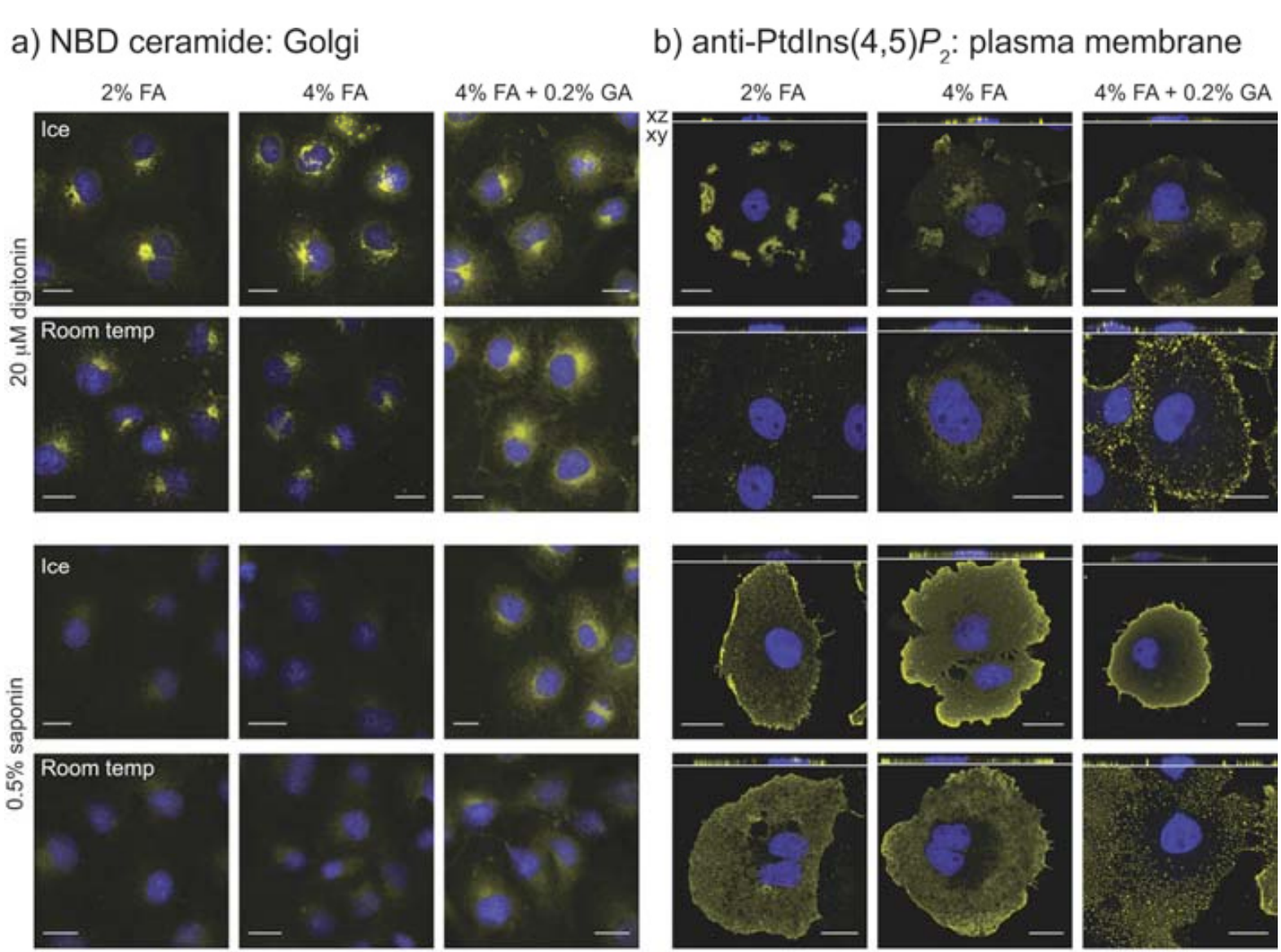
suppl Figure 2



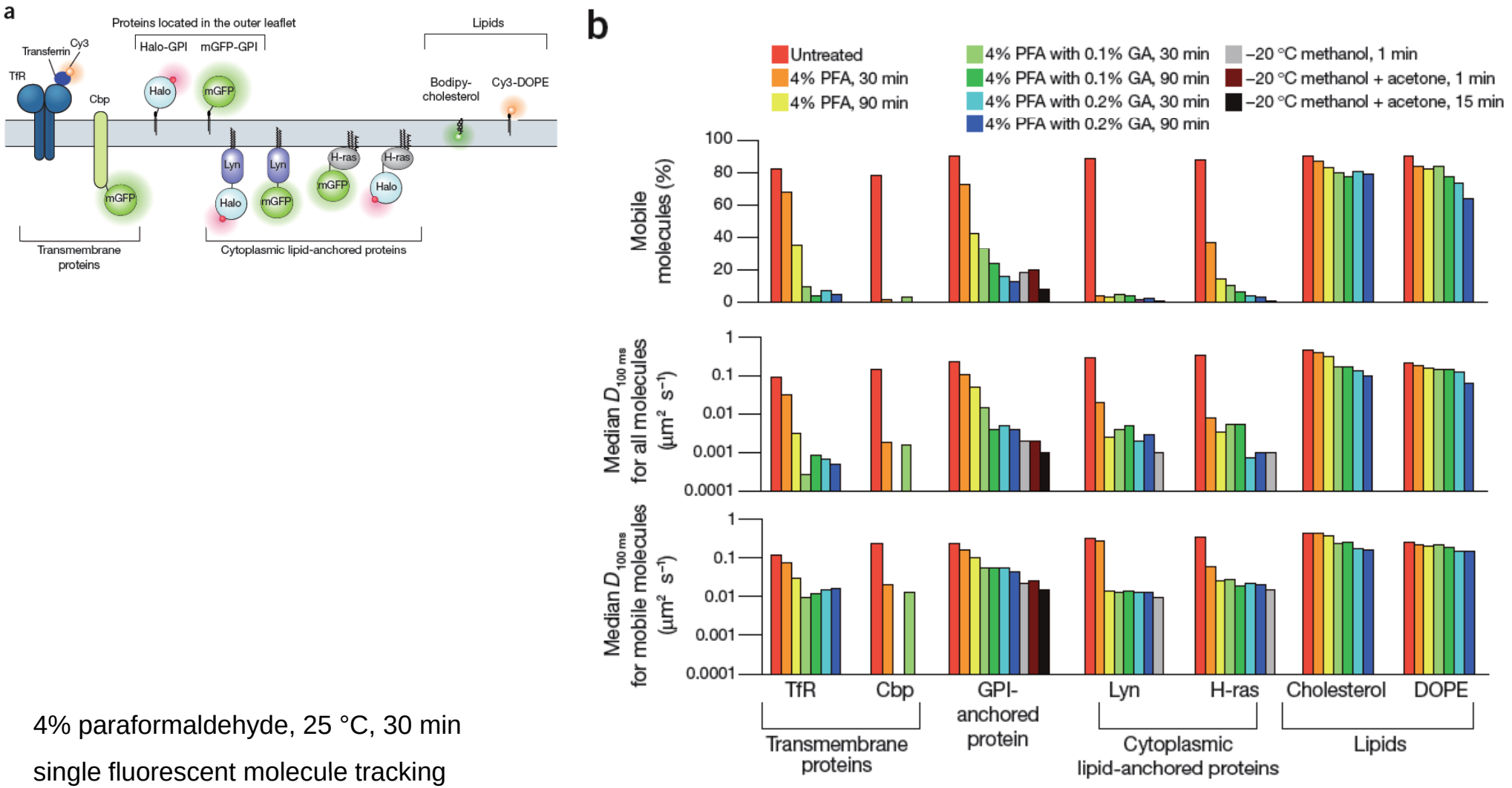
Suppl Figure 3



suppl Figure 7



Gerald R. V. Hammond et al.
Biochem J. 2009 :422(Pt 1): 23–35.

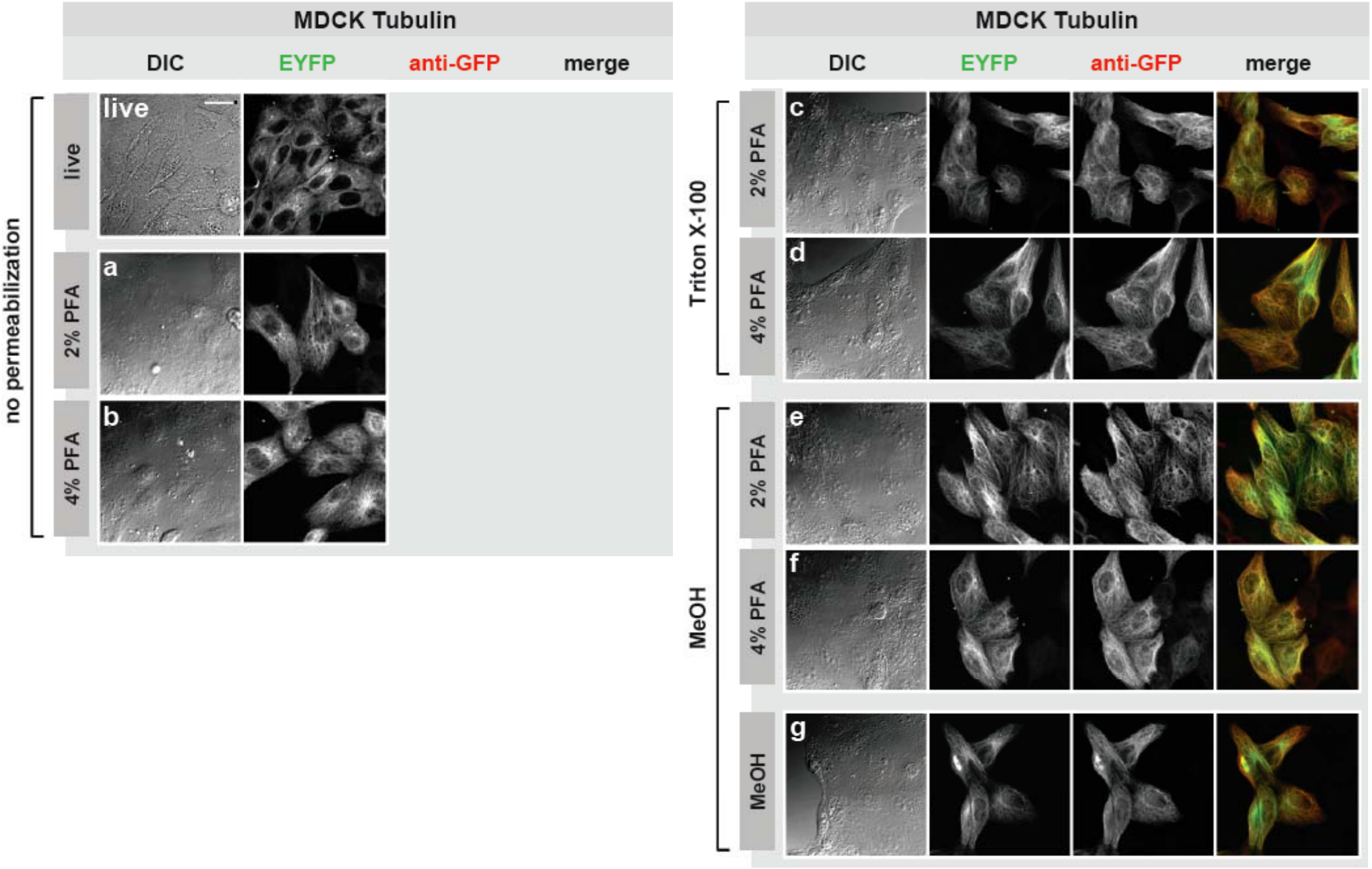


- 4% paraformaldehyde, 25 °C, 30 min
- single fluorescent molecule tracking

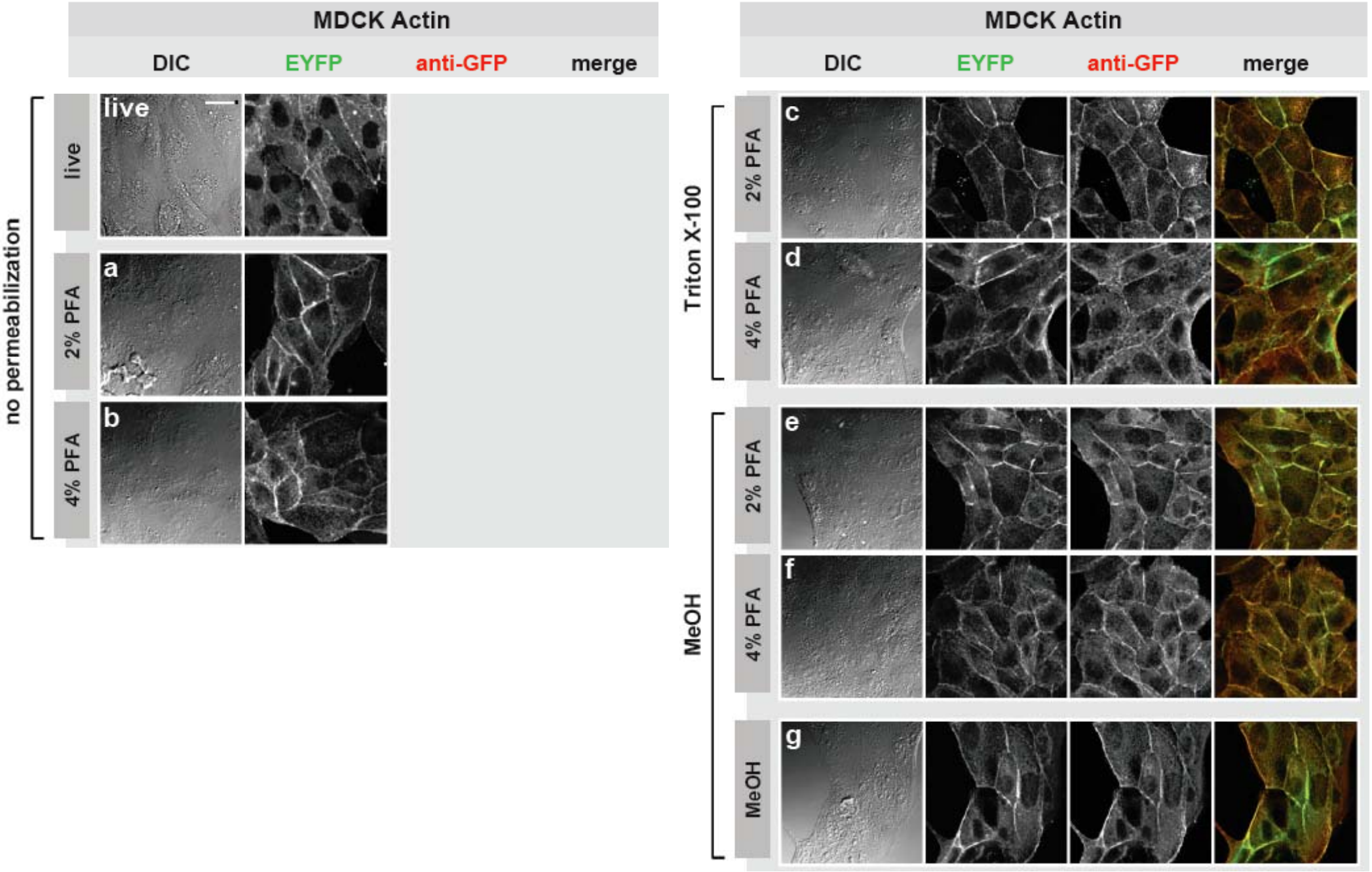
NATURE METHODS | VOL.7 NO.11 | NOVEMBER 2010 | 865

Tanaka et al., Kyoto, Japan

Membrane molecules mobile even after chemical fixation



suppl Figure 8



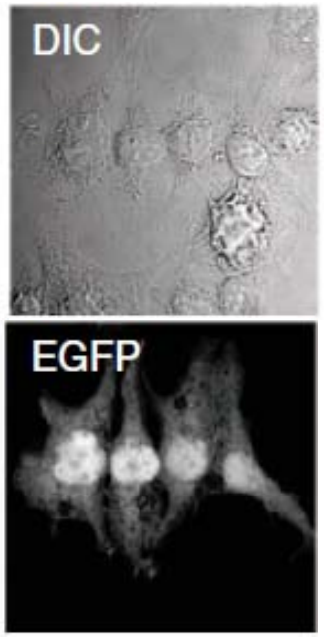
suppl Figure 9

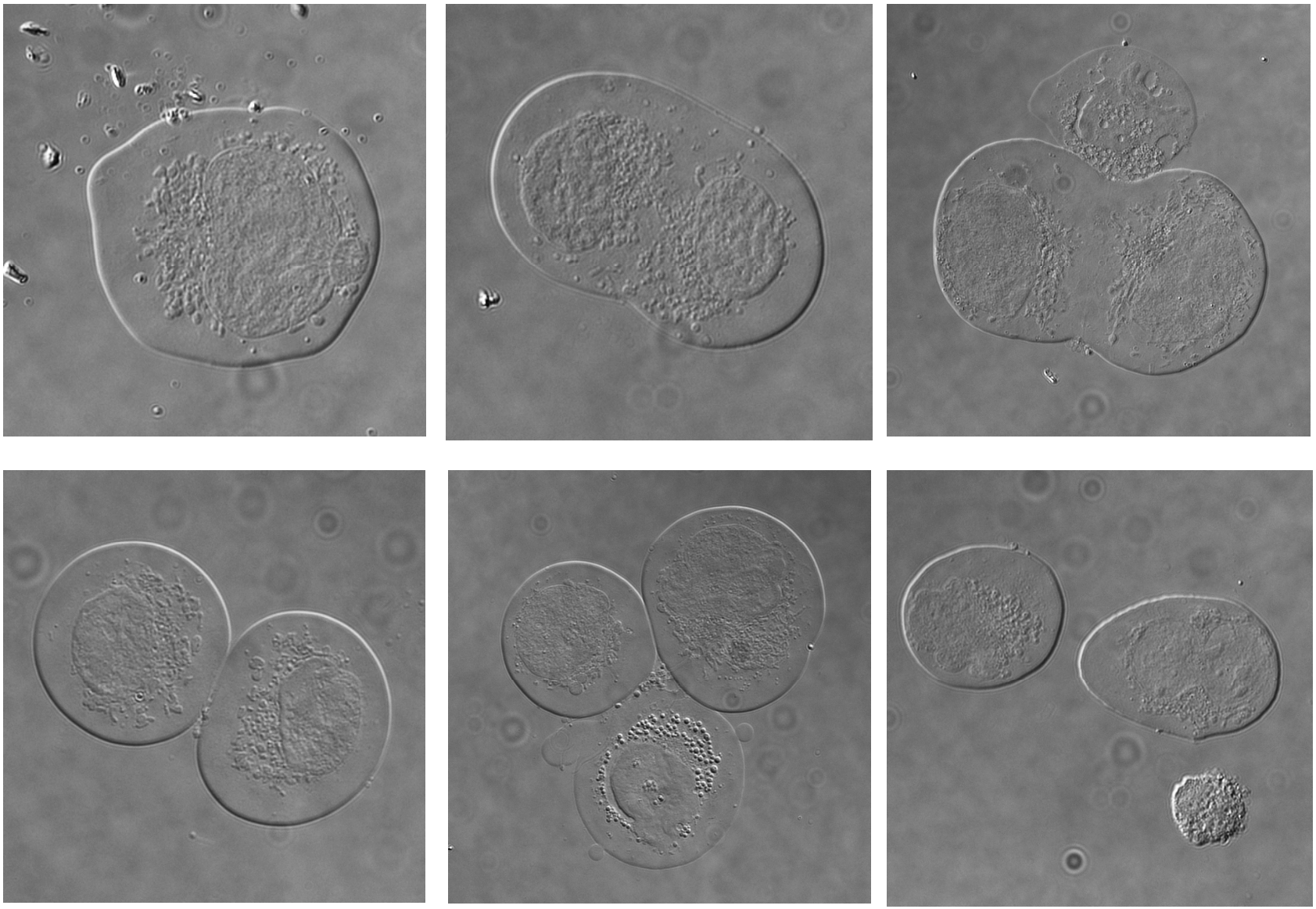
How to avoid artefacts?

- **use fresh chemicals:**
  - prepare PFA freshly, adjust the pH carefully
  - ready-to-use fixatives ('Fix&Perm') contain preservatives (avoiding polymerisation) that can induce artefatcs; work for some samples but not other
  - what's good enough for FACS isn't necessarily good enough for microscopy
- **Controls:**
  - comparison live (fluorescent protein) / fixed (antibody staining)

    → *caution: fluorescent proteins tend to dimerise / oligomerise and can interfere with protein functions / interactions*
  - comparison various fixation / permeabilisation chemicals, protocols and conditions
  - comparison various cell types
  - positive control: known physiological effect (change in expression and / or localisation of protein of interest)
- **Observation:**
  - optimise cell viability before fixation
  - observe samples throughout fixation / permeabilisation process
  - good brightfield images! (DIC, phase contrast, darkfield)

good brightfield images!





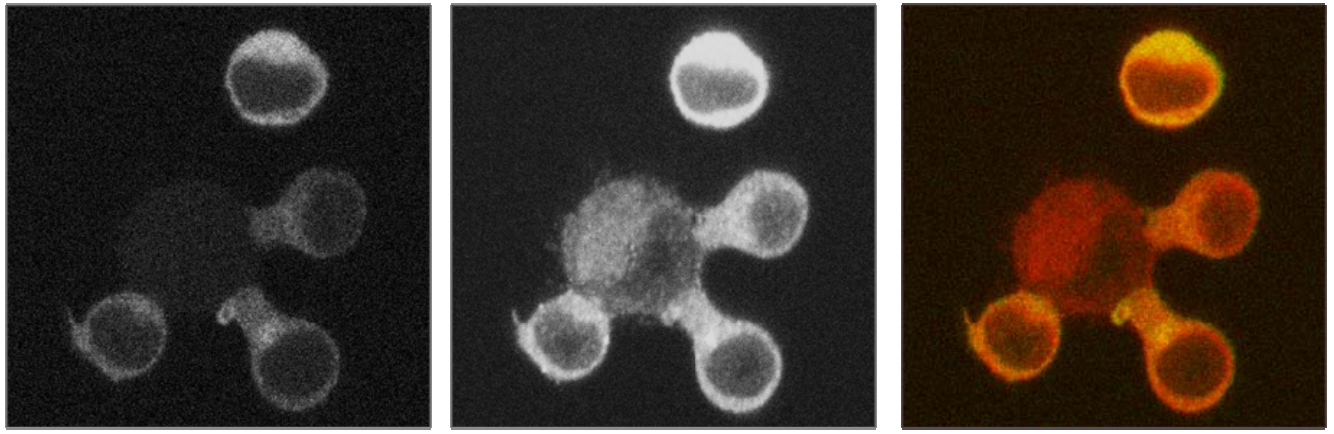
DIC images of Jurkat cells

control: physiological effect

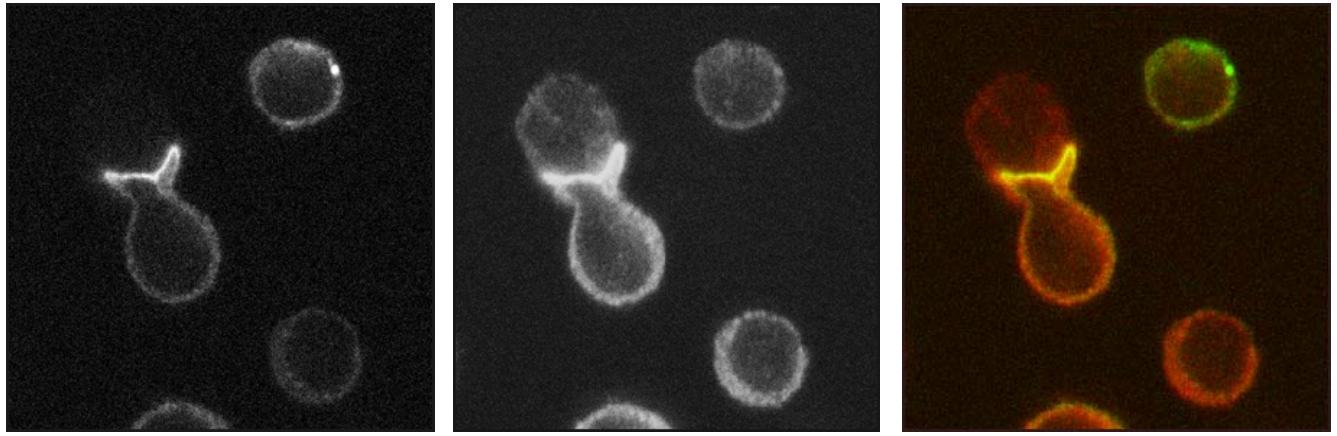
GFP

anti-GFP
Alx-633

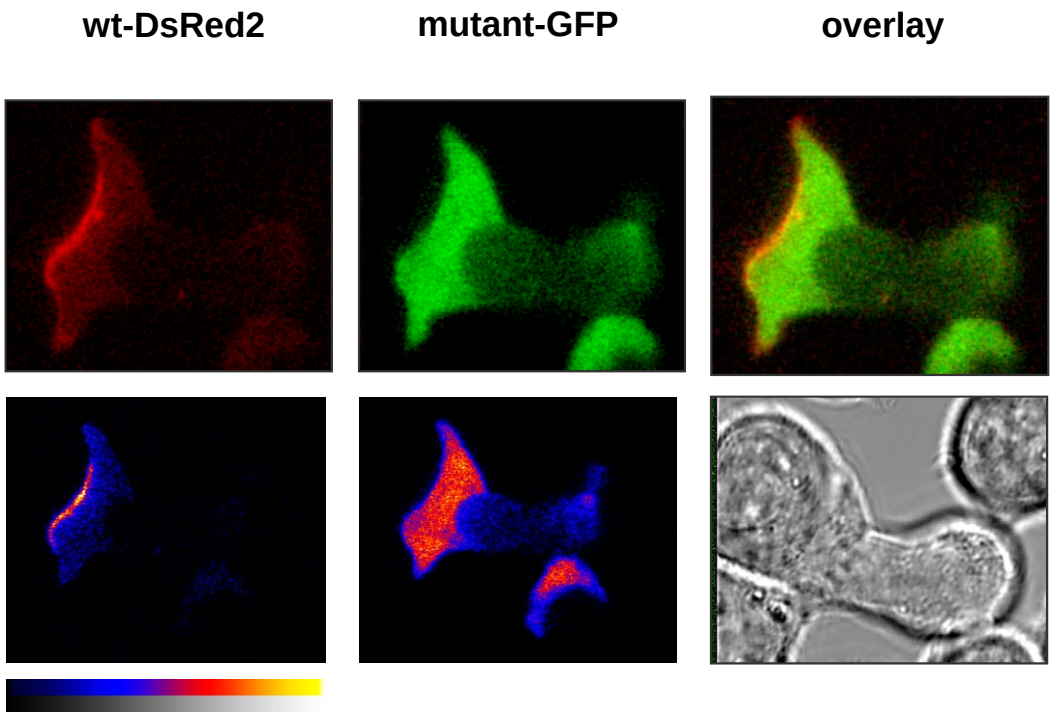
neg control
(unstimulated)

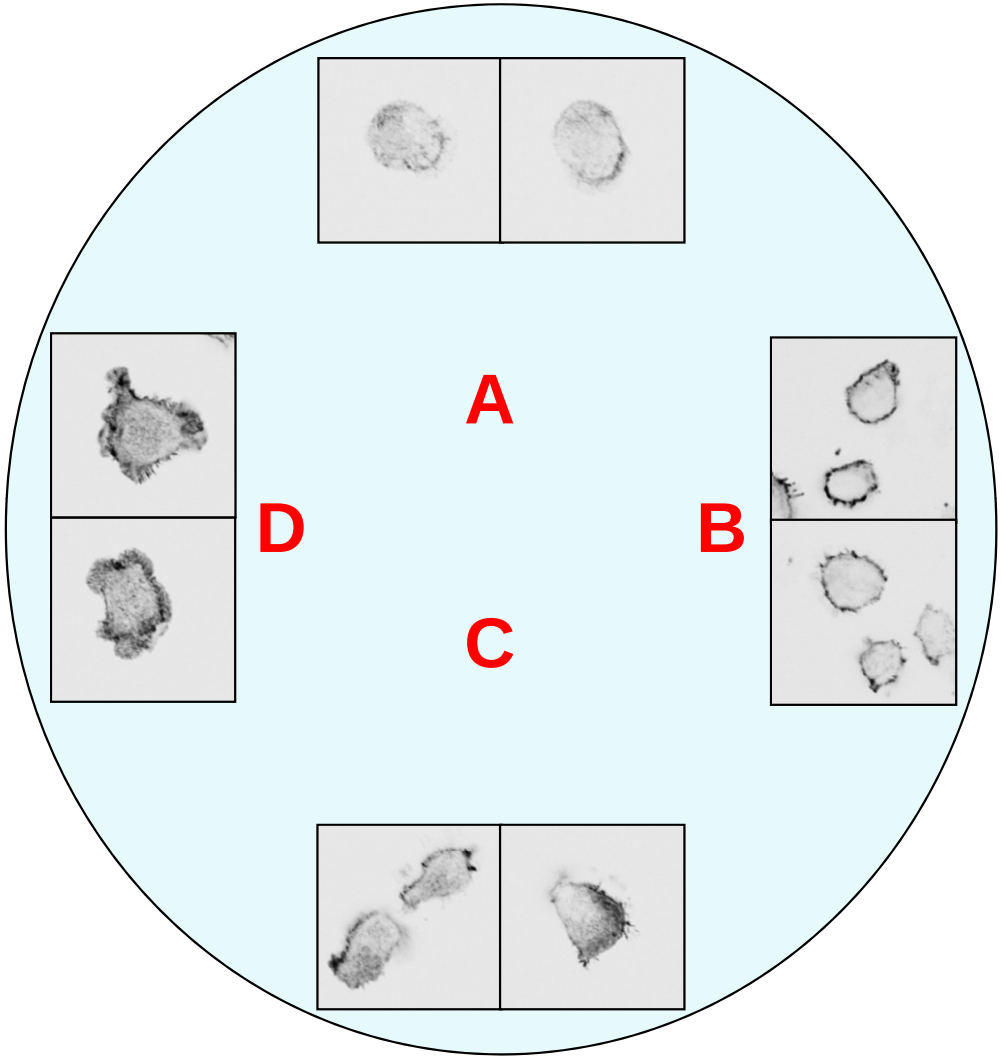


stimulation
(PKD translocation)



double-transfection wildtype / mutant




A
B
C
D

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Date	Presenter	Title
27 Jan 2012	Kieran McGourty	pH measurements by fluorescence for Salmonella studies
24 Feb 2012	Martin Spitaler	fixation
23 Mar 2012	Marguerite Wasowicz	quantification of confocal images with clinical precision
27 Apr 2012		
25 May 2012		
29 Jun 2012	FILM et al.	MICROSCOPY DAY