Improvements on expansion microscopy: protein-retention expansion microscopy (proExM) and expansion FISH (exFISH)

Technical Journal Club
19.07.2016.
Orsolya Török

Microscopy-history

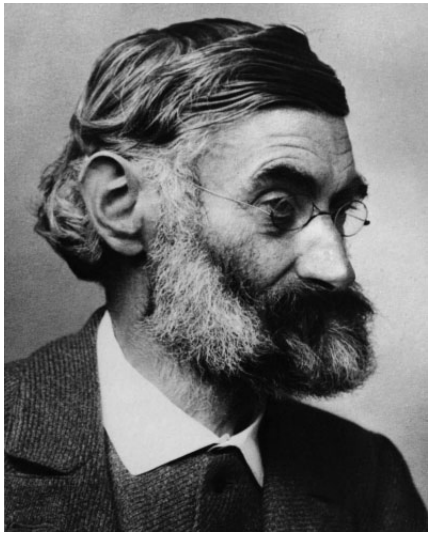
μικρός, *mikrós*, "small" and σκοπεῖν, *skopeîn*, "to look" or "see"

Zacharias Janssen (1585-1632?)

Robert Hooke (1635-1703)

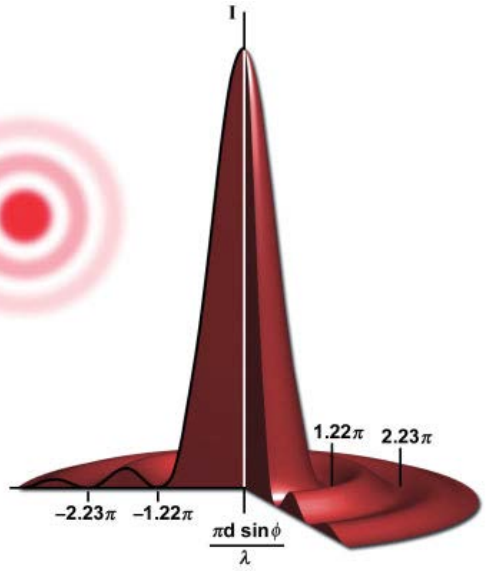
www.wikipedia.org

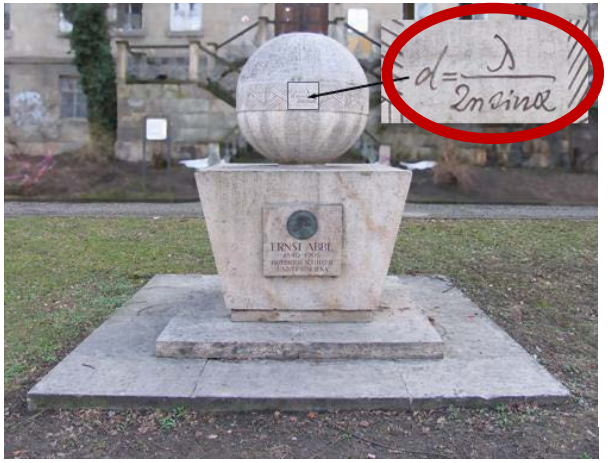
Diffraction and resolution of a point object



Ernst Abbé (1840-1905)

The diffraction pattern of a point source of light





Abbé's diffraction limit ≈ 250 nm

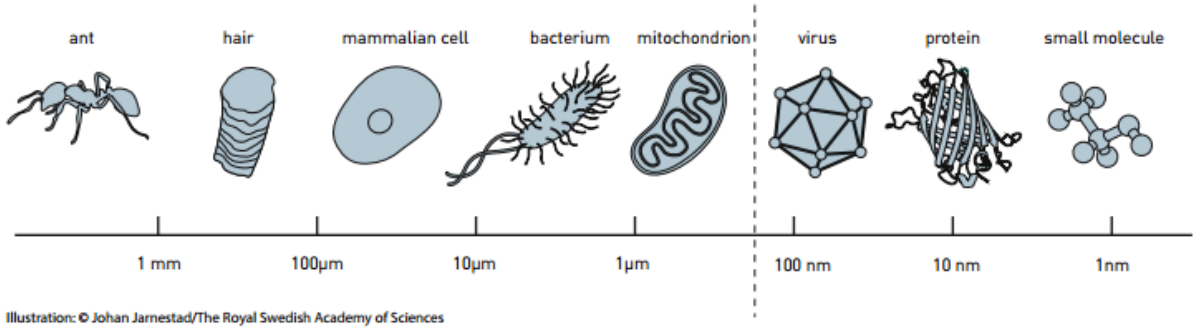
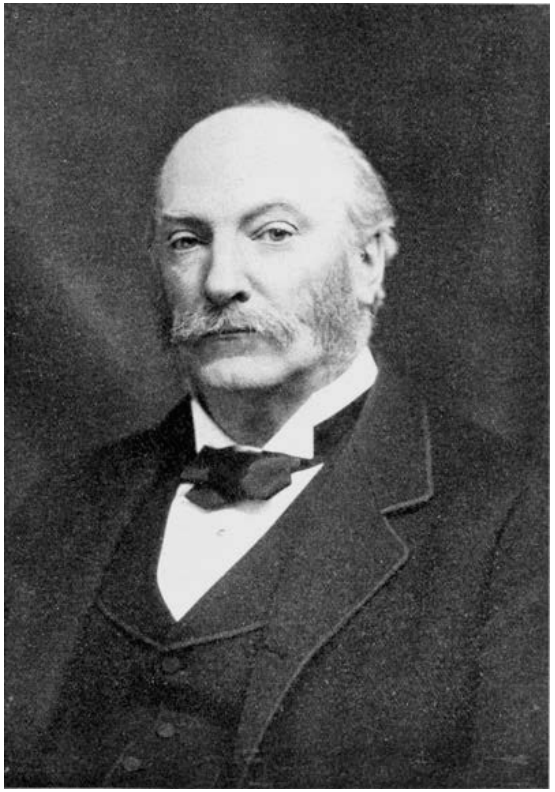


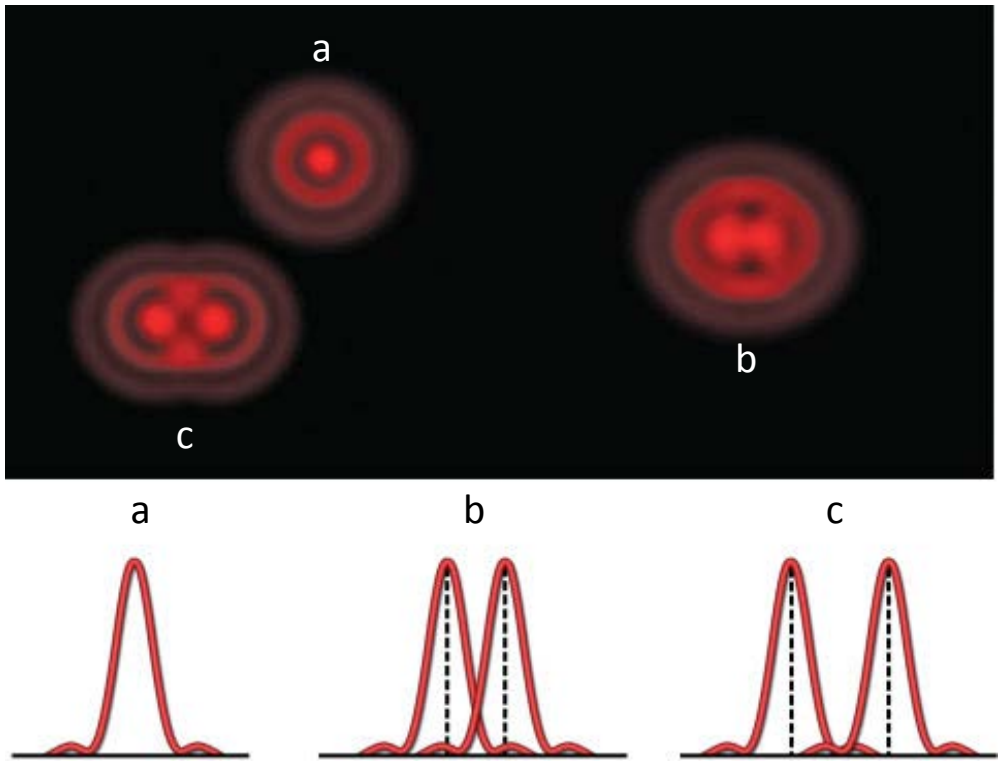
Illustration: © Johan Jarnestad/The Royal Swedish Academy of Sciences

Douglas B. Murphy, Michael W. Davidson: Fundamentals of Light Microscopy and Electronic imaging

Resolution of a point object – Rayleigh-criteria



John William Strutt Lord Rayleigh (1842-1919)



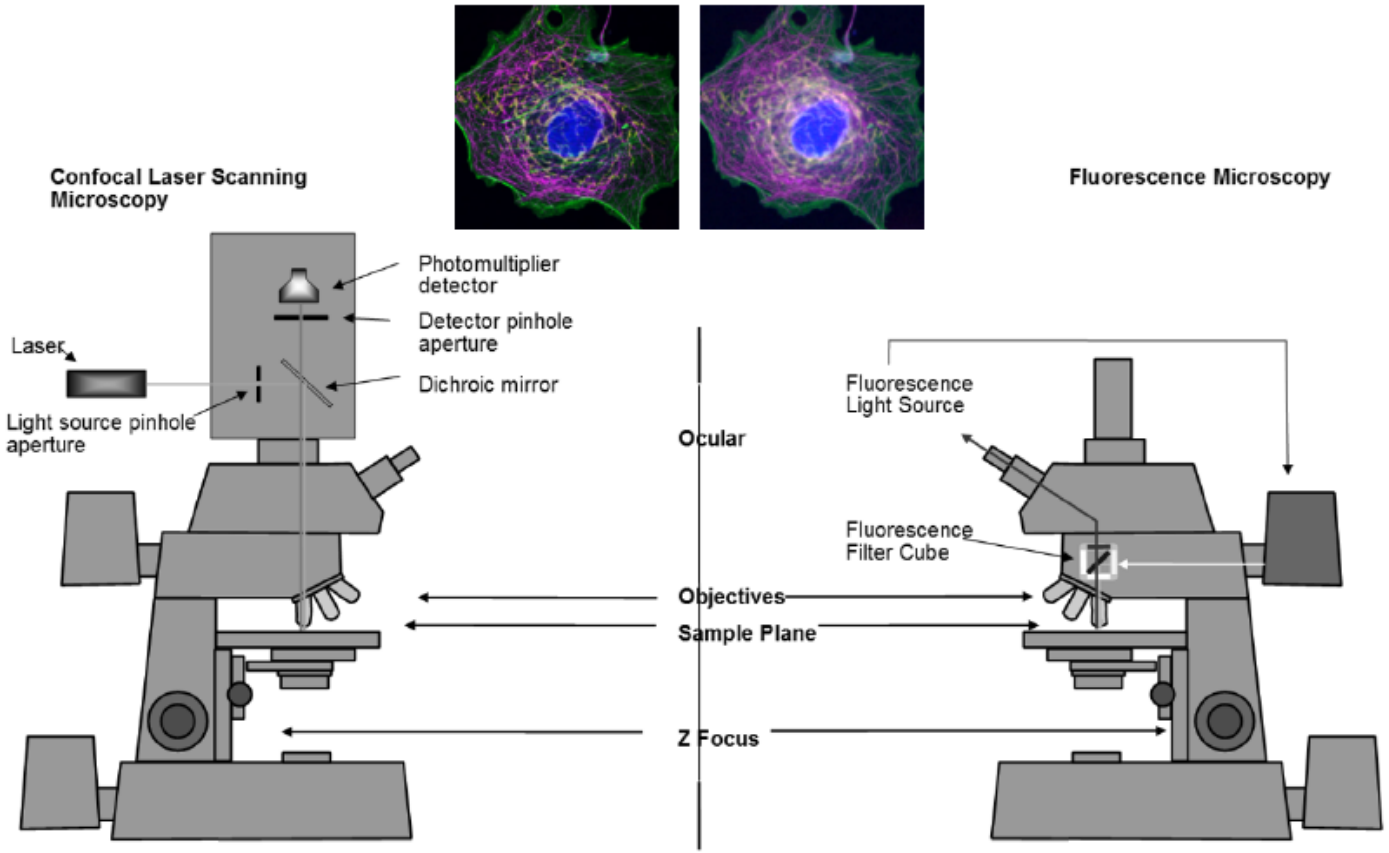
d = 0.61 λ/NA

By this criterion, two adjacent object points are defined as being resolved when the central diffraction spot (Airy disk) of one point coincides with the first diffraction minimum of the other point in the image plane.

Douglas B. Murphy, Michael W. Davidson: Fundamentals of Light Microscopy and Electronic imaging

Confocal laser scanning microscope

The sample is illuminated with laser light focused to a diffraction limited spot.
The spot is scanned in a raster fashion over the sample to illuminate fluorescent dyes.
Emission of fluorescent light is detected through a pinhole located in a confocal plane relative to the plane of focus.



Super-resolved fluorescent microscopy

All microscopy techniques that achieve a resolution higher than that defined by Ernst Abbé (approx. 250nm in x,y axis and 450-750nm in z axis).

The Nobel Prize in Chemistry 2014

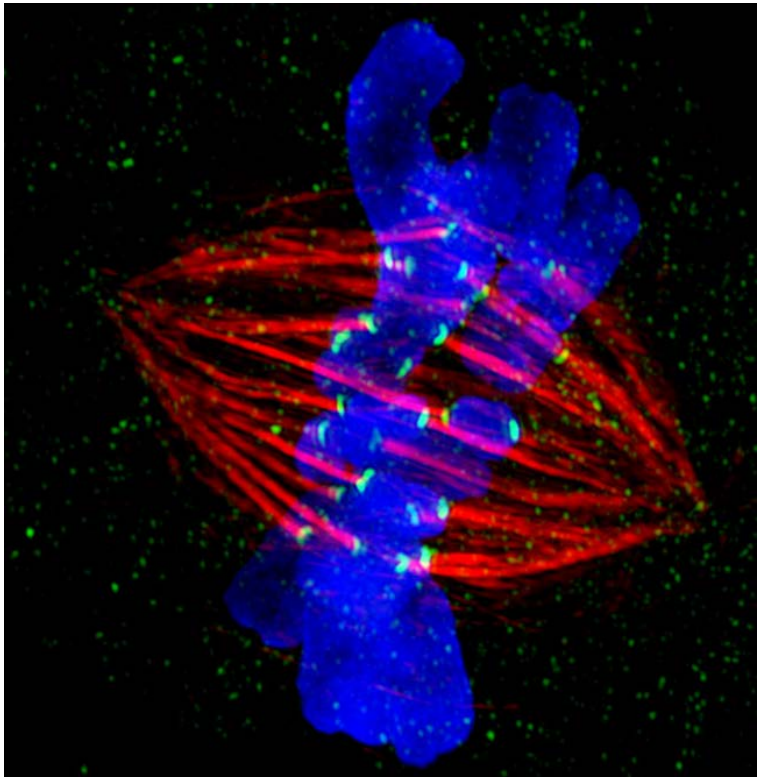
Eric Betzig | Stefan W. Hell | William E. Moerner

2 types: “super-resolved *ensemble* fluorophore microscopy” and “super-resolved *single* fluorophoremicroscopy”.

www.nobelprize.org

Disadvantages of superresolution microscopy:

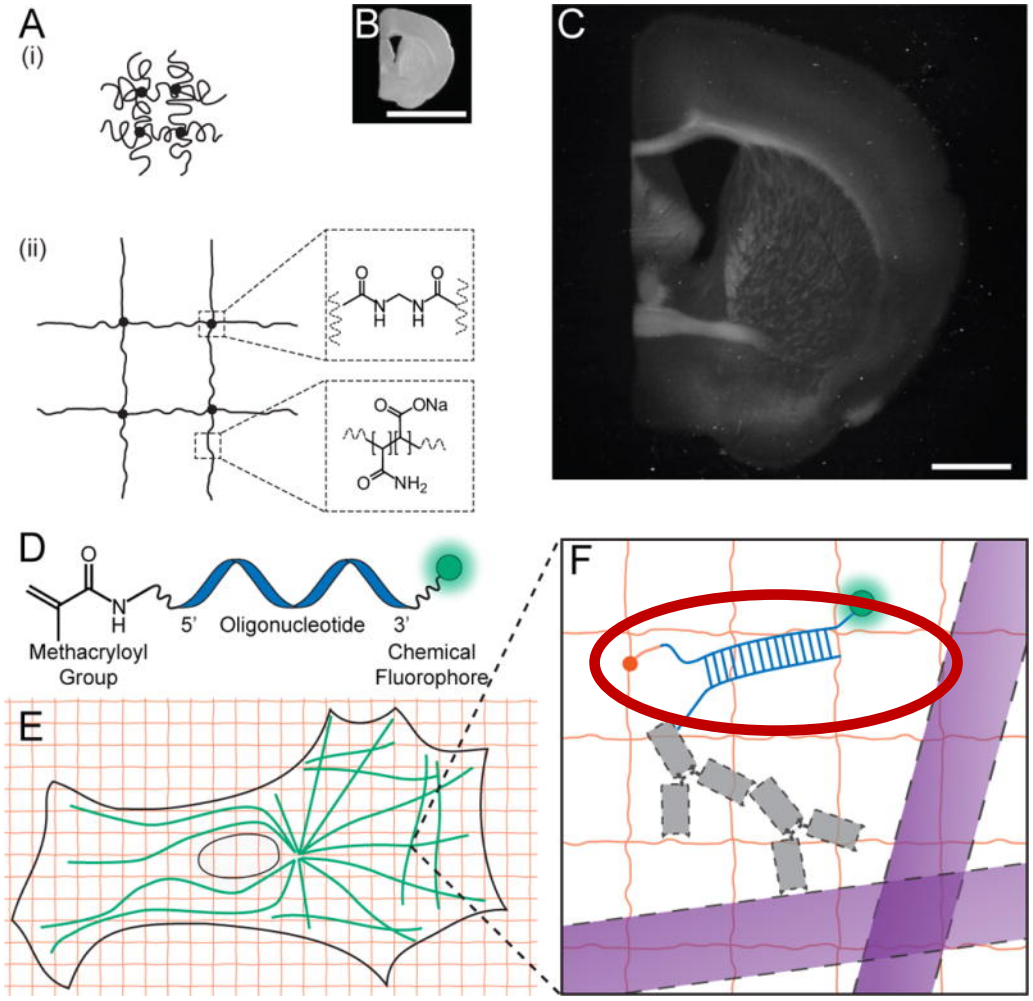
- requires specialized equipment
- long acquisition time
- high illumination intensities



http://www.imaging-git.com

Expansion microscopy (ExM)

Fei Chen, Paul W. Tillberg, Edward S. Boyden



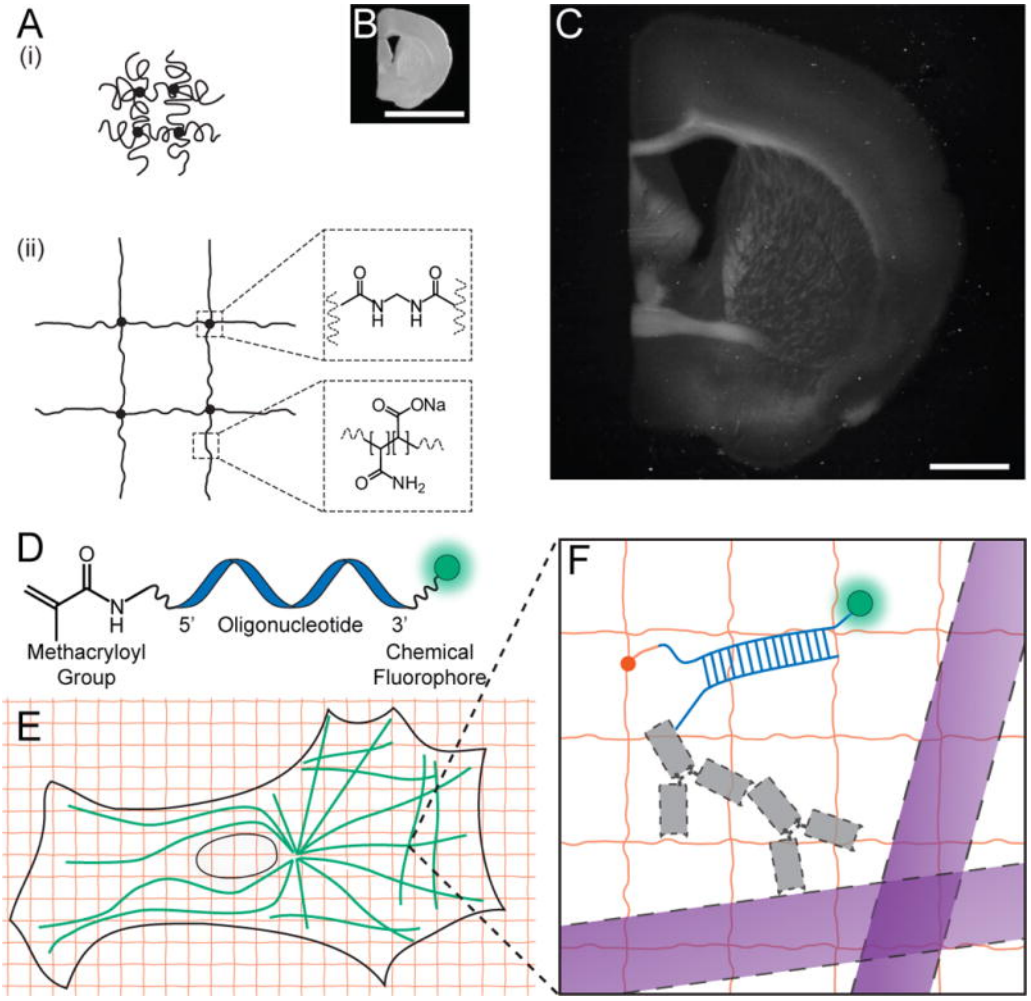
- ≈70 nm lateral resolution
- compatible with conventional diffraction-limited microscopes
- imaging at the voxel-rates of a diffraction-limited microscope and with the voxel-sizes of a superresolution microscope

1. Labelling with a gel-anchorable fluorophore
2. Swellable polyelectrolyte gel is synthesized in the sample
3. Treatment with non-specific protease.
4. Dialysis in water.

Expansion microscopy , F. Chen, P. W. Tillberg, E. S. Boyden, Science 2015. Jan 30;347(6221):543-8.

Expansion microscopy (ExM)

Fei Chen, Paul W. Tillberg, Edward S. Boyden



1. Gel-anchorable label: custom shynthesis and therefore can not be widely adopted among researchers.
2. Genetically encoded fluorophores can not be imaged without antibody labeling.

Expansion microscopy , F. Chen, P. W. Tillberg, E. S. Boyden, Science 2015. Jan 30;347(6221):543-8.

NATURE BIOTECHNOLOGY | RESEARCH | LETTER

Protein-retention expansion microscopy of cells and tissues labeled using standard fluorescent proteins and antibodies

Paul W Tillberg, Fei Chen, Kiryl D Piatkevich, Yongxin Zhao, Chih-Chieh (Jay) Yu, Brian P English, Linyi Gao, Anthony Martorell, Ho-Jun Suk, Fumiaki Yoshida, Ellen M DeGennaro, Douglas H Roossien, Guanyu Gong, Uthpala Seneviratne, Steven R Tannenbaum, Robert Desimone, Dawen Cai & Edward S Boyden

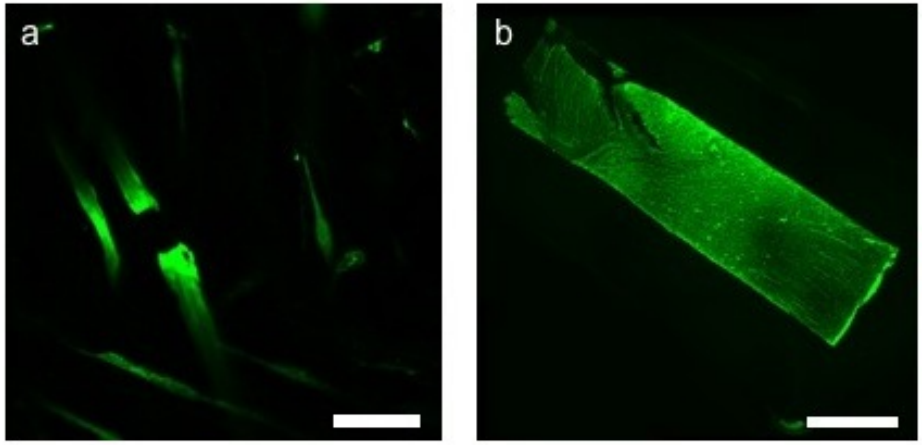
Is it possible to incorporate native proteins into the polymeric gel instead of labels?

1. Reduced protelysis to preserve epitopes:
   - succinimidyl ester of 6-((acryloyl)amino)hexanoic acid (acryloyl-X, SE; abbreviated **AcX**)
   - alkaline detergent-rich buffer for 1 hour in an autoclave
   - ~4 × expansion of Thy1-YFP mouse brain samples
2. Exposed the tissue to LysC enzyme.

Highly variable staining and incomplete homogenization

↓

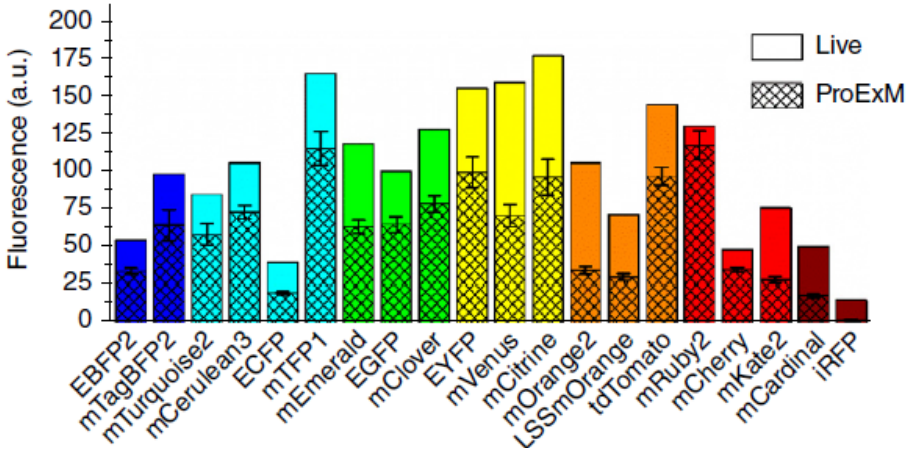
strong proteolysis is neccessary

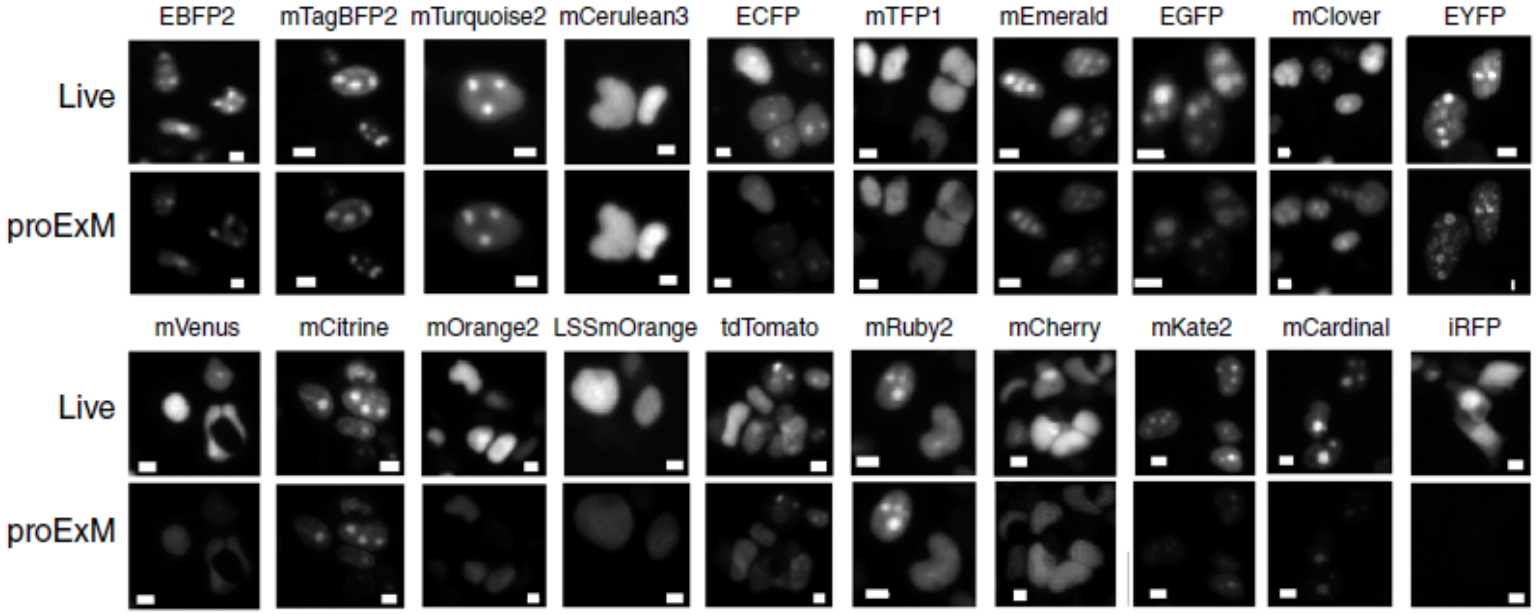


Fluorescence images of Thy1-YFP expressing mouse cerebral cortex, with YFP stained with anti-GFP

Combining direct protein anchoring with strong digestion

- AcX treatment of fixed specimen
- Gelation
- Strong digestion (proteinase-K)
- Expansion
- Imaging





Scale bars: 5 µm

Combining direct protein anchoring with strong digestion

- AcX treatment of fixed specimen
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- Strong digestion (proteinase-K)
- Expansion
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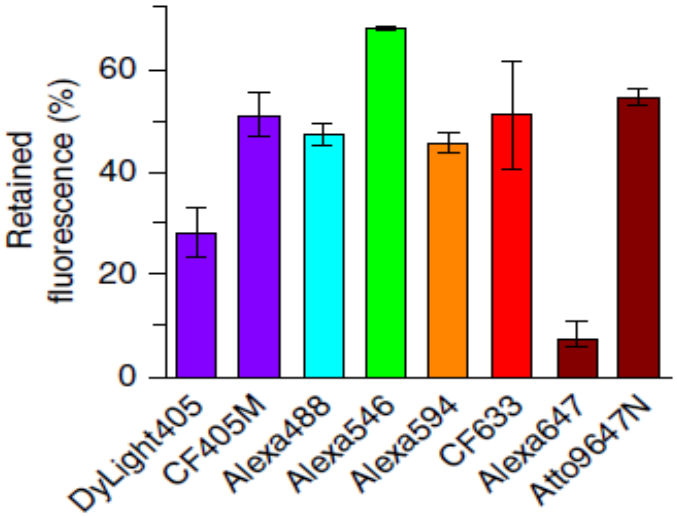


Performance of selected secondary antibody dyes in proExM

Dye	Ex max, nm	Em max, nm	Brightness in proExM as % of post antibody stain	Source
DyLight405	400	421	28±5	Life Technologies
CF405M	408	452	51±4	Biotium
Alexa488	495	519	48±2	Life Technologies
Alexa546	556	573	68±3	Life Technologies
Alexa594	590	617	46±2	Life Technologies
CF633	630	650	51±10	Biotium
Alexa647	650	668	7±3	Life Technologies
Atto647N	644	669	55±2	Sigma

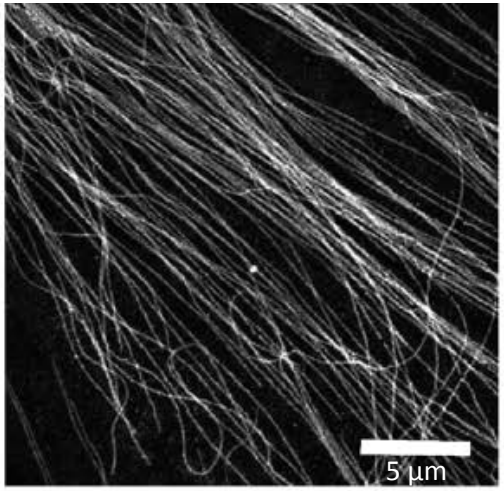
Validation in cultured cells

Imaging immunostained microtubules in cultured cells with super-resolution structured illumination microscopy (SR-SIM).

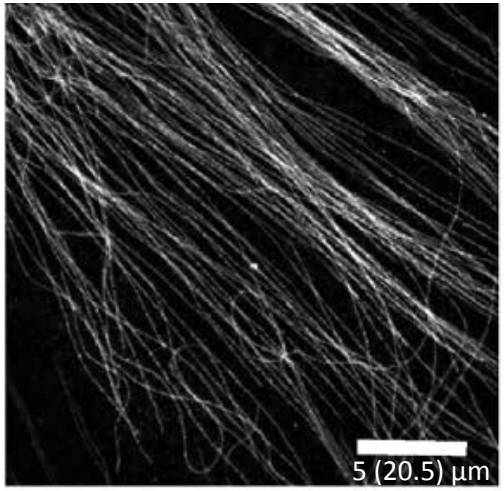
Quantified the root-mean square (r.m.s.) error after proExM over length scale between 0 and 20 µm.

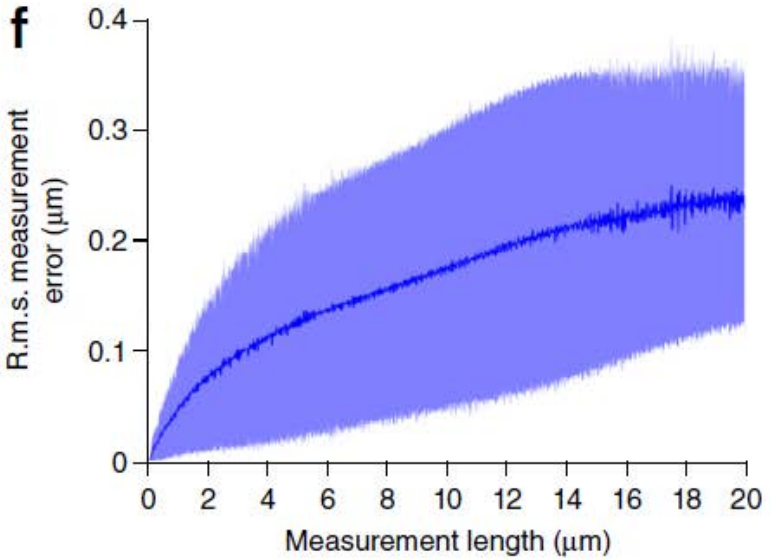
≈1-2% of the measurement distance

SR-SIM image pre-expansion



Post-expansion (spinning-disk confocal microscope)



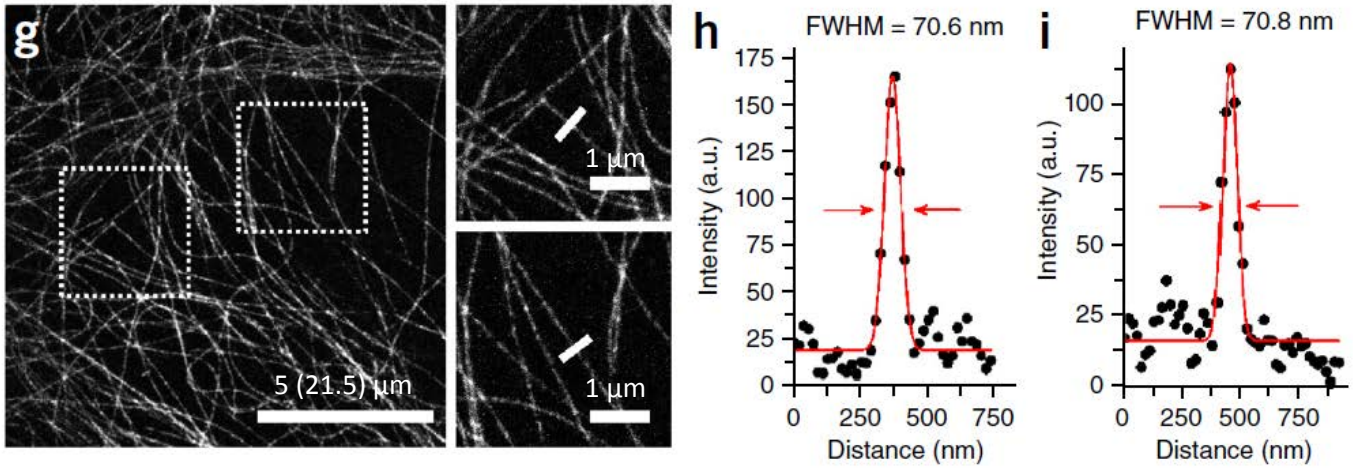


Validation in cultured cells

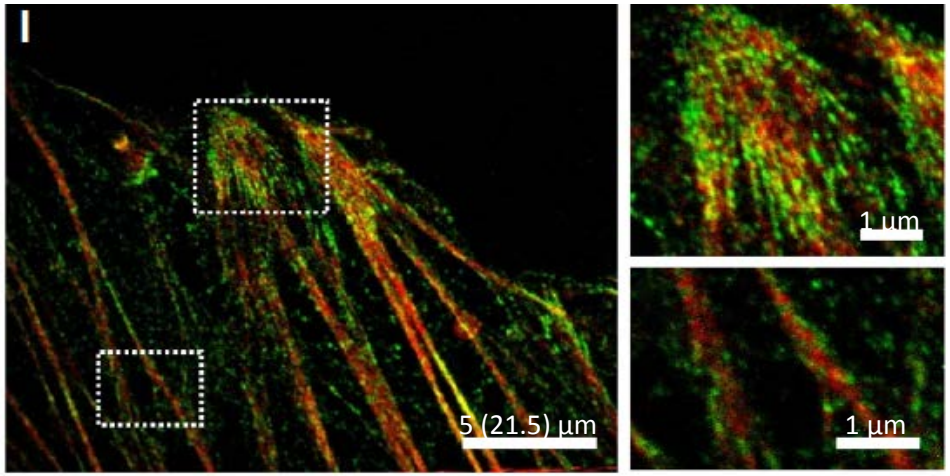
Imaging fusion proteins bearing genetically encoded flourophores in culture HeLa cells.

Calculation of the full-width at half maximum (FWHM).

mClover-α-tubulin fusion



mEmerald-paxillin fusion (green) and mRuby2-actin fusion (red)

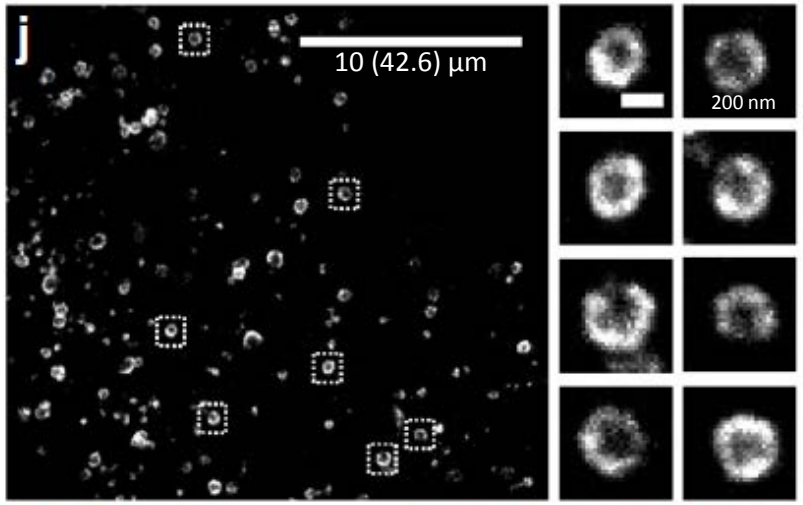


Validation in cultured cells

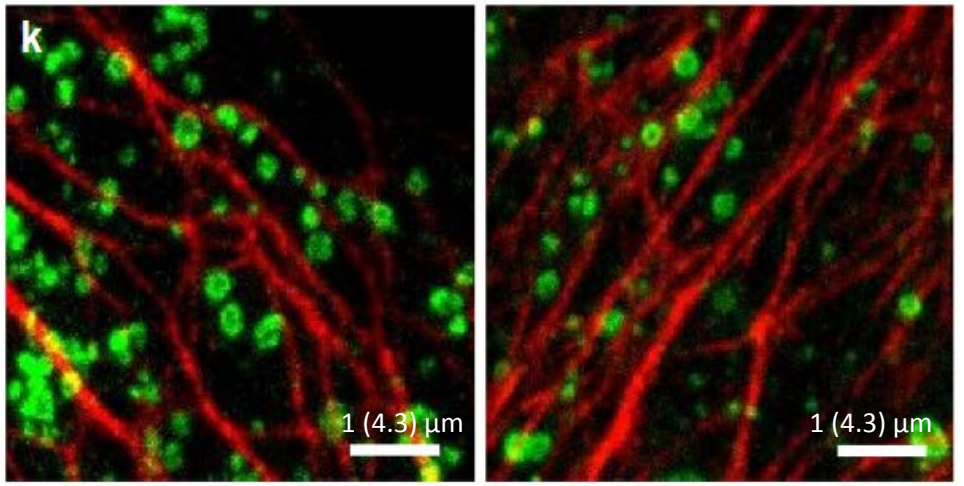
Imaging fusion proteins bearing genetically encoded flourophores in culture HeLa cells.

Calculation of the full-width at half maximum (FWHM).

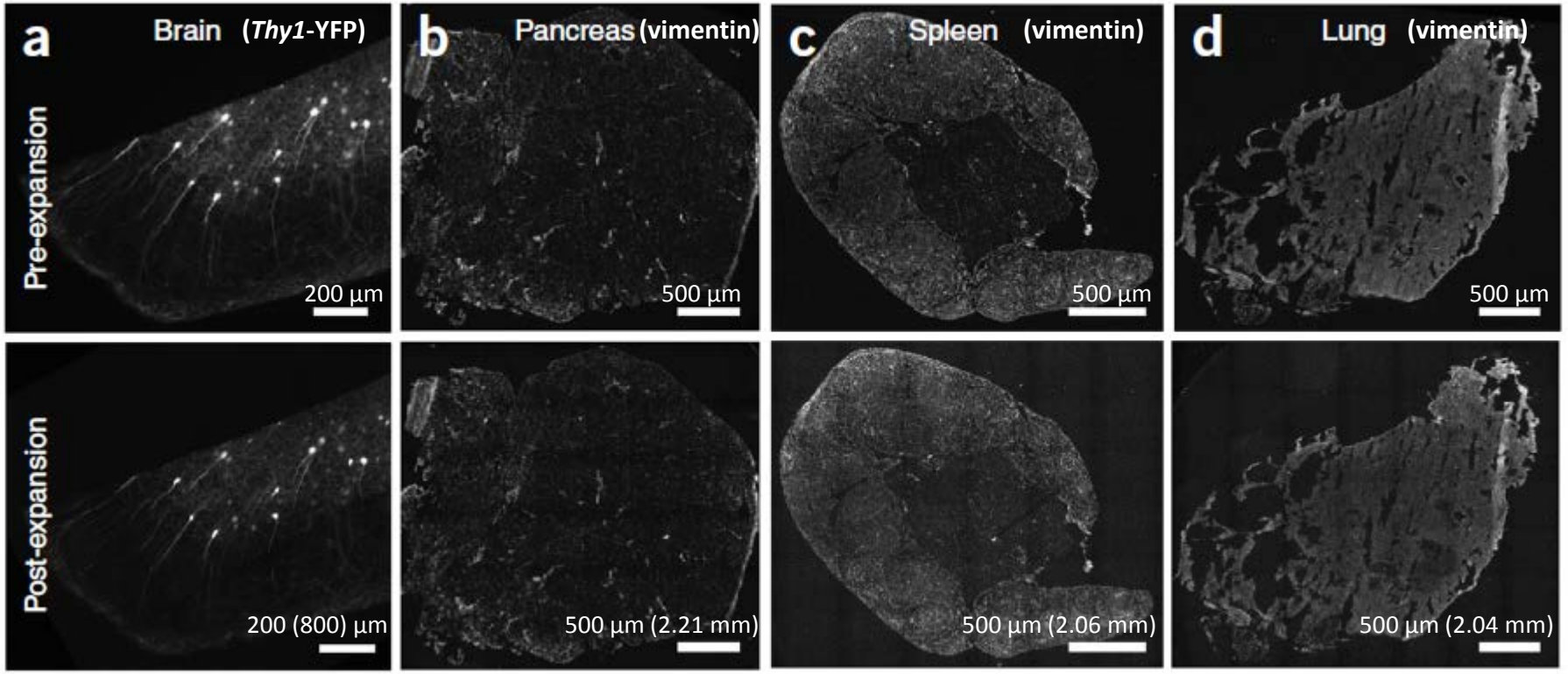
mEmerald-clathrin fusion



mEmerald-clathrin fusion (green) and mRuby2-keratin fusion (red)



Performance in 3D tissues

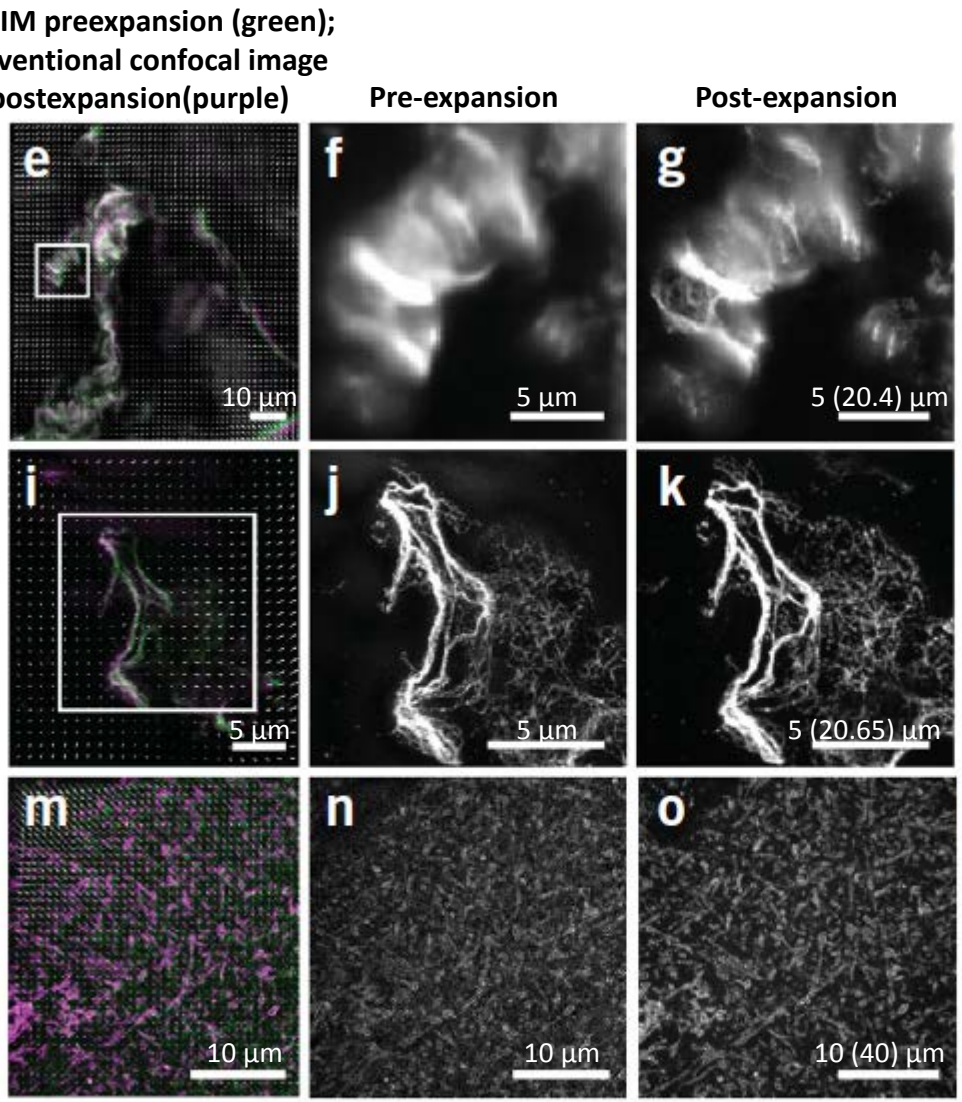


For tissues with different mechanical propeties (i.e.:more connective tissue)

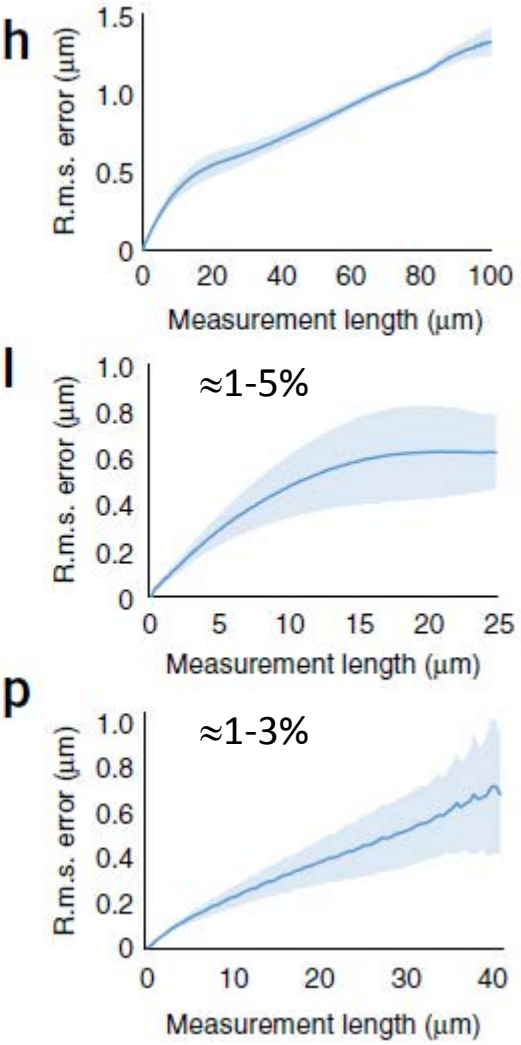
Slight modification in digestion temerature: 60 ^{o}C for 4 hours

Performance in 3D tissues

Quantified the root-mean square (r.m.s.) error after proExM at the microscale (<100 µm) and nanoscale (0-25 µm and 0-40 µm).



≈1-3% of the measurement distance

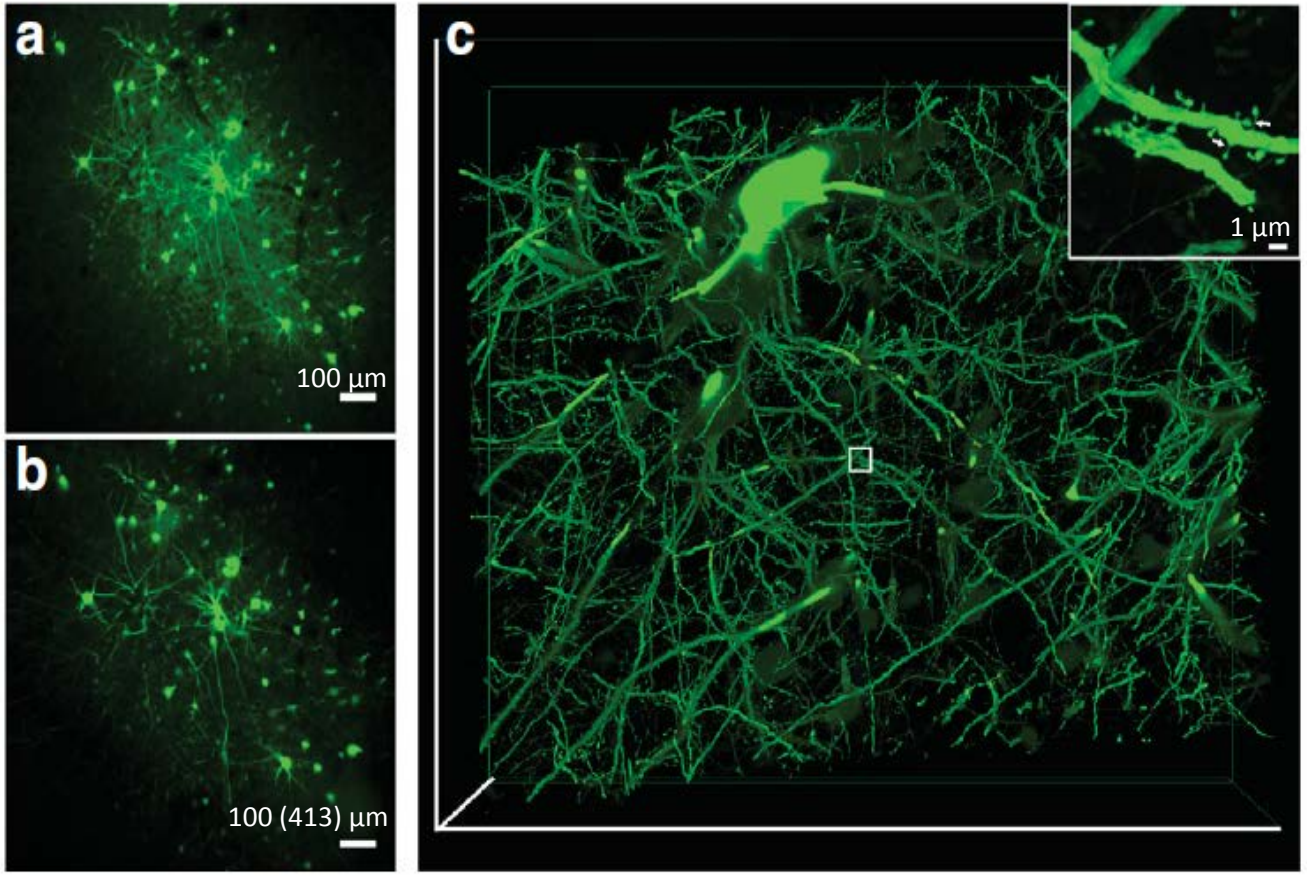


Performance in flourescent protein expressing transgenic animals

Imaging of GFP fluorescence in virally injected *rhesus macaque* cortex.

Pre-expansion and postexpansion widefield image

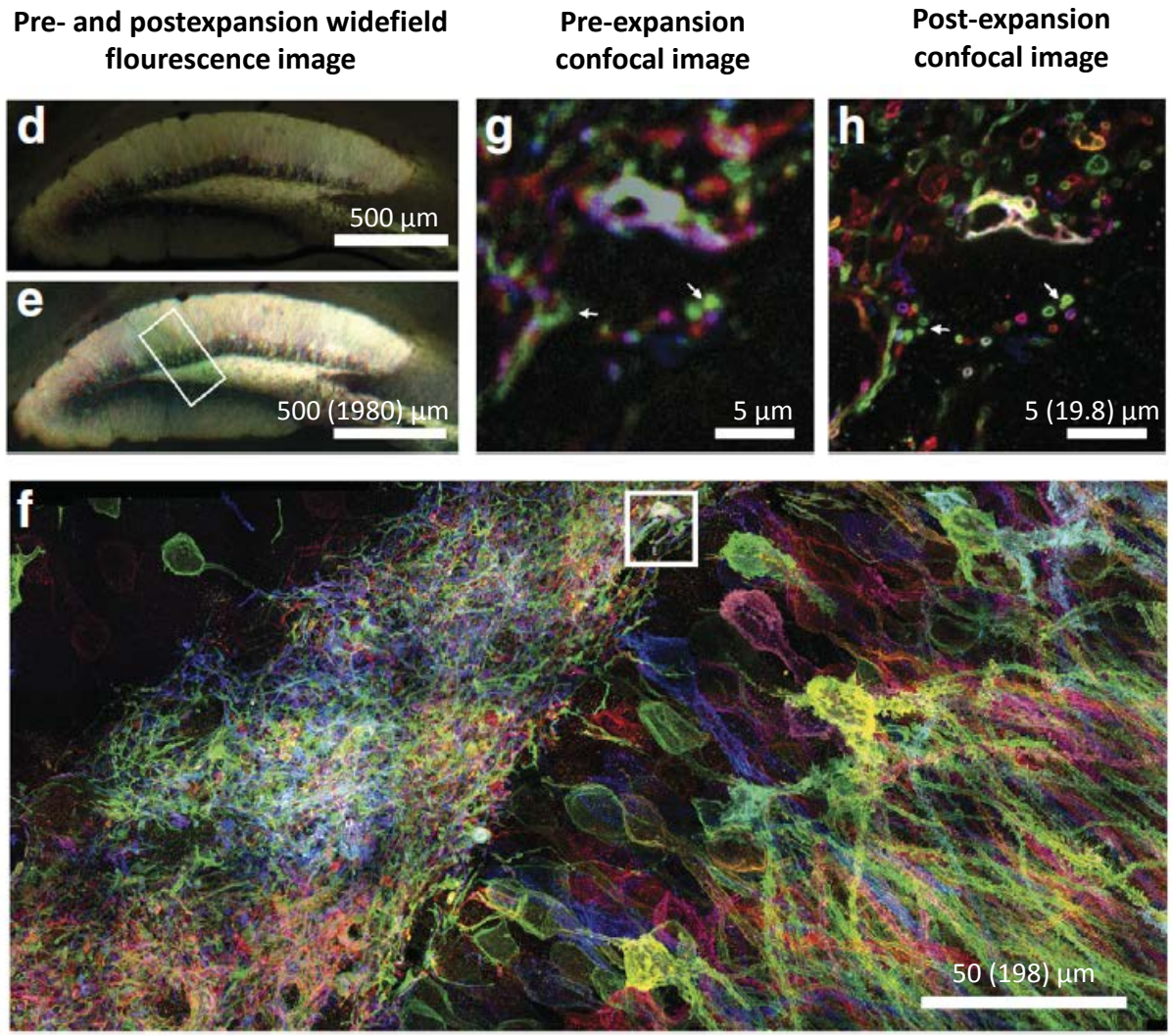
Postexpansion overlay and volume rendering of confocal microscopical image



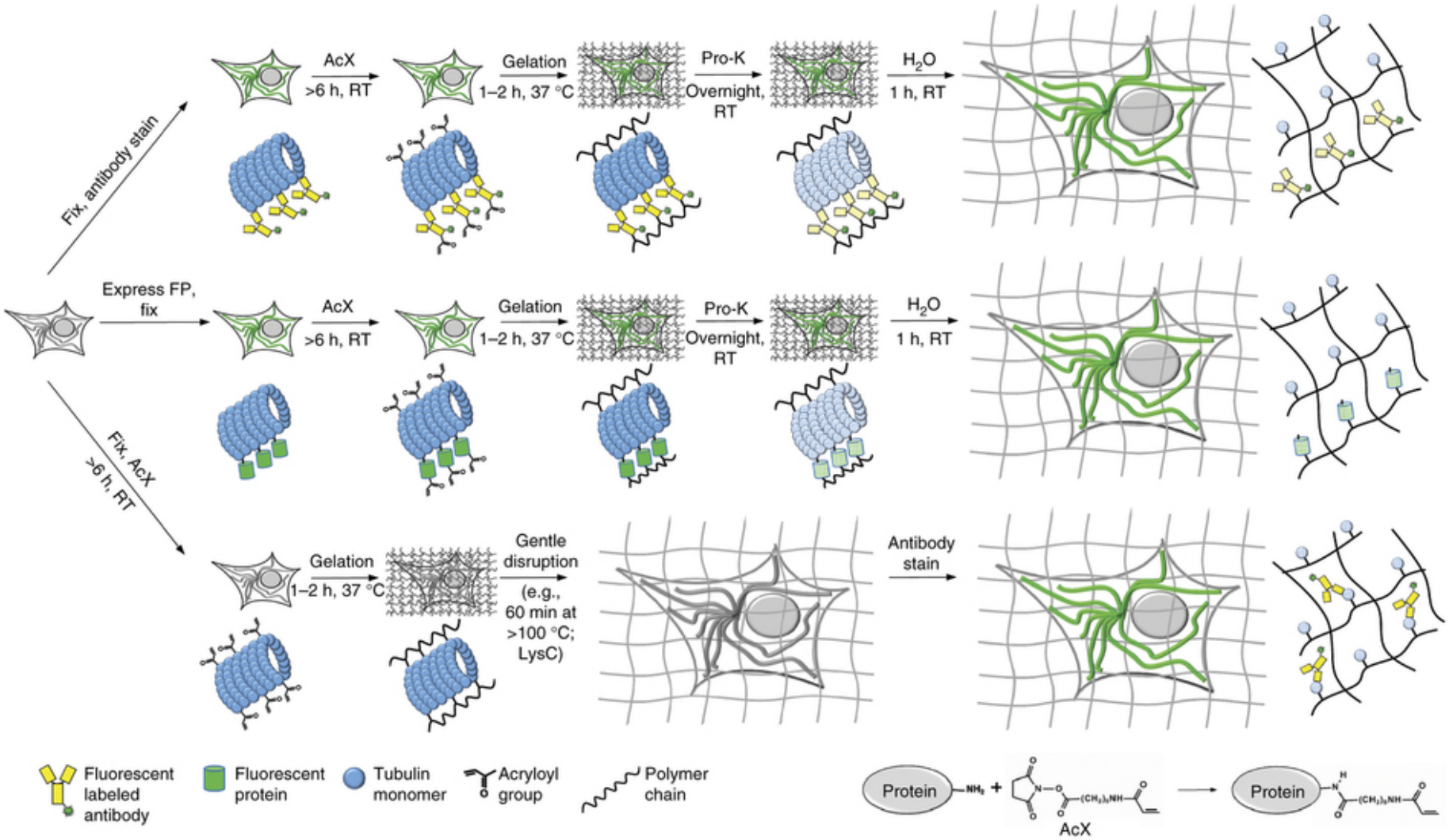
Performance in flourescent protein expressing transgenic animals

Imaging of brain circuitry in mouse hippocampus expressing virally delivered Brainbow 3.0

large volume, multicolorsuper-resolved imaging



ProExM variants



- **Improvements:**
  - flourescent proteins (FP)and antibodies delivered using standard methods are retained in the gel;
  - preservation of endogenous fluorescence -> allows the usage of transgenic animals, viral expression vectors and transfection of FP constructs;
  - multicolor, large volume capability (i.e.: Brainbow staining, useful for circuit studies);
  - optically clear and index-matched samples -> suitable for superresolution imaging.

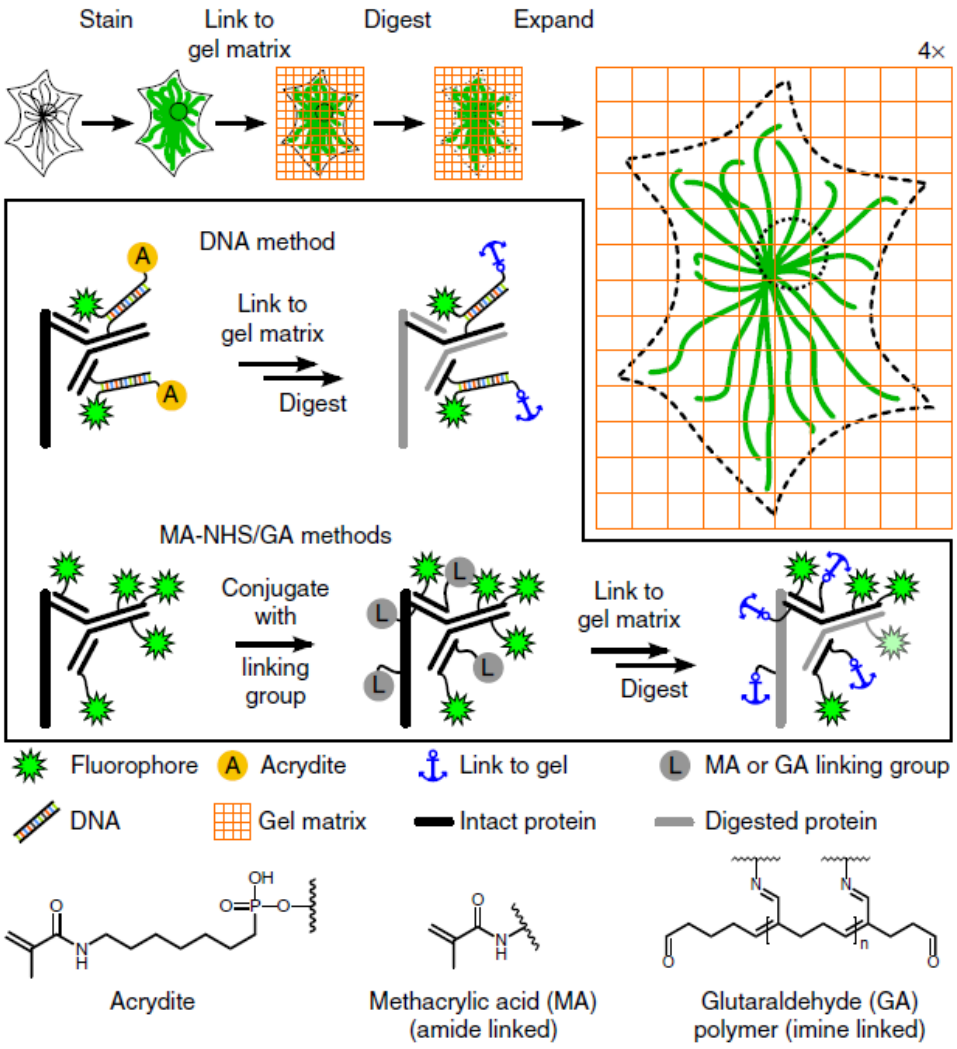
- **Limitations:**
  - samples are large in size -> limited by the working distance of the objective and requires tiled acquisition;
  - voxels are smaller -> contain fewer flouorophores -> dimmer signals and longer exposure time.

NATURE METHODS | BRIEF COMMUNICATION

Expansion microscopy with conventional antibodies and fluorescent proteins

Tyler J Chozinski, Aaron R Halpern, Haruhisa Okawa, Hyeon-Jin Kim, Grant J Tremel, Rachel O L Wong & Joshua C Vaughan

Modifications compared to the original method



Fixed and conventionally immunostained cultured cells:

1. 60 minutes with a 25 mM solution of the amine-reactive small molecule ***MA-NHS*** (methacrylic acid *N*-hydroxysuccinimidyl ester) at room temperature;
2. 10 minutes with 0.25% **glutaraldehyde (GA)** at room temperature.

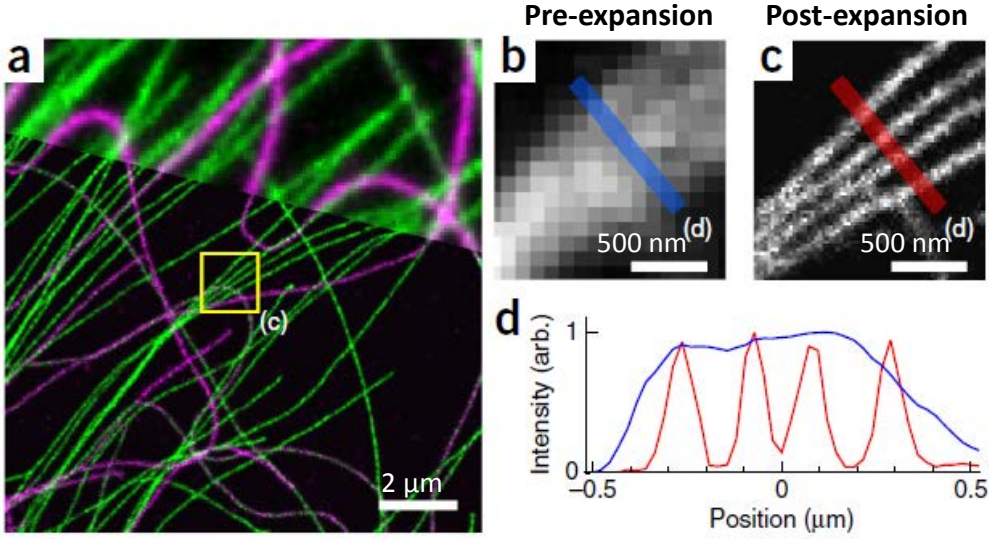
Conferred excellent retention of fluorescent signal after digestion and expansion.

Imaging cultured cells

BS-C-1 cell immunostained for **tyrosinated tubulin (green)** and **detyrosinated tubulin (magenta)** using conventional secondary antibodies, imaged with conventional confocal microscope.

Digestion time: ≈12-18 h

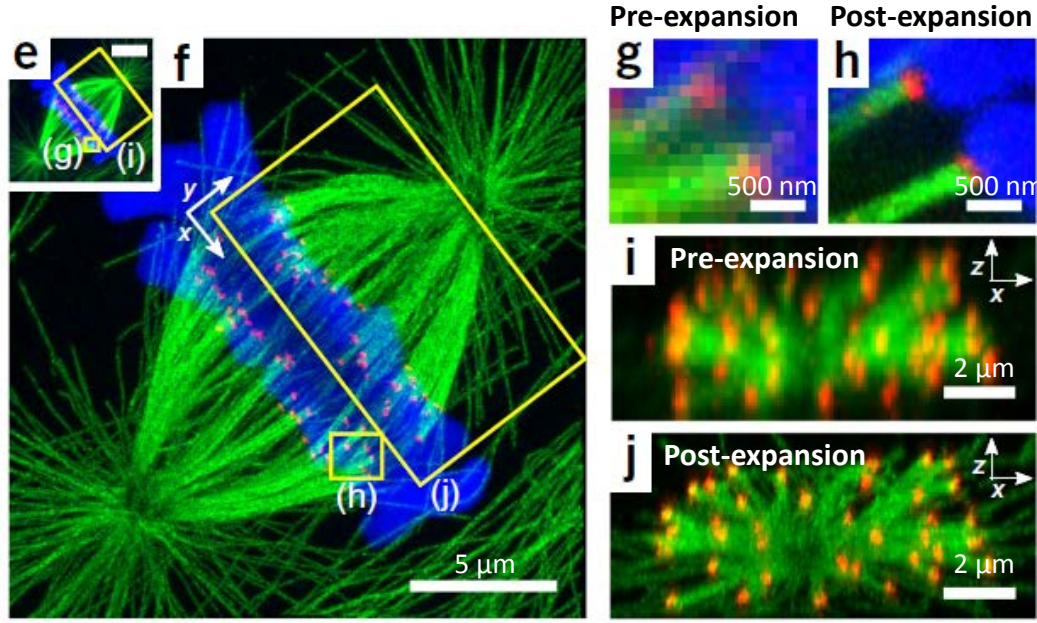
MA-NHS treatment



Dividing PtK1 cell immunostained for **tubulin (green)** and the **kinetochore protein HEC1 (red)** using conventional secondary antibodies and also stained for **DNA (blue)** using **TO-PRO-3**, imaged with conventional confocal microscope.

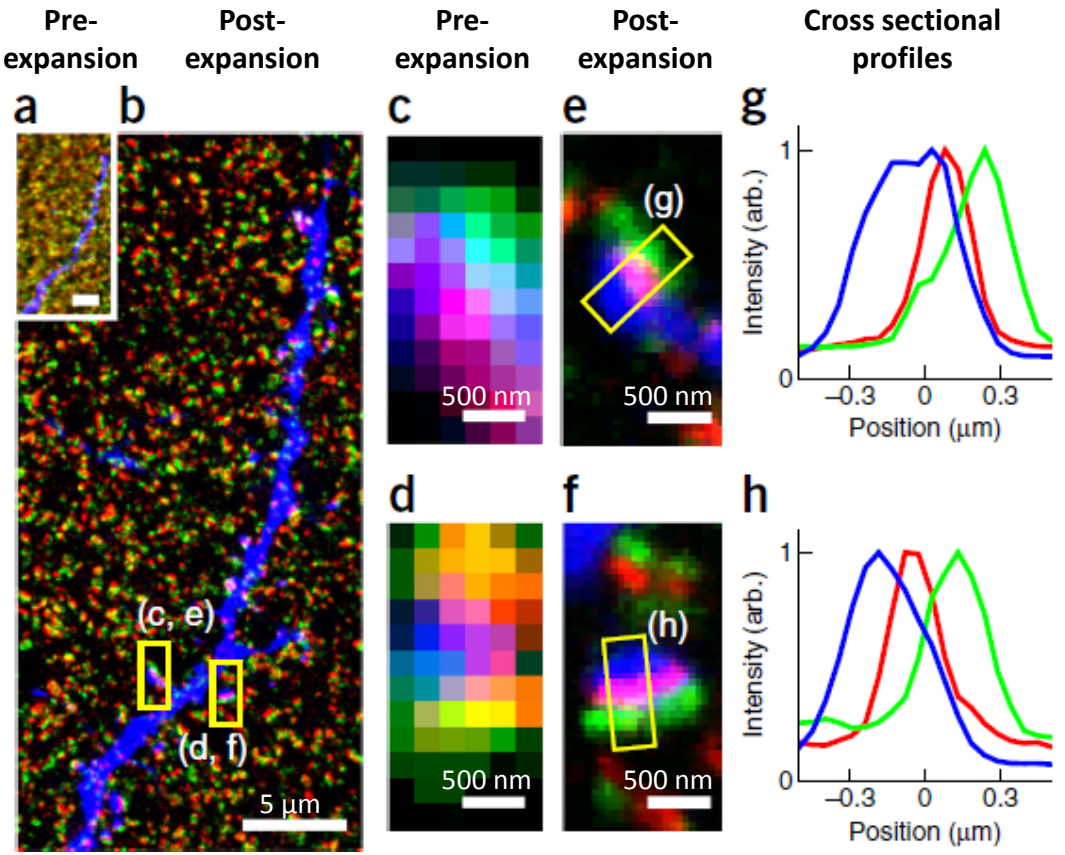
Digestion time: ≈30 min

GA treatment



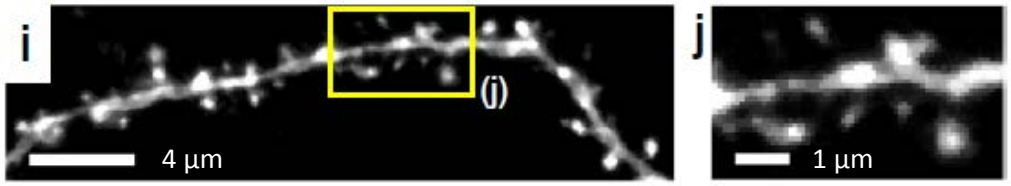
Imaging brain slices
MA-NHS treatment

Thy1-YFP-H mouse brain slice indirectly immunostained for **YFP (blue)**, the presynaptic marker **Bassoon (green)**, and the postsynaptic marker **Homer (red)** using conventional secondary antibodies.



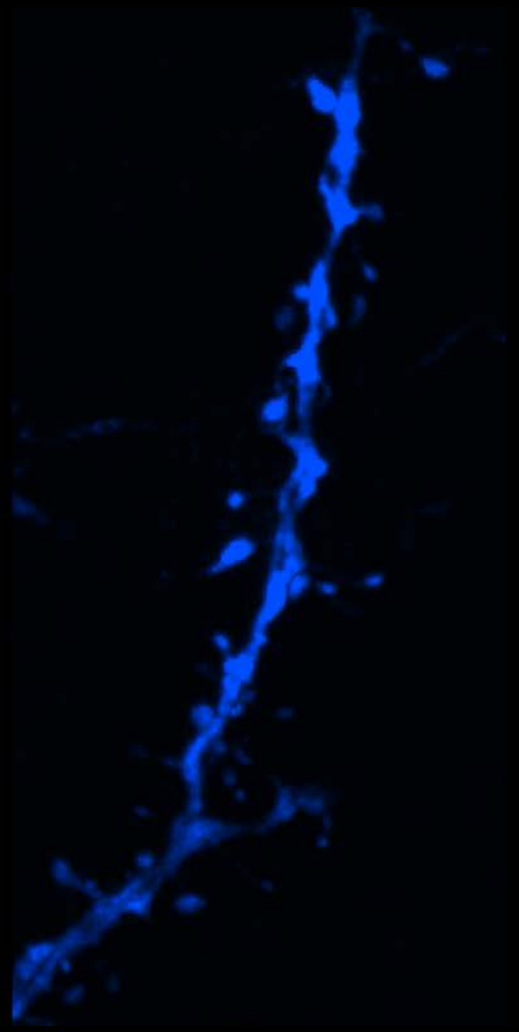
Epifluorescence image of a neuron in an expanded *Thy1*-YFP-H mouse brain slice using YFP itself as the fluorescence reporter.

Digestion time: ≈1 h



Imaging brain slices

MA-NHS treatment



- **<u>Improvements:</u>**
  - faster procedure-incubation time ≈60 minutes or less;
  - flourescent proteins (FP)and antibodies delivered using standard methods are retained in the gel;
  - MA-NHS is incorporated covalently into the polymer, but the linking mechanism of GA is less obvious.

- **<u>Limitations:</u>**
  - limitations by the working distance of the objective and requires tiled acquisition;
  - quenching and photobleaching of the sample can happen during polymerization.

NATURE METHODS | ARTICLE

Nanoscale imaging of RNA with expansion microscopy

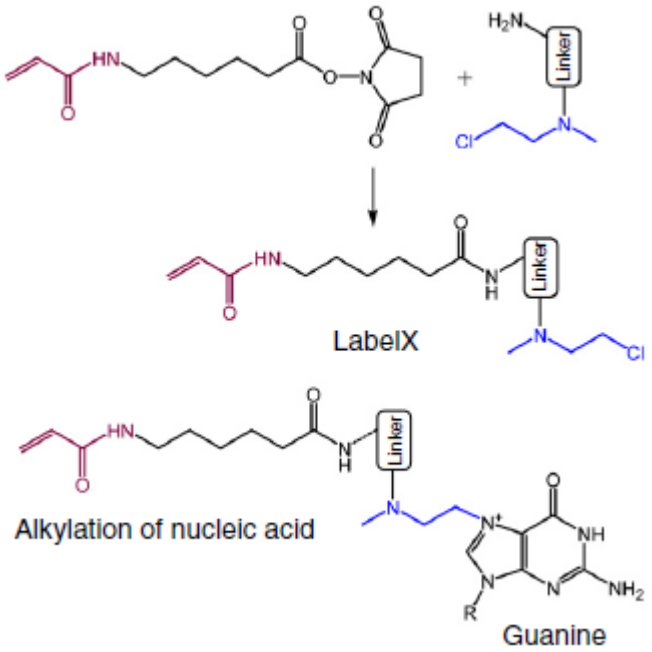
Fei Chen, Asmamaw T Wassie, Allison J Cote, Anubhav Sinha, Shahar Alon, Shoh Asano, Evan R Daugharthy, Jae-Byum Chang, Adam Marblestone, George M Church, Arjun Raj & Edward S Boyden

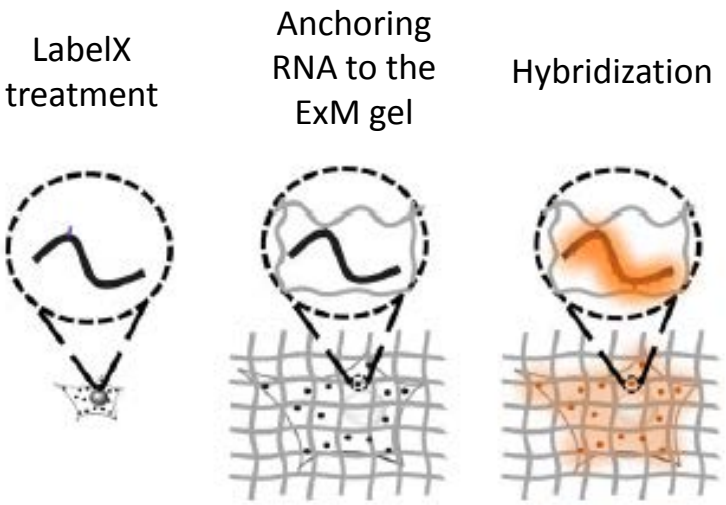
Affiliations | Contributions | Corresponding author

Covalent binding of RNAs to the ExM gel

Reagent of 2 main component:

1. a molecule containing an amine and an alkylating group, which reacts to the N7 of guanine
2. a molecule containing an amine-reactive succinamide-ester and a polymerizable acrylamide

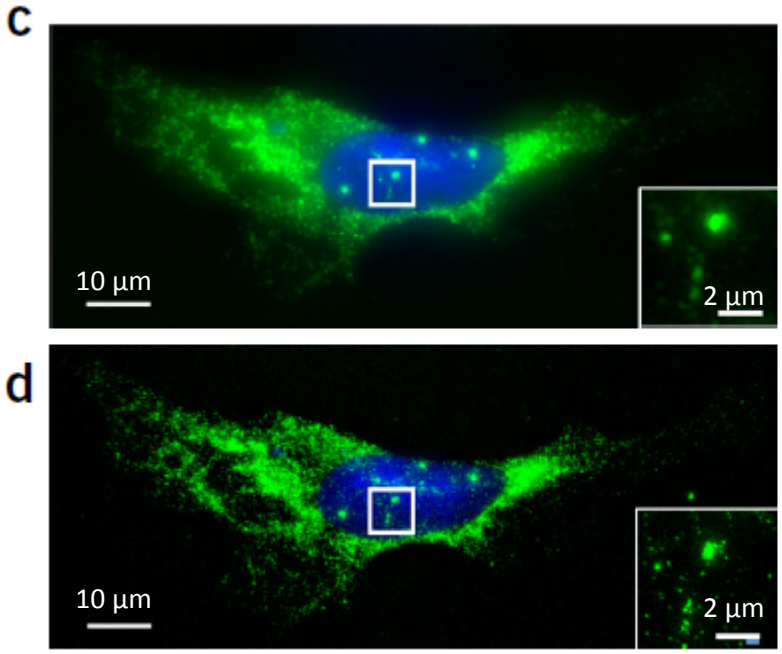


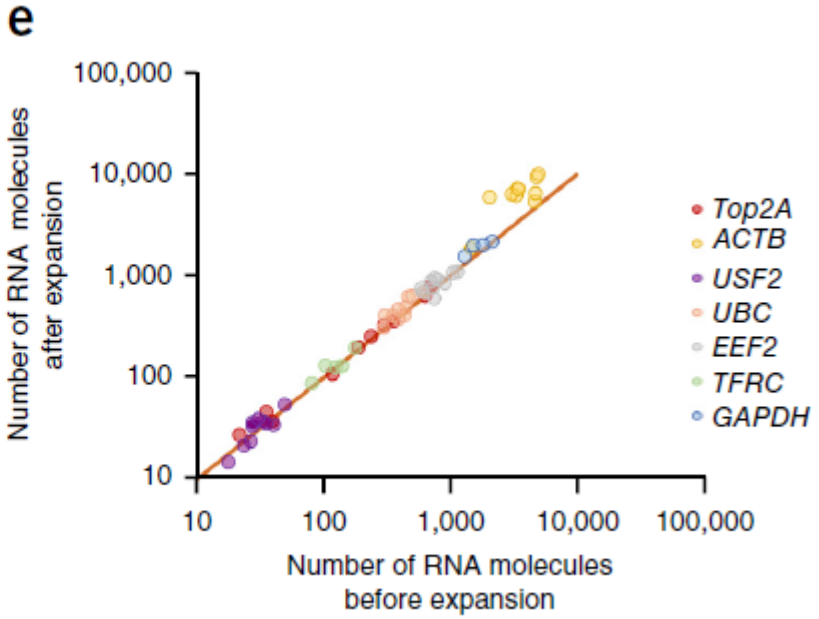


Quantification of RNA-transcript anchoring yield

- smFISH probes targeting mRNAs of varying copy numbers in cultured HeLa cells.
- Results: more transcripts were detectable for highly expressed mRNAs.

smFISH against *ACTB*



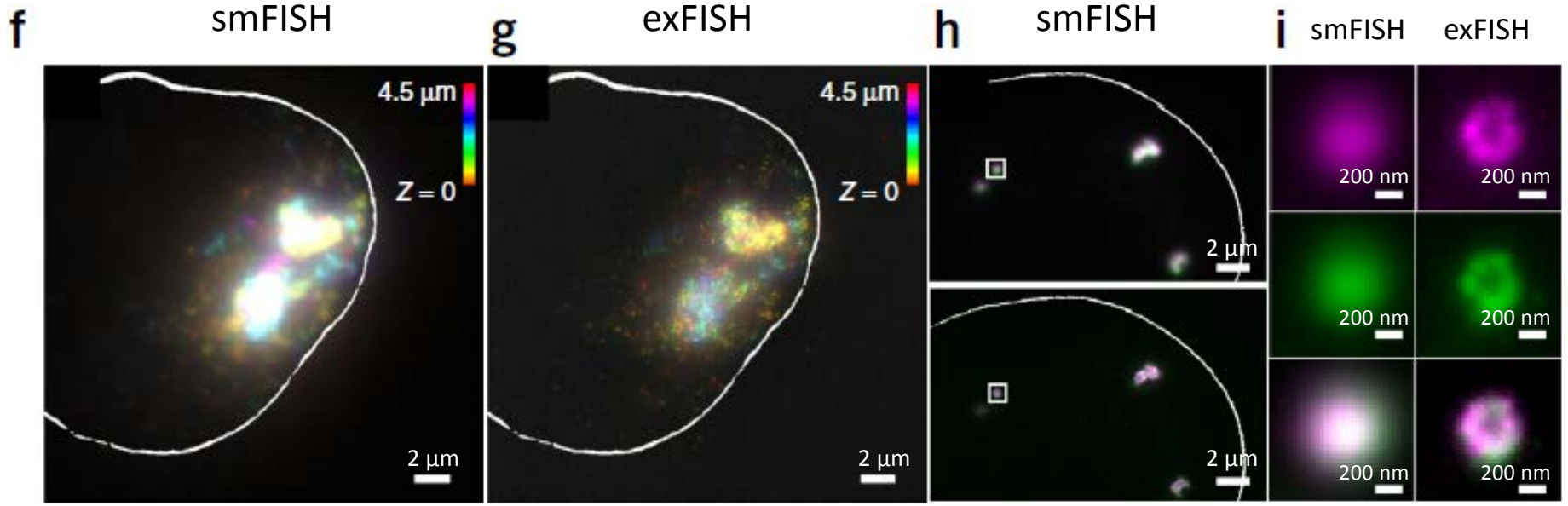


Imaging of lncRNAs with exFISH

- Imaging of lncRNAs, which play sturctural roles in cell biology
- 2 candidates:
  1. XIST: possible role in inactovating the X-chromosome
  2. NEAT1: play a role in gene expression and nuclear mRNA retention

XIST

NEAT1



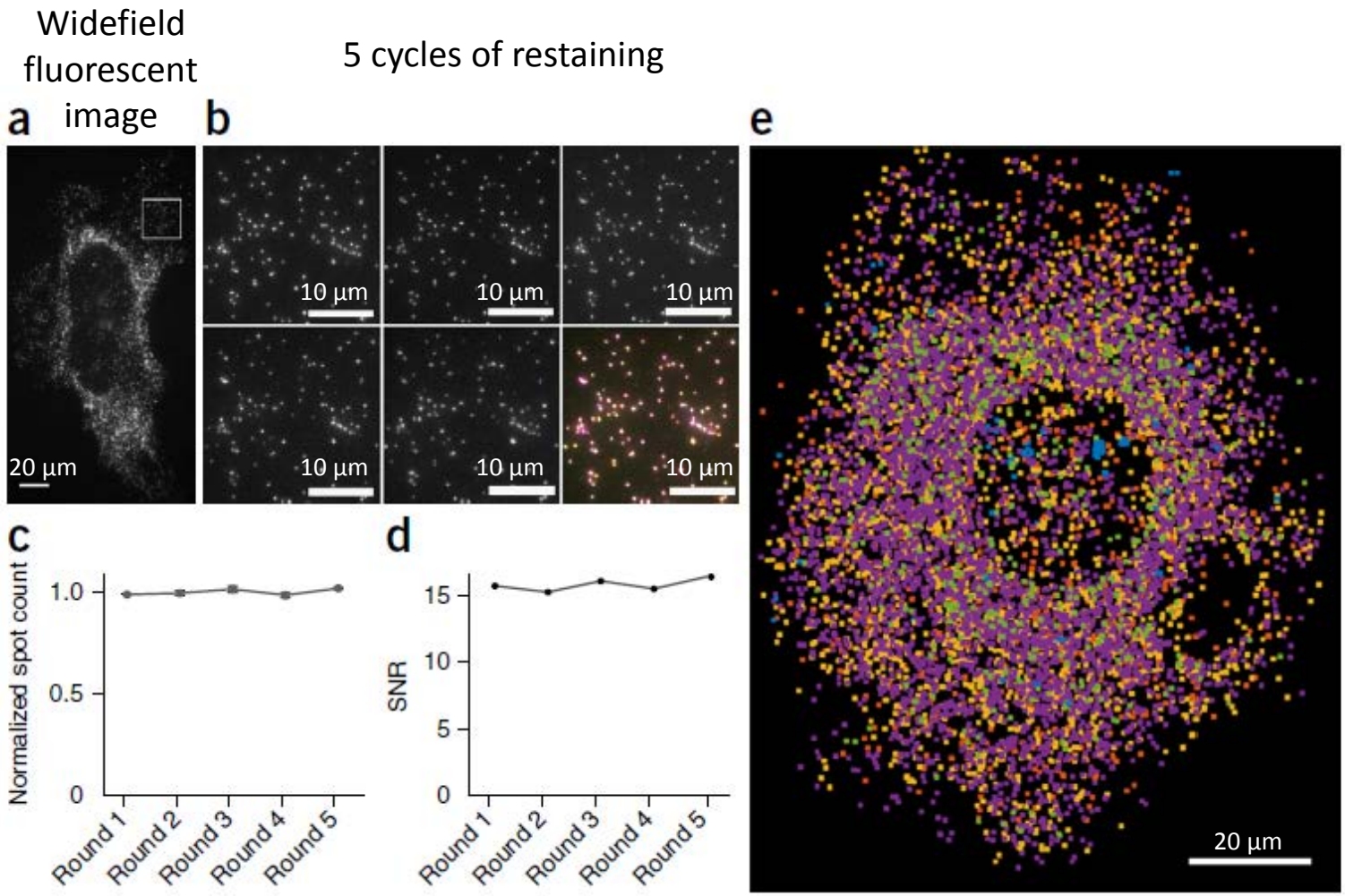
exFISH

Expansion factor: 3.3X

Multiplex imaging of RNAs with exFISH

Facilitate multiplex cycles of FISH -> re-embedded expanded specimens in charge-neutral polyacrylamide for immobilization.

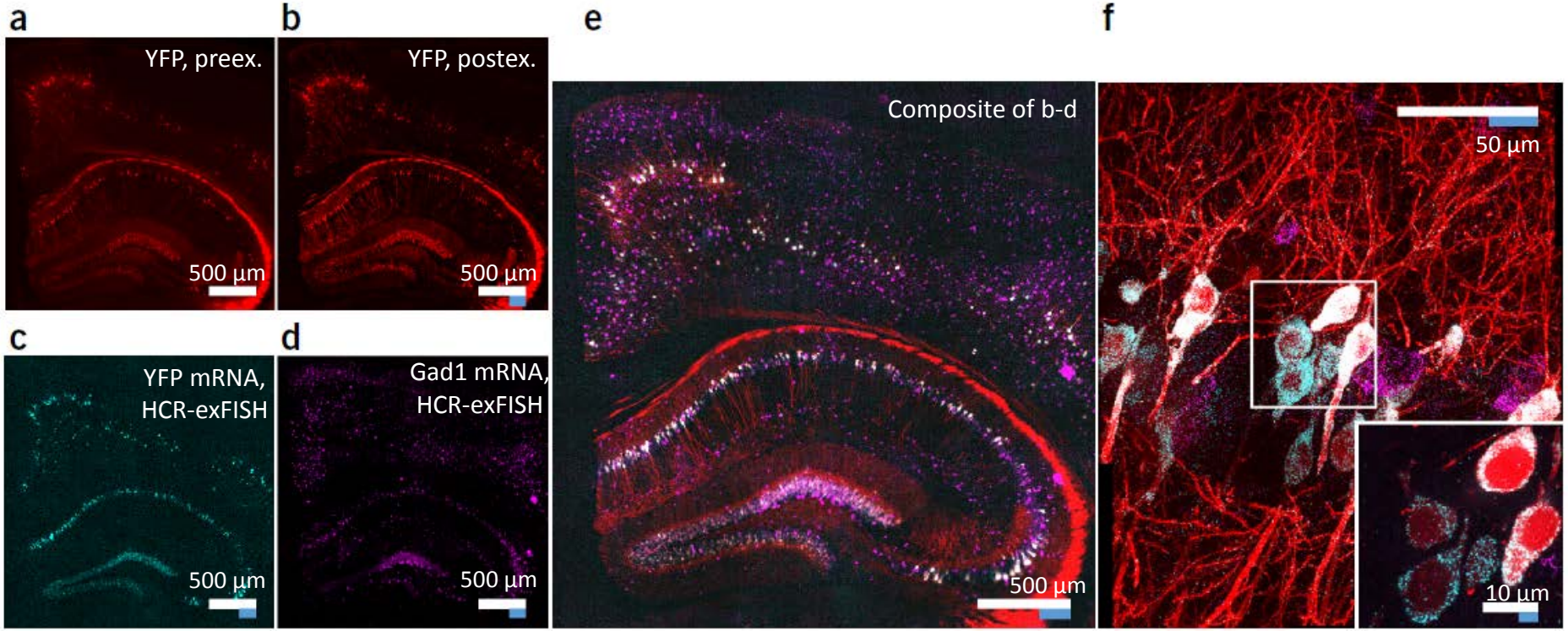
Target:
GAPDH



NEAT1 (blue); EEF2 (orange); GAPDH (yellow); ACTB (purple); UBC (green); USF2 (light blue)

3D imaging of RNA in mouse brain

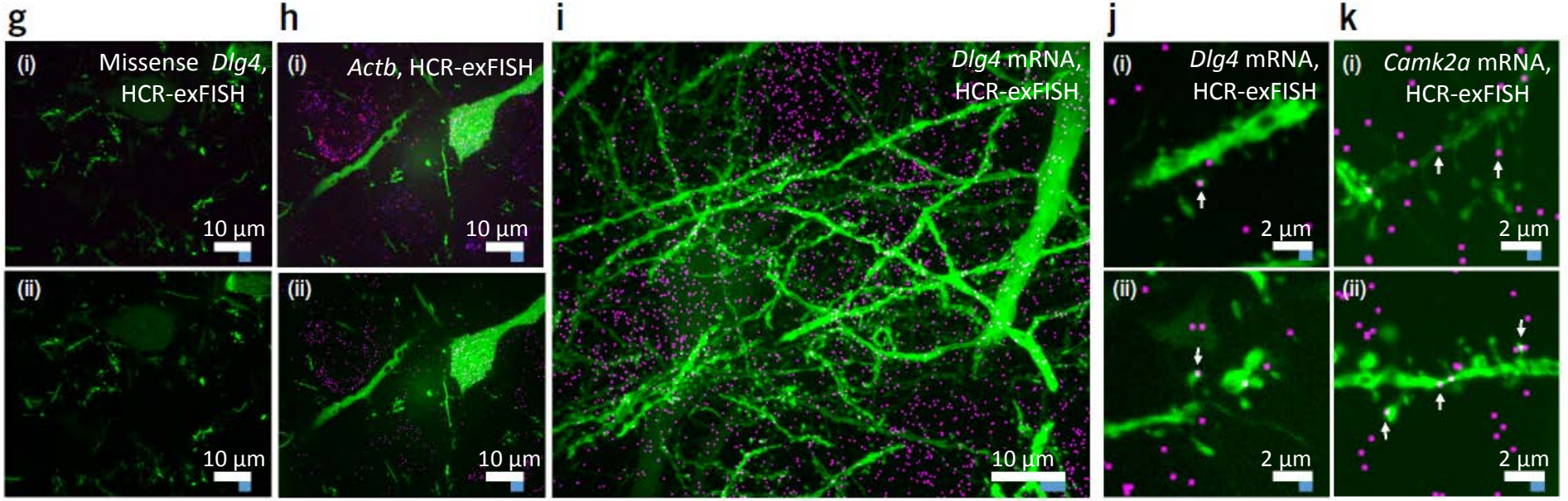
- samples: *Thy1*-YFP mouse brain tissue
- YFP protein was anchored: AcX, proExM protocol
- RNA: LabelX, exFISH protocol
- Hybridization chain reaction (HCR) technique



Expansion factor: 2.9X

3D imaging of RNA in mouse brain

- samples: *Thy1*-YFP mouse brain tissue
- YFP protein was anchored: AcX, proExM protocol
- RNA: LabelX, exFISH protocol
- Hybridization chain reaction (HCR) technique



Green: YFP protein,
Red: *Dlg4* missense even,
Blue: *Dlg4* missense odd,
Magenta: collocalization

Green: YFP protein,
Red: *Actb* even,
Blue: *Actb* odd,
Magenta: collocalization

Green: YFP protein,
Magenta: *Dlgb4* collocalization

Green: YFP protein,
Magenta: *Camk2a* collocalization

Expansion factor: 3X

- **Improvements:**
  - RNA is possible to covalently anchor for expansion microscopy;
  - excellent yield for more accurate counts and localization;
  - robust enough to perform serial smFISH;
  - covalent anchoring might possibly support enzymatic reactions to be performed and expanded;
  - other methods could be implemented to achieve brighter signal: quantom dots, bottlebrush fluorophores.

- **Limitations:**
  - more validation is needed.

Thank you for your attention!