Multifuntional linkers for Expansion Microscopy

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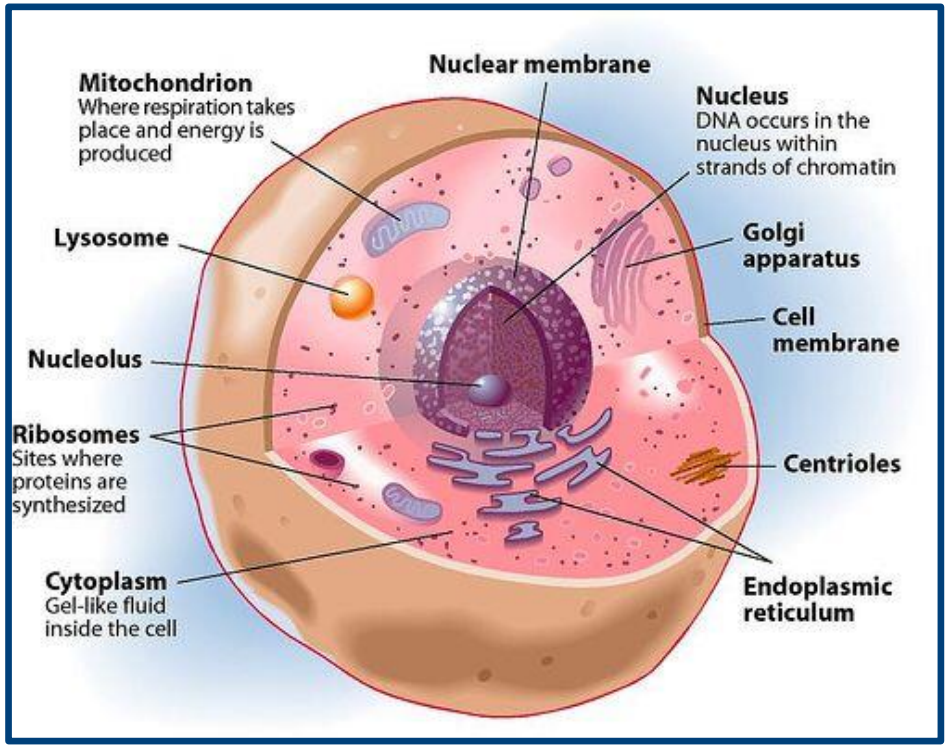
KU LEUVEN

From your old biology textbook,

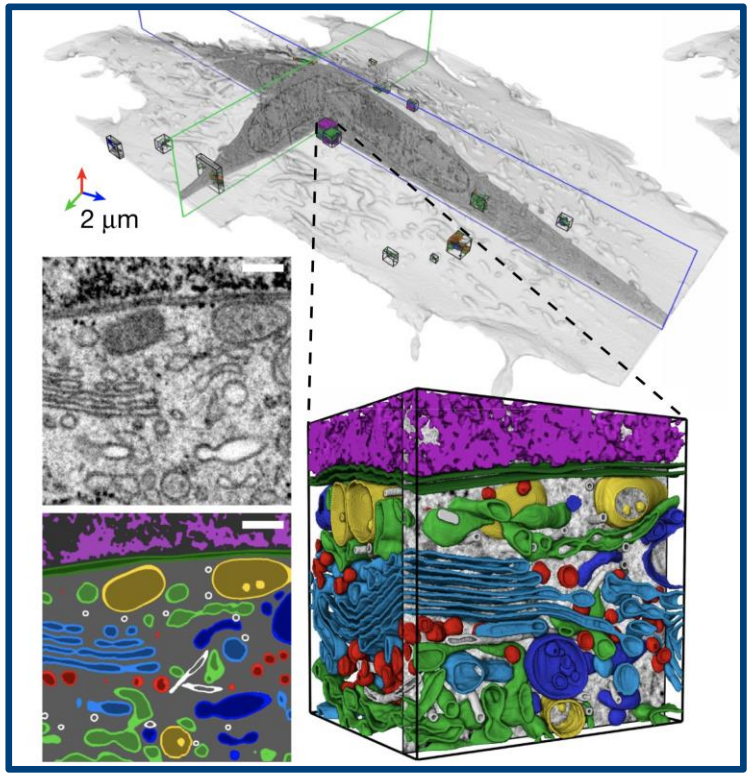
Function floating freely



VS

To what it looks like under the electron microscope:

Condensed & Crowded



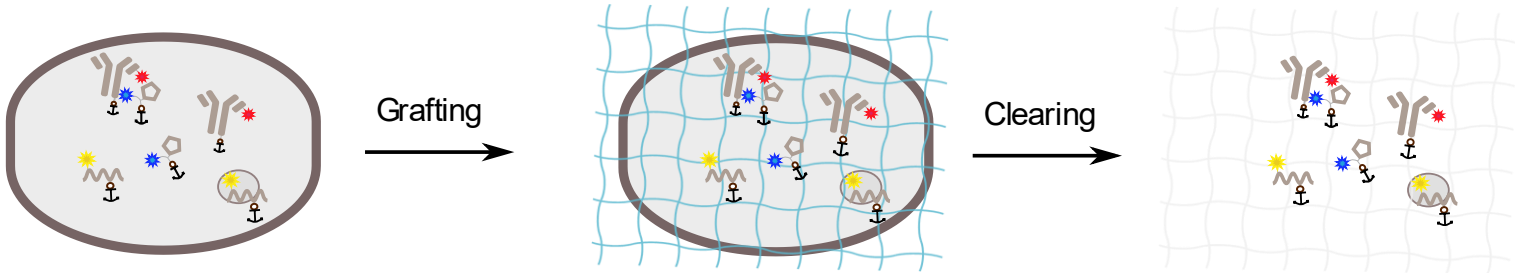
Nature **volume 599**, pages 147–151 (2021)

Very dense 3D information with an enormous degree of differentiation
Organized and interactive

The development of ExM

Grafting/Clarity ⟶ ***Expansion Microscopy***

In CLARITY, hydrogel crosslinks with protein (in the brain) to preserve structural and molecular information for further imaging and analysis at reduced background.



And that looks like this: adult mouse brain imaging

Chung et al., *Nature.,2013,* ***497*** *(7449): 332*

The unconfirmed tale of how ExM came about:

It was around the years of 2013-2014 when someone in Ed Boyden's lab at MIT probably said :
"Now, while perfectly clear after two days, those hydrogels have an annoying tendency to swell…"

"Wait, can we not use that?"

Expansion microscopy (ExM)

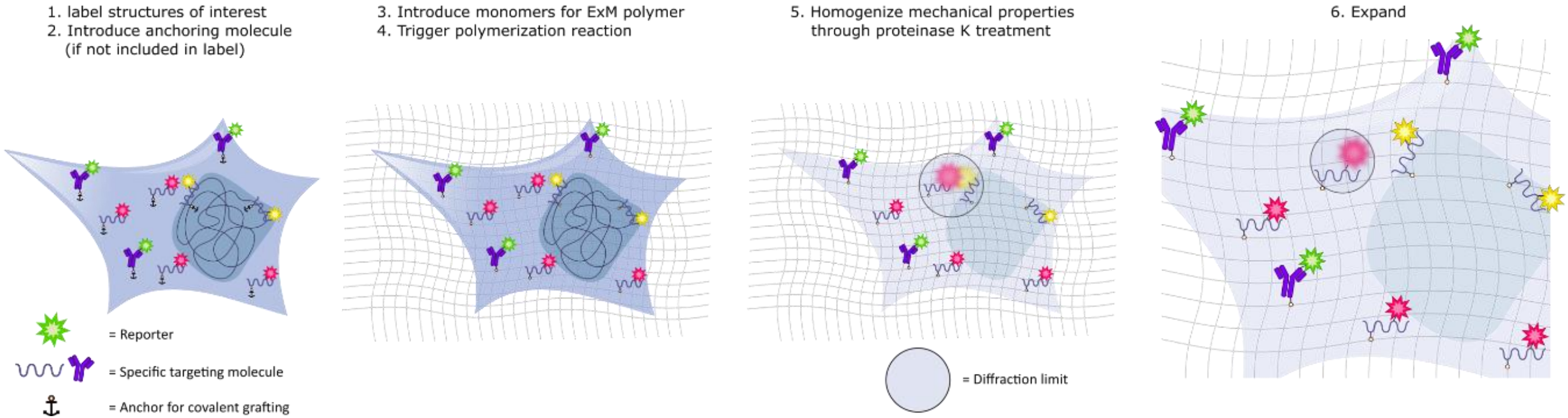
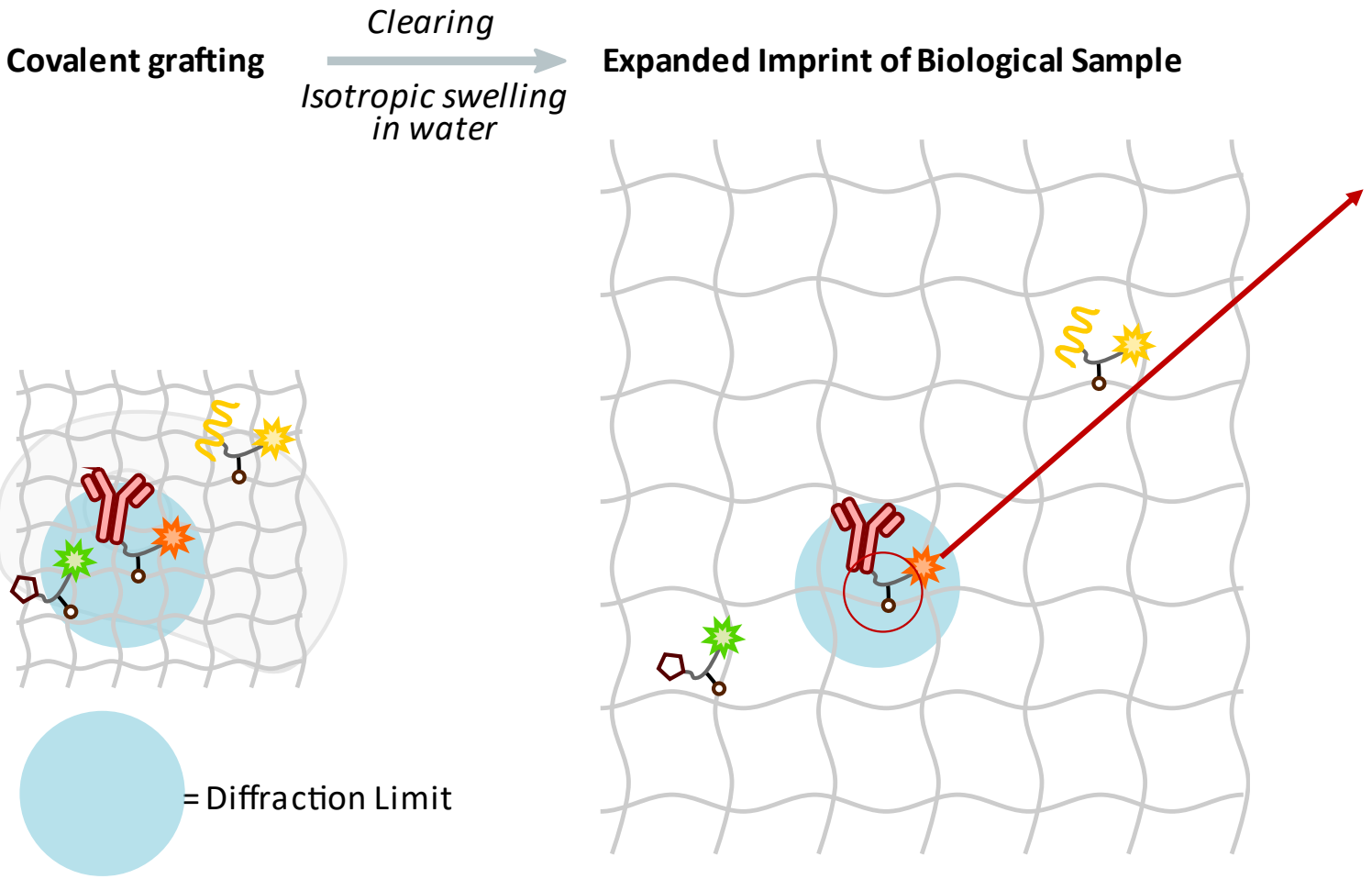


***Figure 1**. Concept figure explaining the ExM protocol.*

- **Labeling**: Biological samples (e.g., proteins) are tagged with fluorescent markers.
- **Anchoring**: The labeled molecules are anchored to a polymer network, typically a hydrogel.
- **Digestion**: Enzymes partially digest the sample to allow it to expand evenly.
- **Expansion**: The hydrogel is swollen with water, enabling microscopes to capture detailed cellular structures.

A perfect case for organic chemistry!



The project is about “linkers”

- Reagents that literally “link” information to signal and matrix
- From a biological sample to a fully imprinted model
- All biological information to be addressed
- Linking is permanent, read-out multiplexed and cycled.

Chen et al., Science. ***347*** *(6221): 543*

A perfect case for organic chemistry!

Super-resolution Microscopy drawbacks

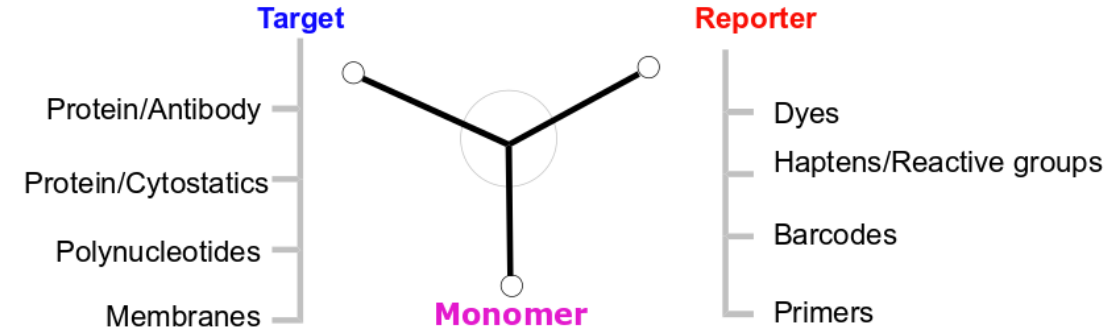
- Expensive hardware
- Specialized operators
- Specific organic dyes/ fluorescent proteins

Advantages of Expansion Microscopy

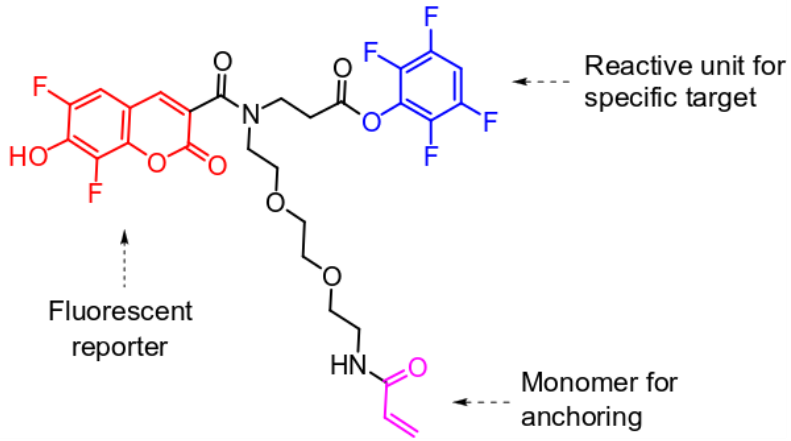
- Enhanced resolution
- Compatibility with various biomolecules and thick tissues
- Multiplexed and high-content imaging

So, at the 14th Conference on Methods and Applications in Fluorescence, in Würzburg, Germany (2015), after seeing a lecture of Boyden on his recent paper, we devised the following:

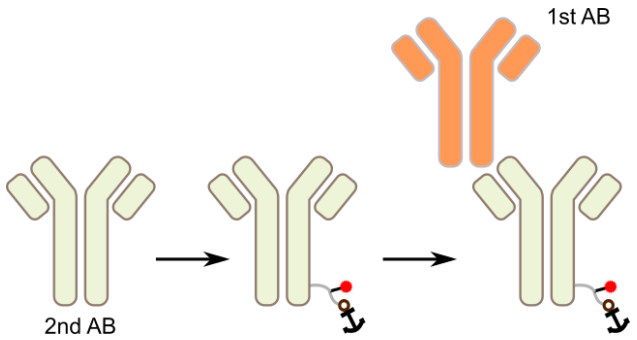
a. TRITON concept

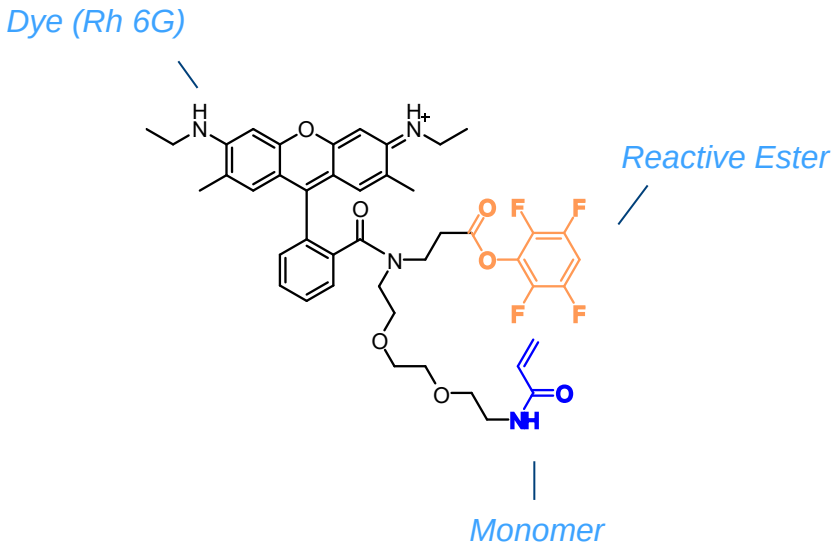


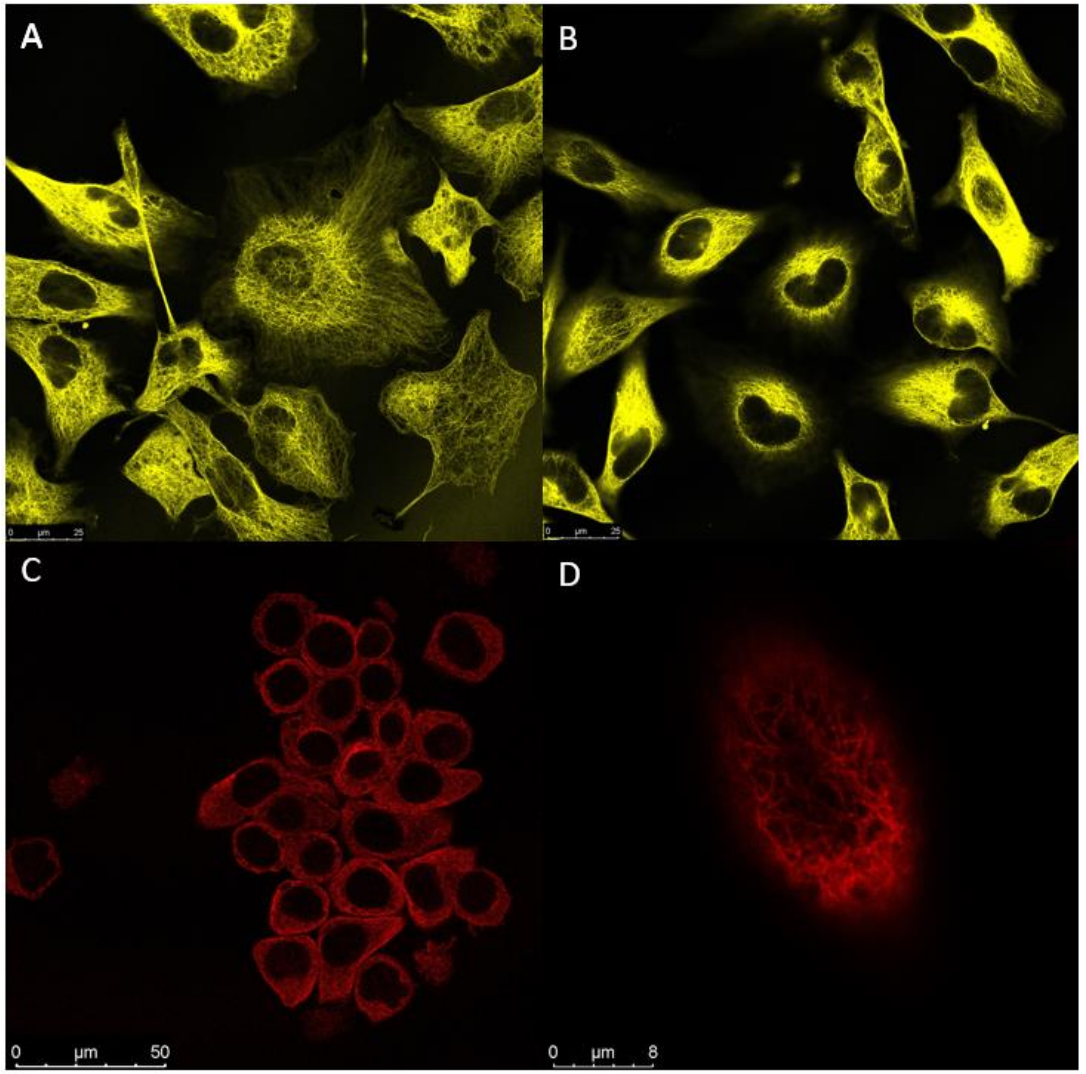
b. Example structure



So somewhere in 2018, this finally got tested



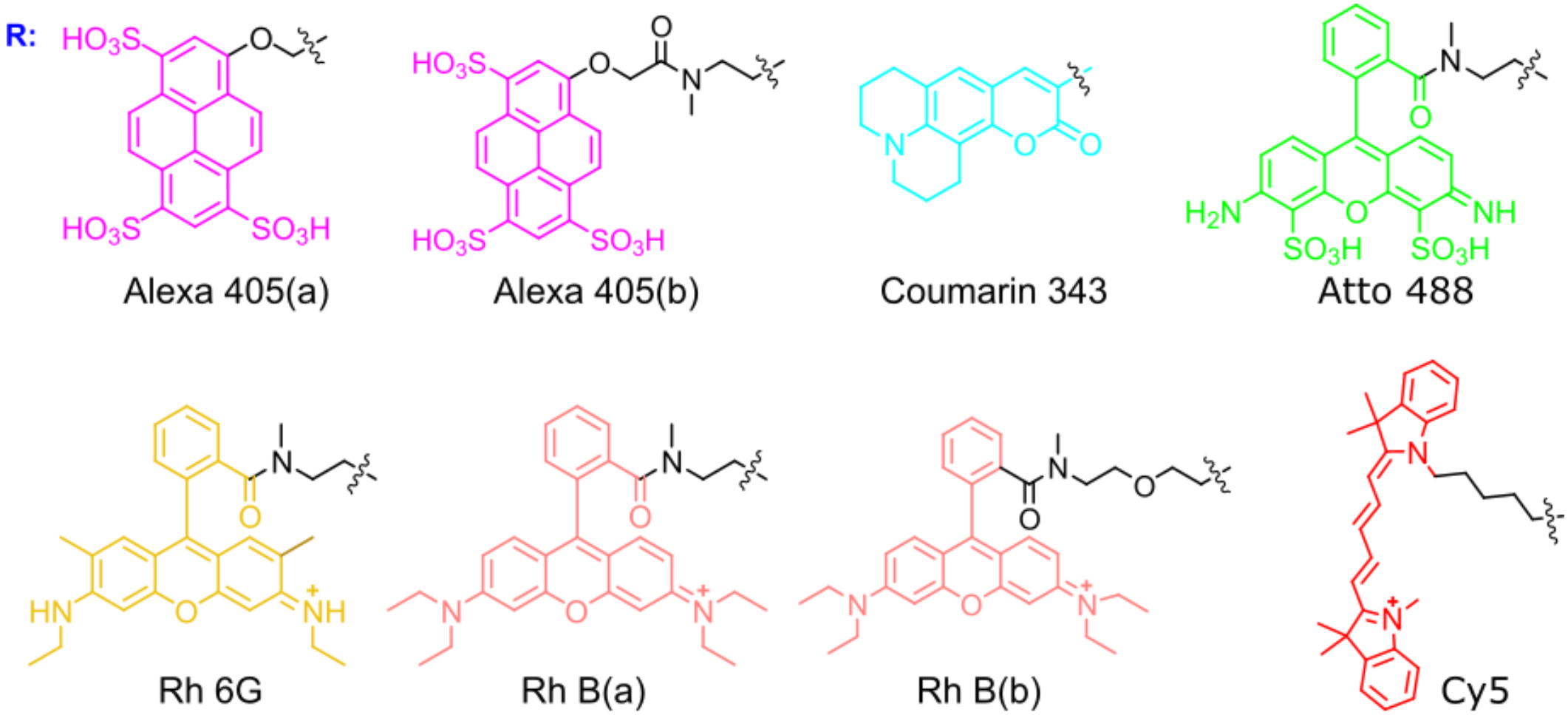




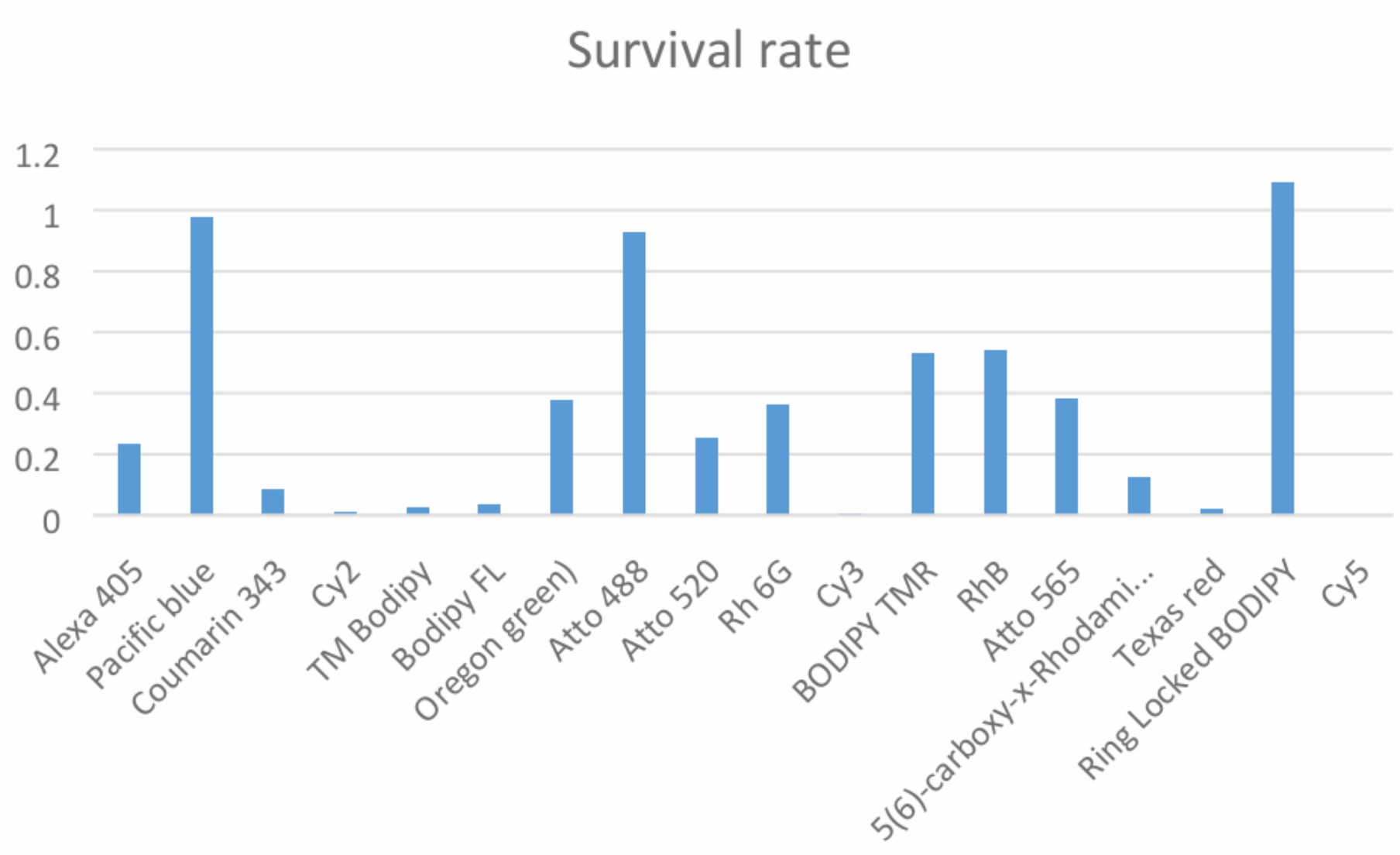
Donato Valli

Pre-ExM GAM (A, B) and Post-ExM GAM (C, D) stained HeLa cells (alfa-tubulin). Images recorded with LEICA TCS SP8 X CM, 63x wobj.

First step: Focus on Dye Synthesis



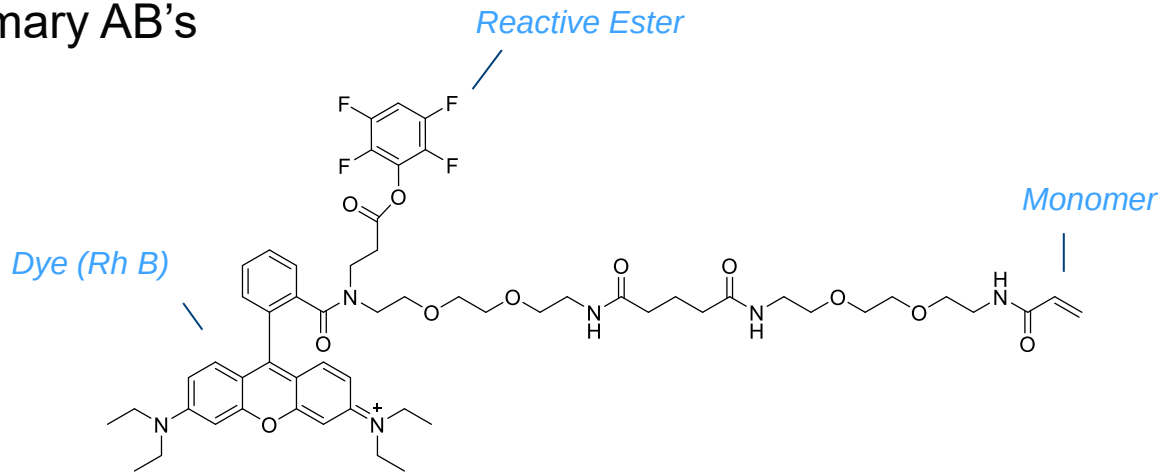
In-depth Analysis of Linker Molecules: Focus on Dyes



Antibody labeling for Expansion Microscopy

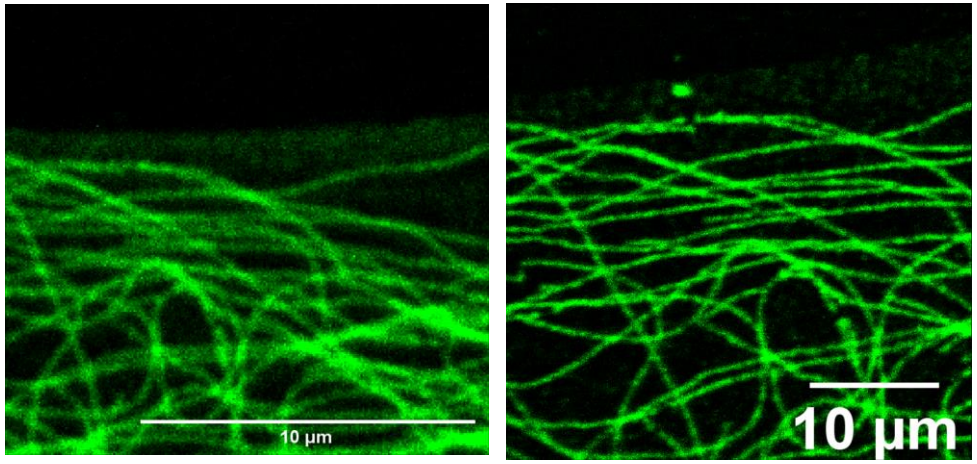
- Readily extended to direct immunostaining with primary AB's
- *Enabling lipid membrane and cytoskeleton staining*

Gang Wen

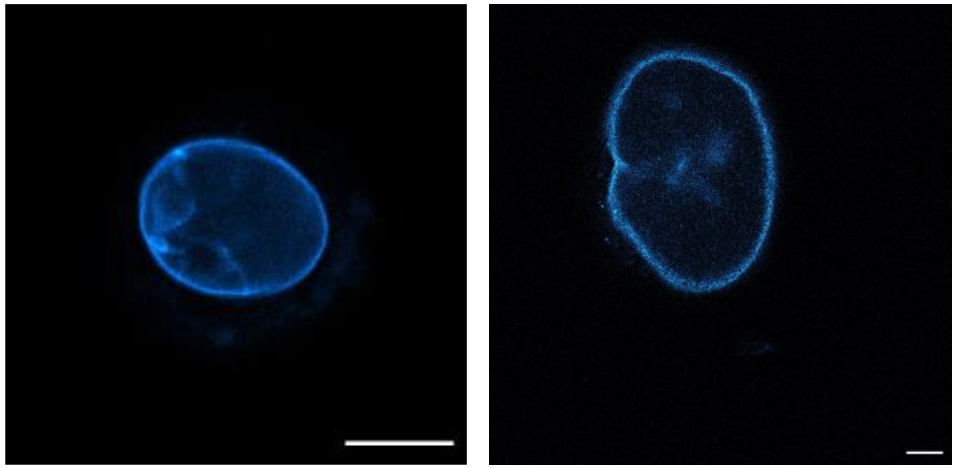


Primary antibodies, ×4 ExM

Wen et al., ACS nano, 2020, 14(7): 7860-7867



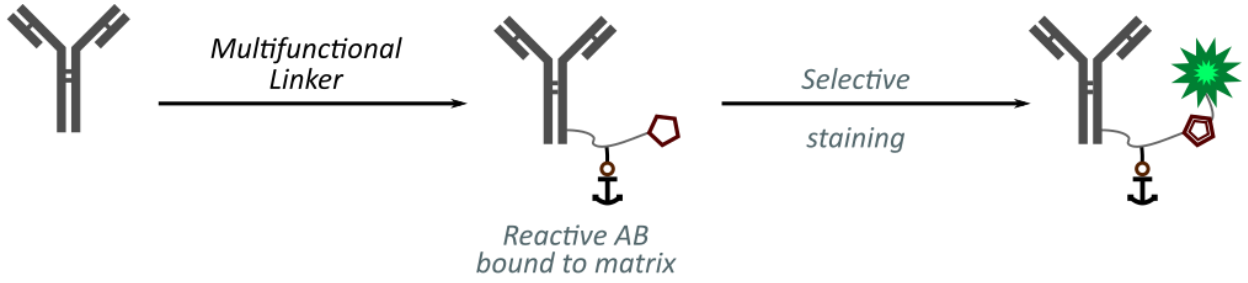
Primary AB anti Tubulin staining.

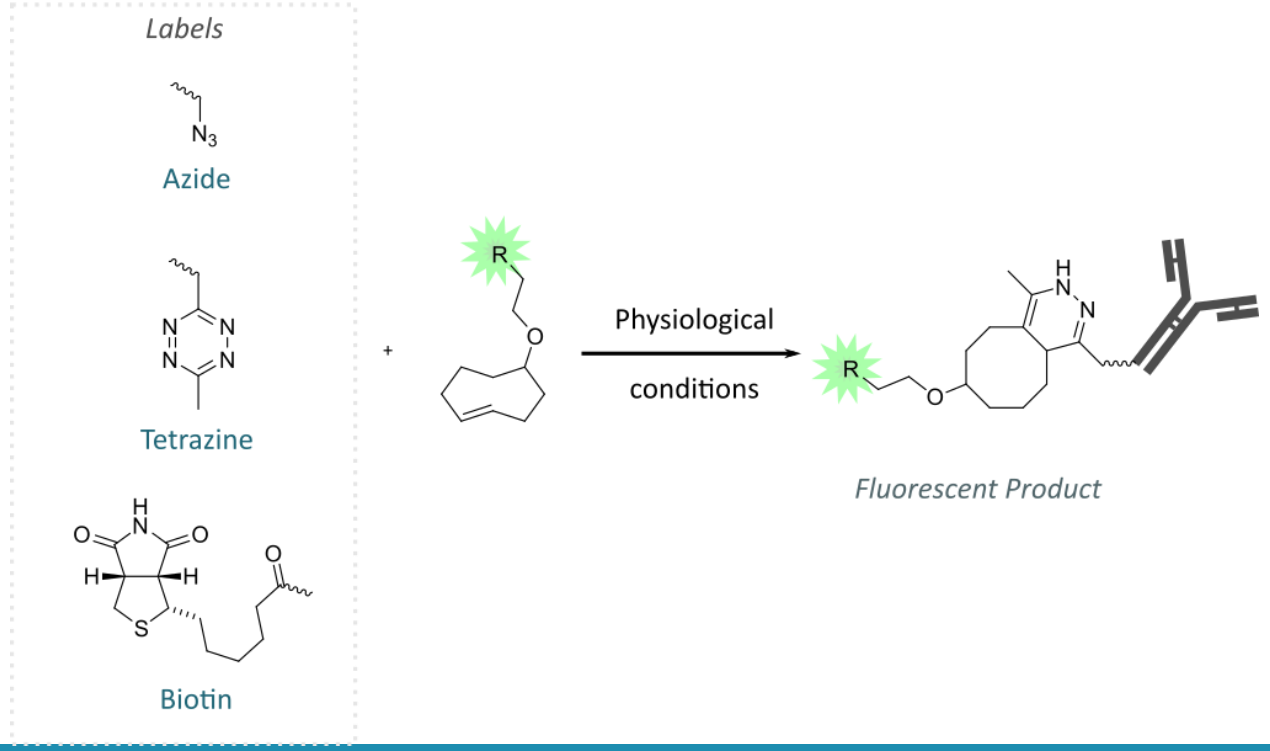


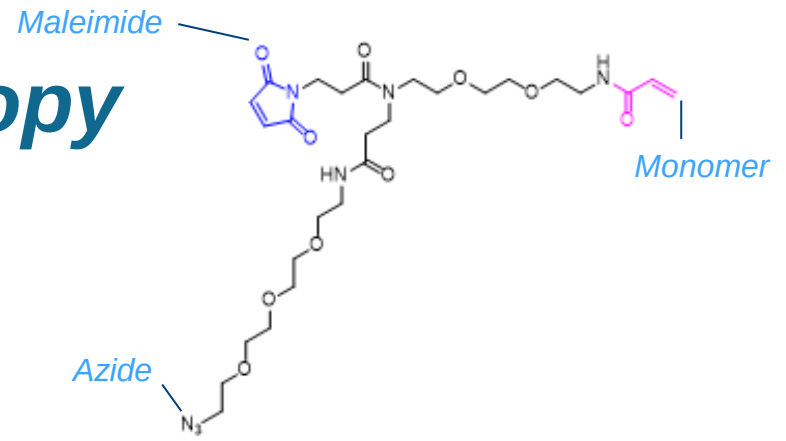
Primary AB anti Lamin staining.

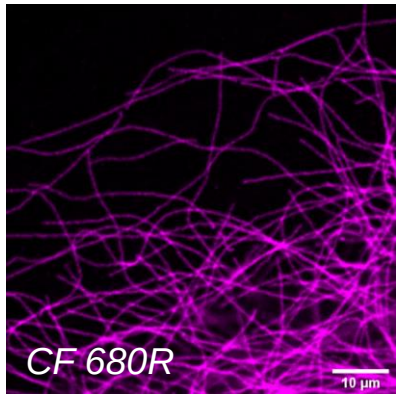
Antibody labeling for Expansion Microscopy

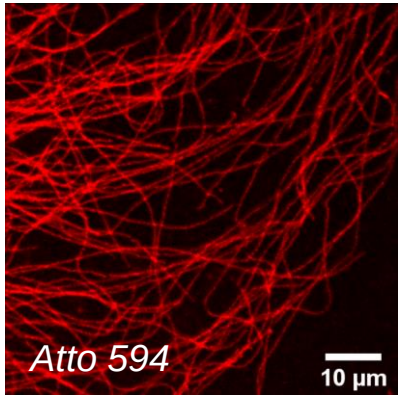
- Post-coupling of the dye allows for flexibility and dye selection

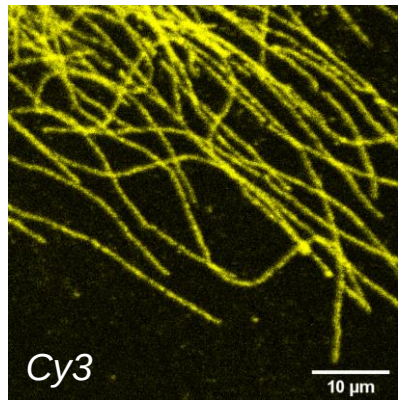


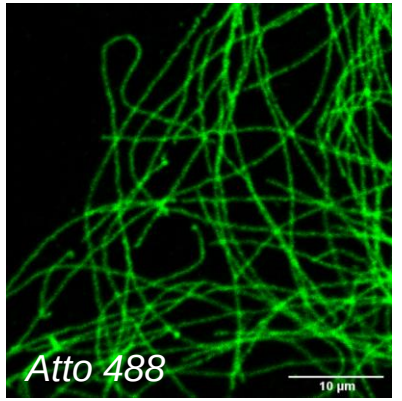






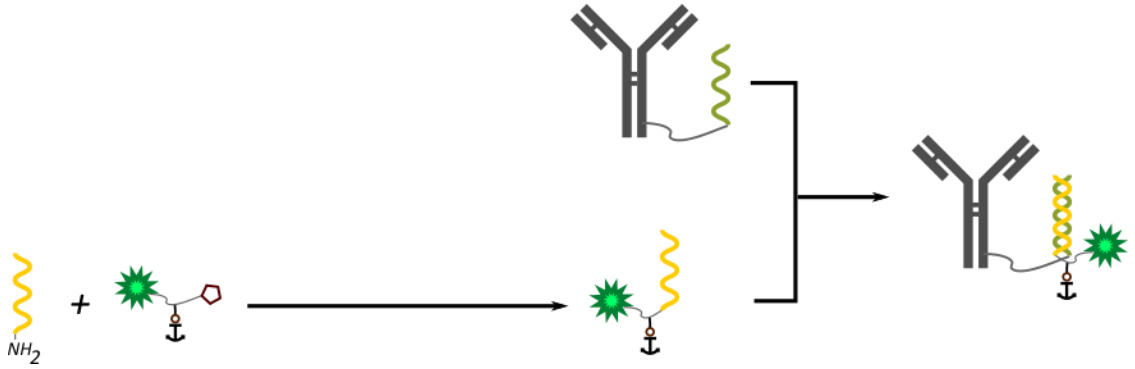


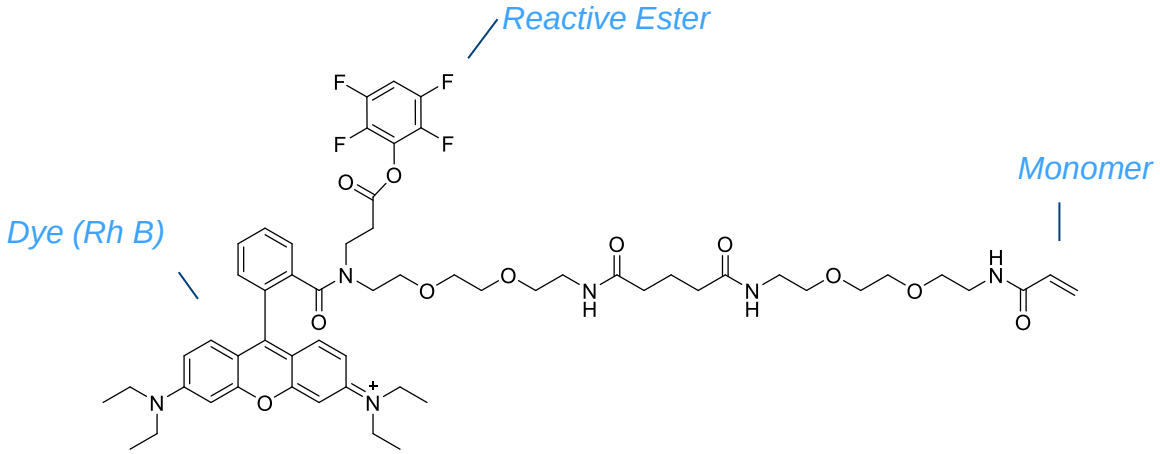


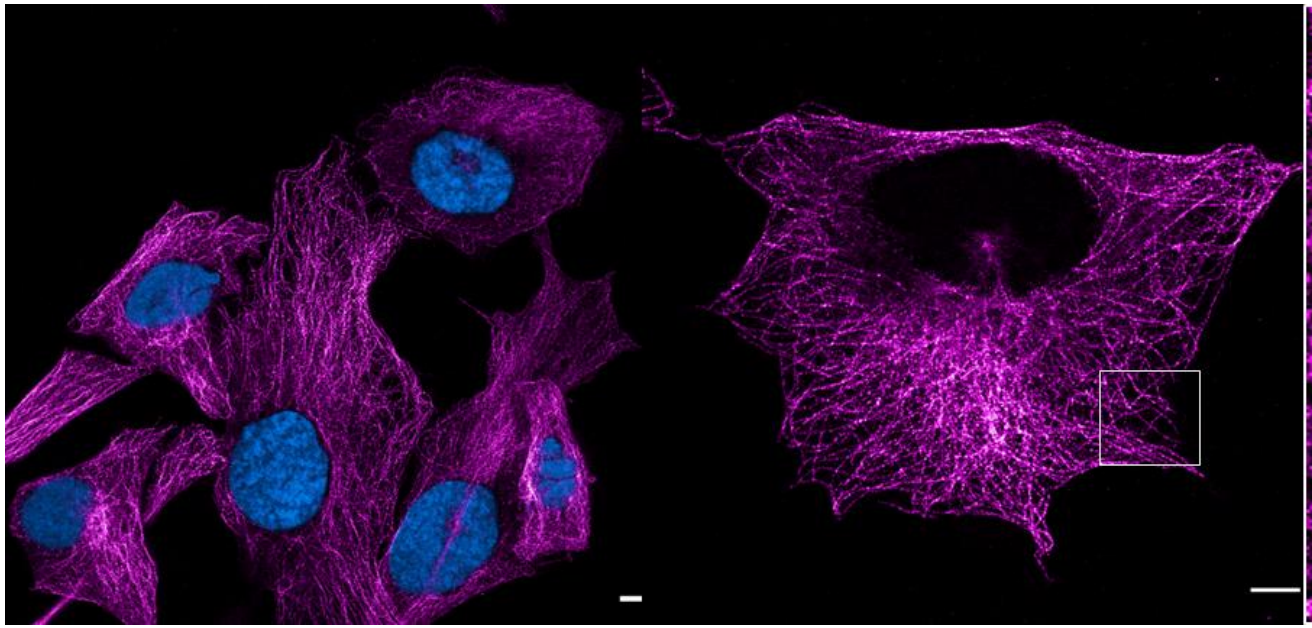


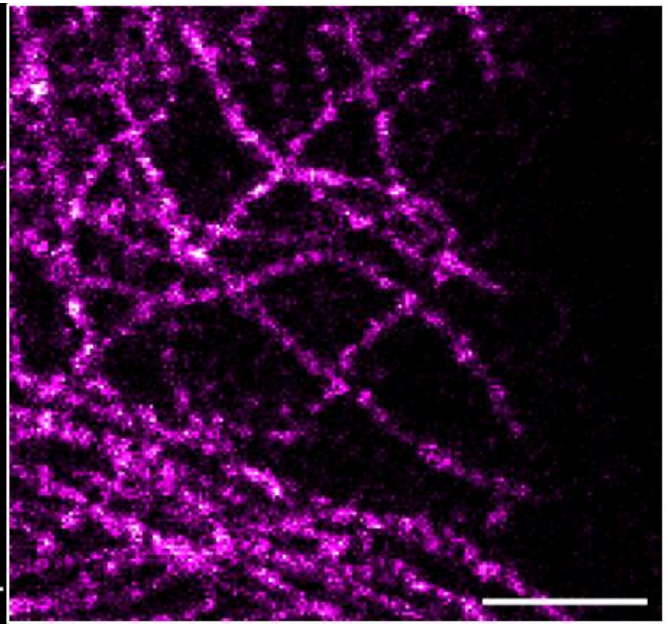
Antibody labeling for Expansion Microscopy

- Short oligonucleotides can replace the dye, for post gelation staining and barcoding



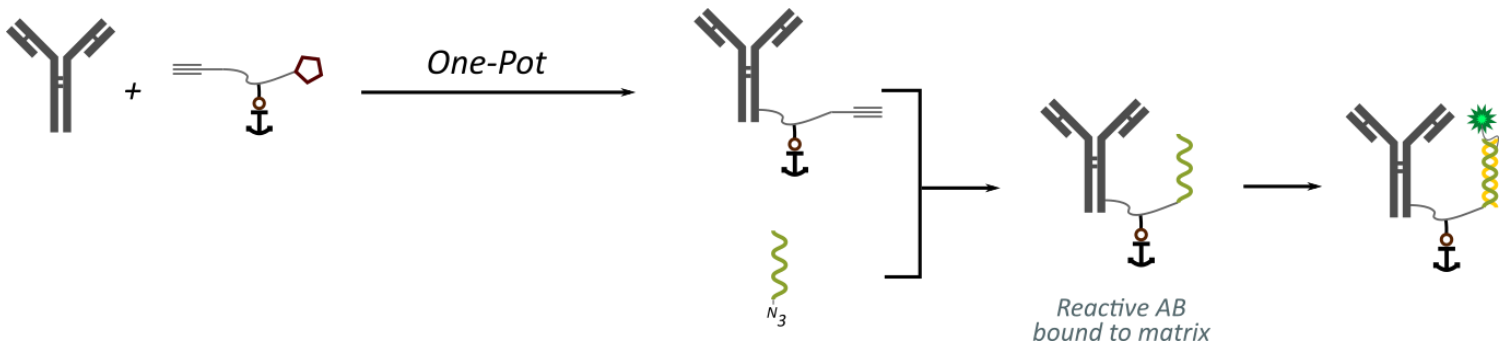


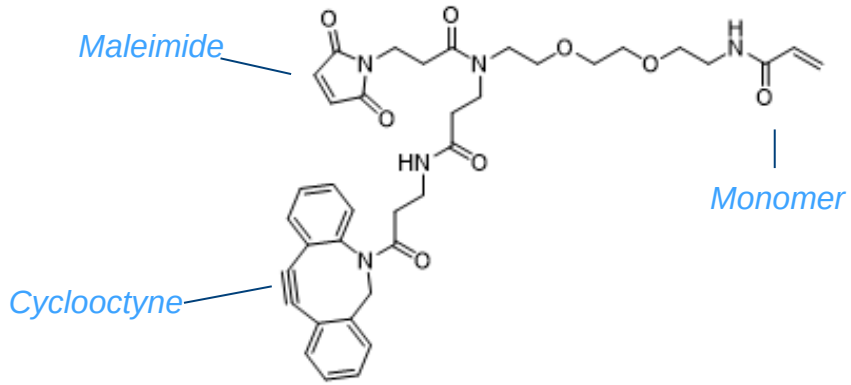


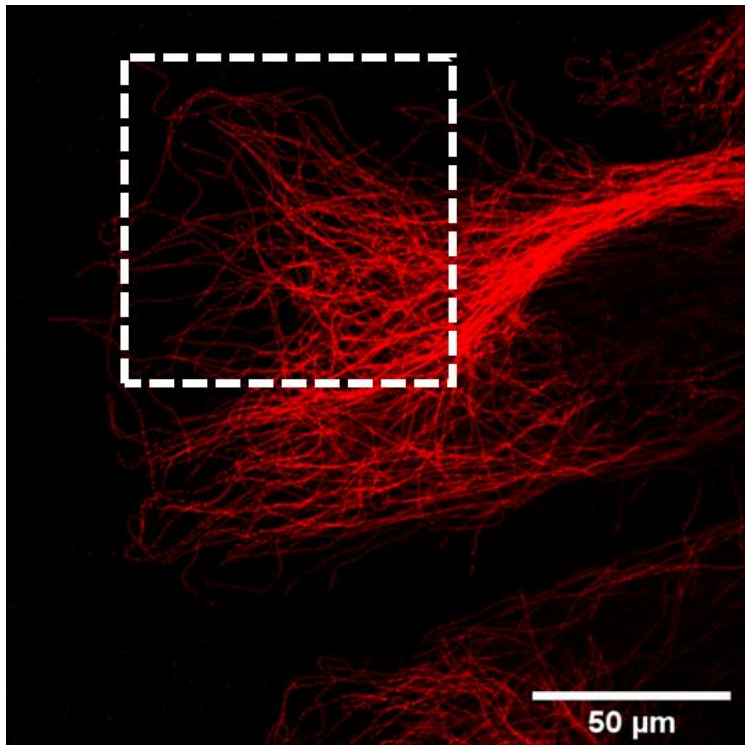


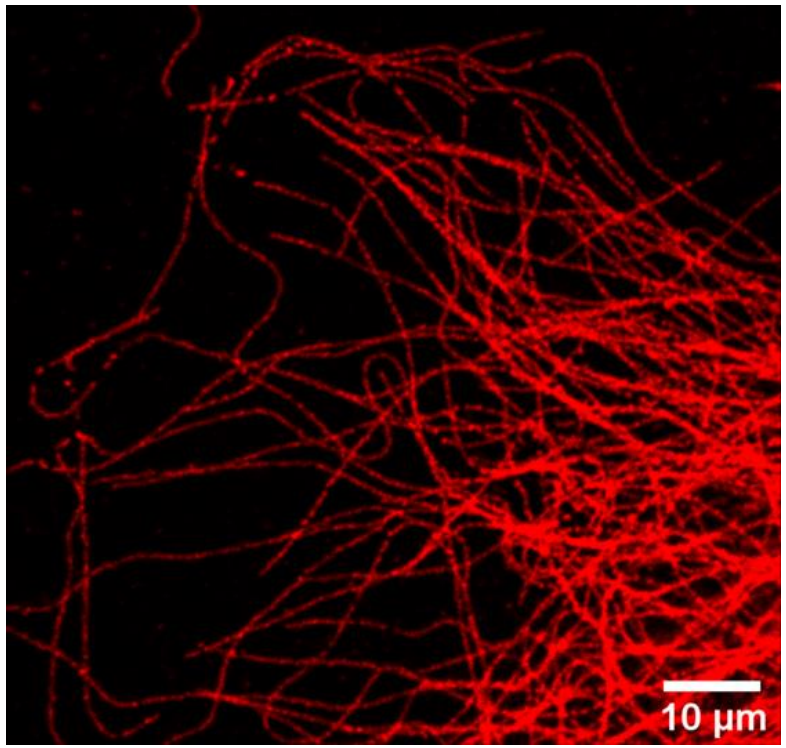
Antibody labeling for Expansion Microscopy

or ➔ precouple the antibody





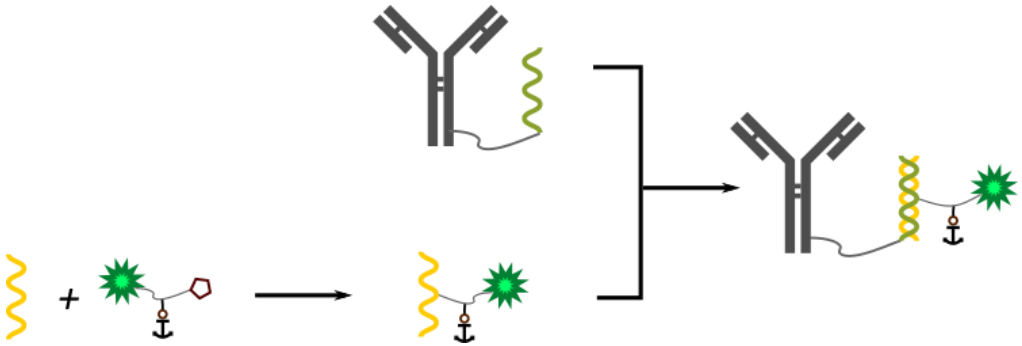




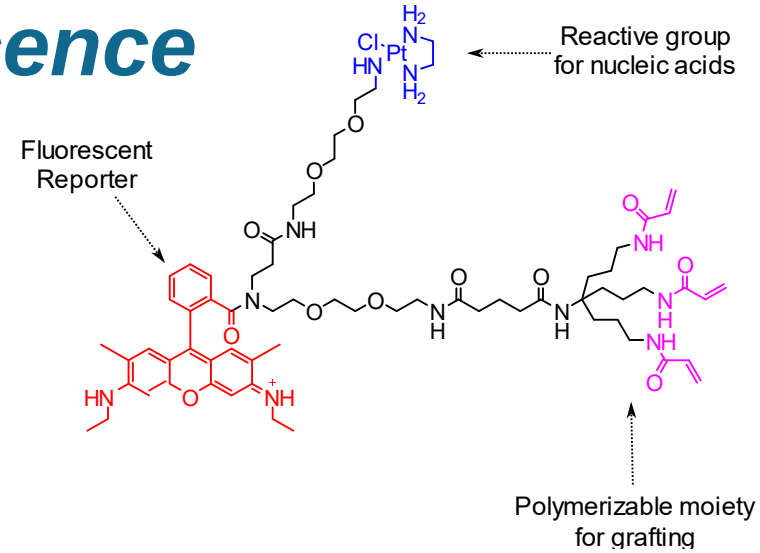
Excellent for split-mix approaches:
Only add reagent and use commercial
Azide Oligonucleotides

Oligo-mediated immunofluorescence

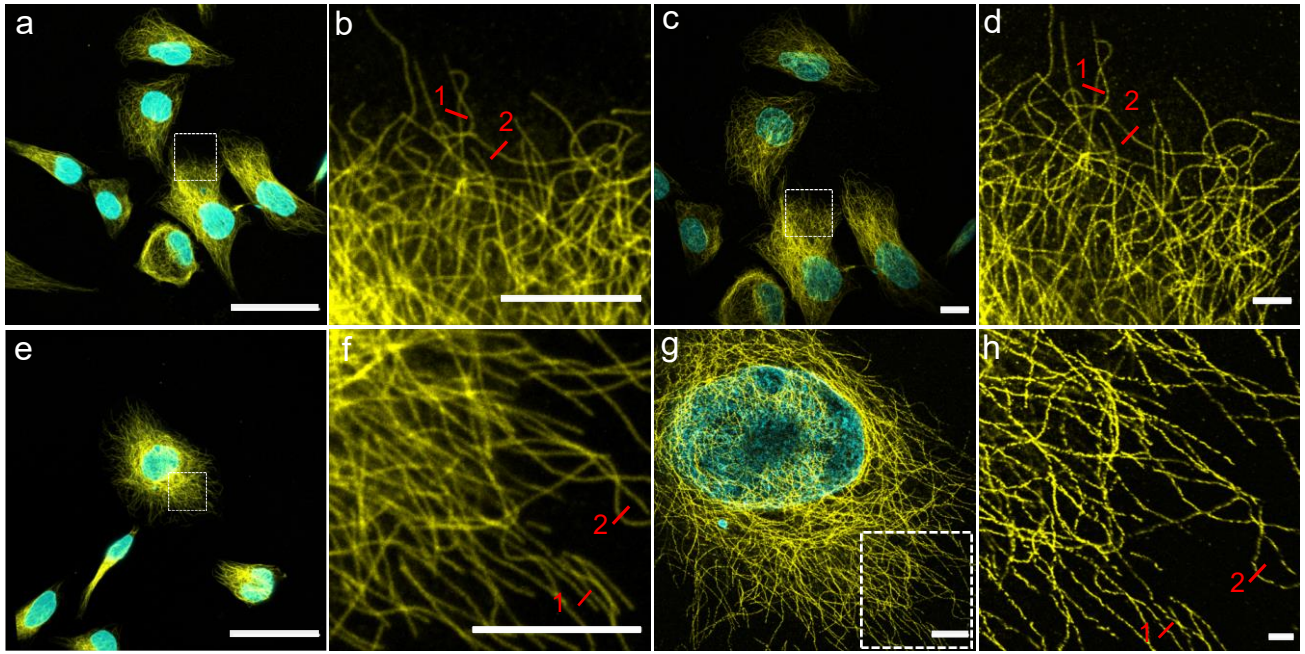
or ➔ graft the docking probe



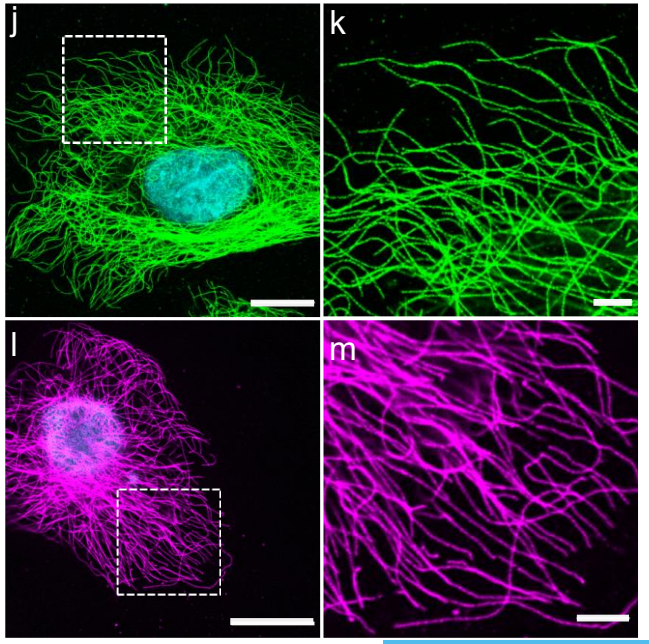
Allows the use of your current AB-Oligo conjugates without modification and with cheap Reporting probes (no mods)



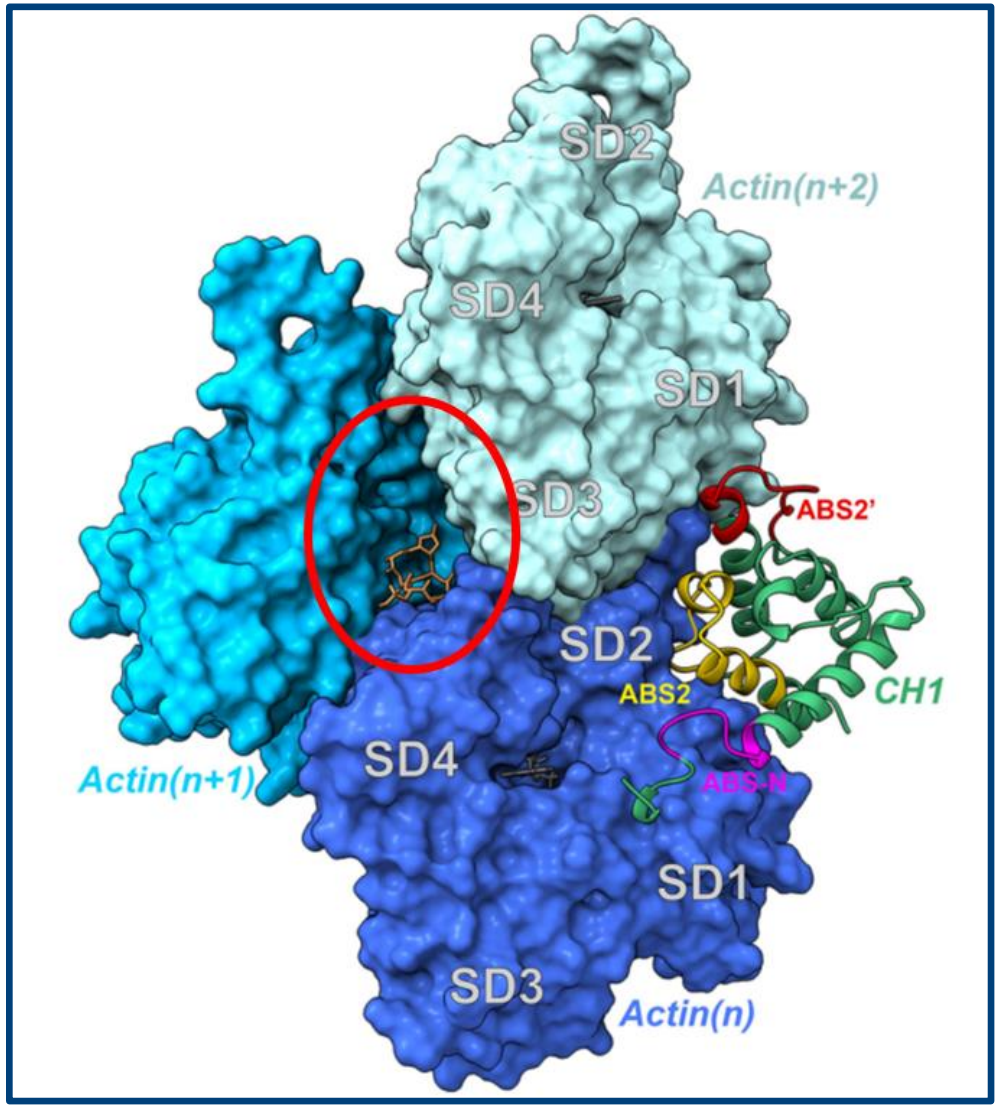
Secondary antibodies, ×4 ExM

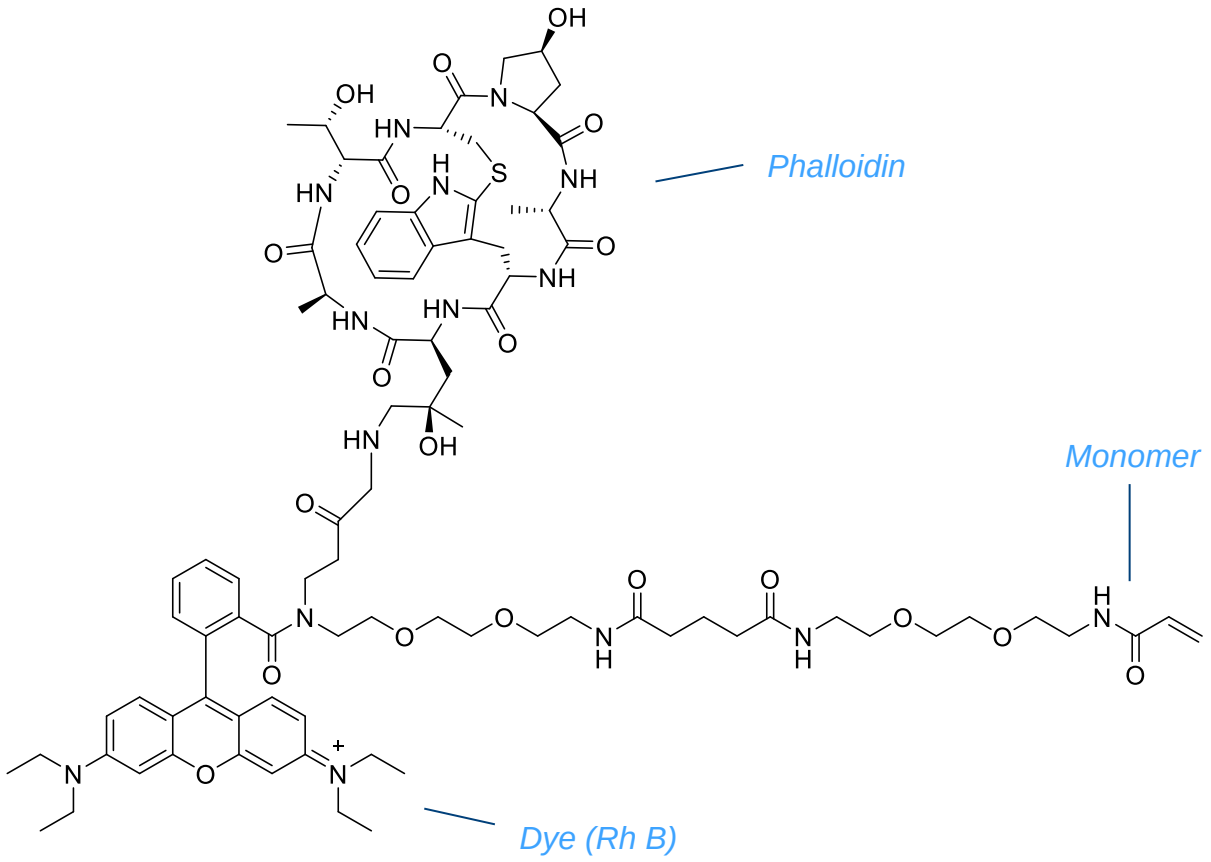


Azide variant for color switching & post gelation staining



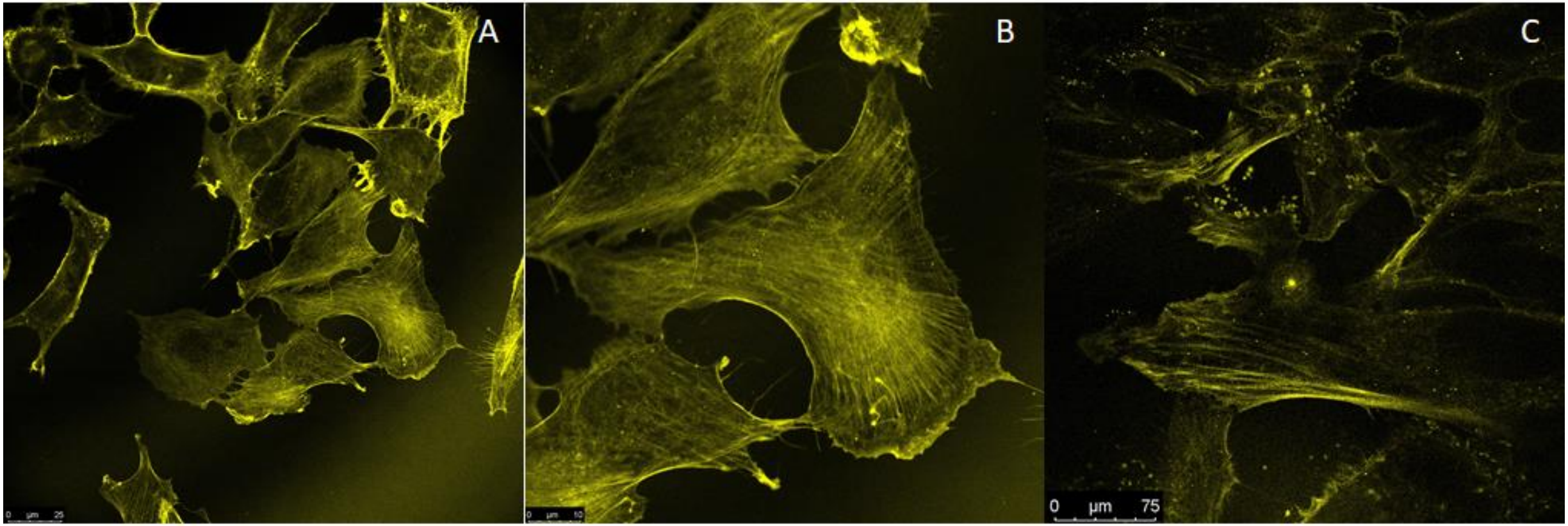
Small-molecule ligands for structural elements/cytoskeleton in ExM





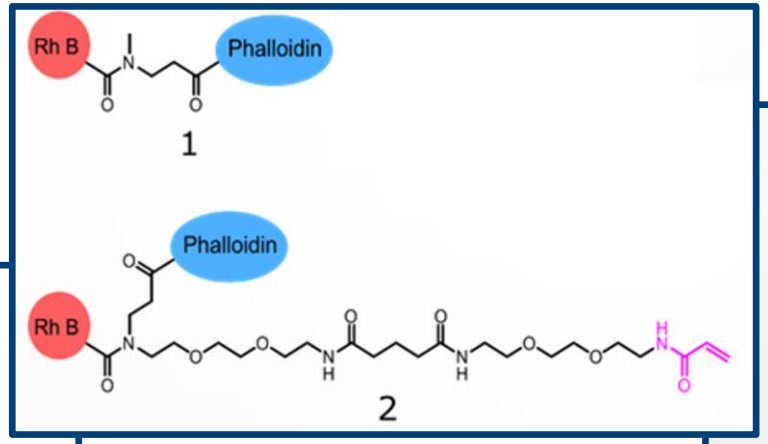
Small-molecule ligands for structural elements/cytoskeleton

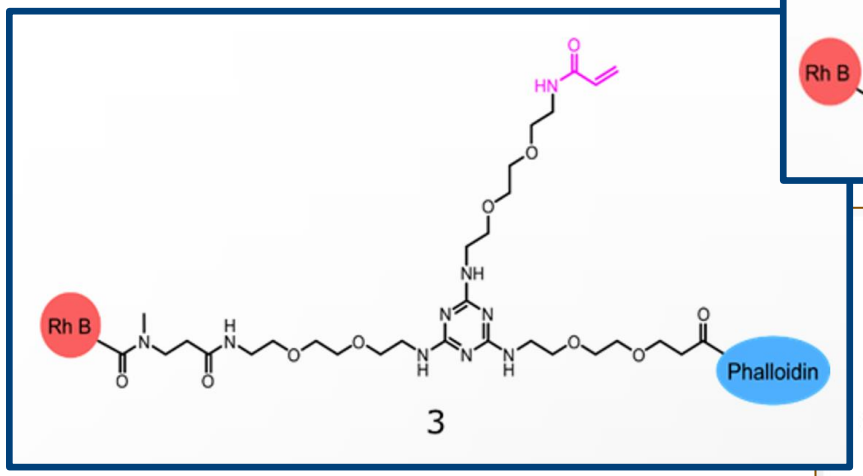
And this is what the first images looked like back in 2019:

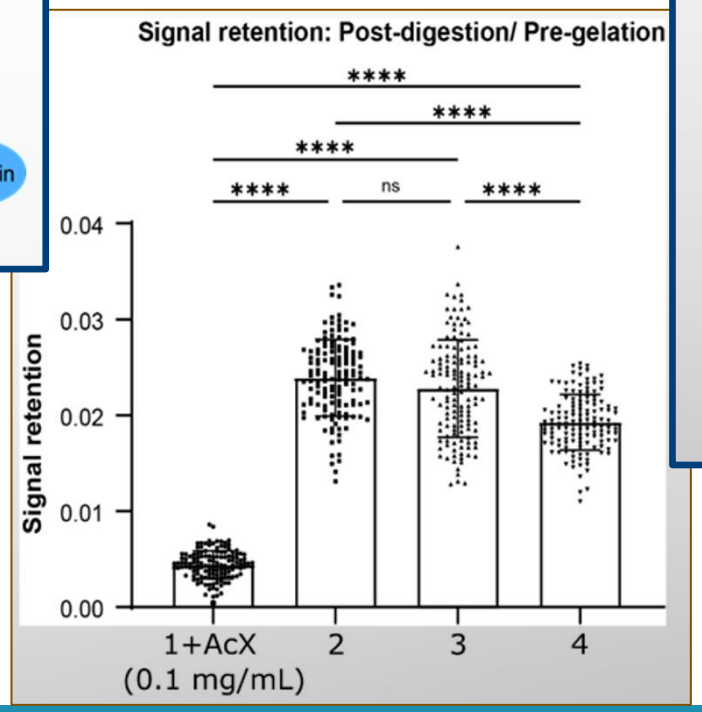


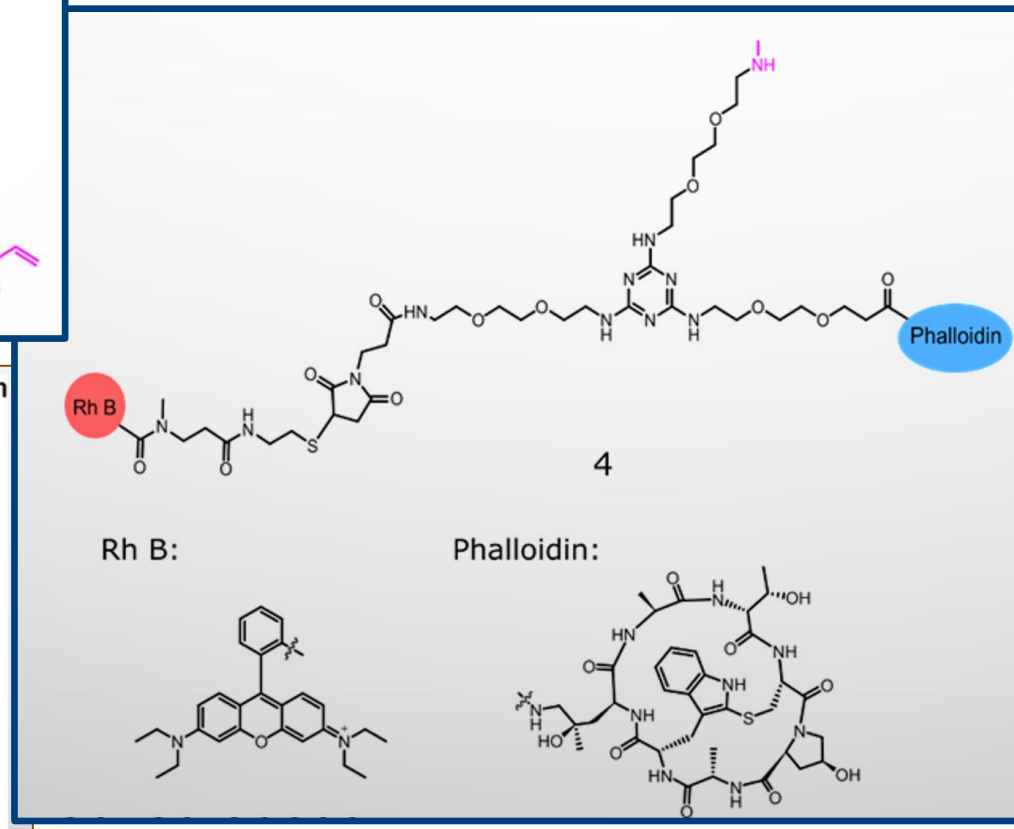
Pre-ExM (A, B) and Post-ExM (C) Rhodamin B Phalloidin stained HeLa cells (F-actin). Images recorded with LEICA TCS SP8 X CM, 63x wobj (A, B), 40x wobj (C).

Insights in structural requirements









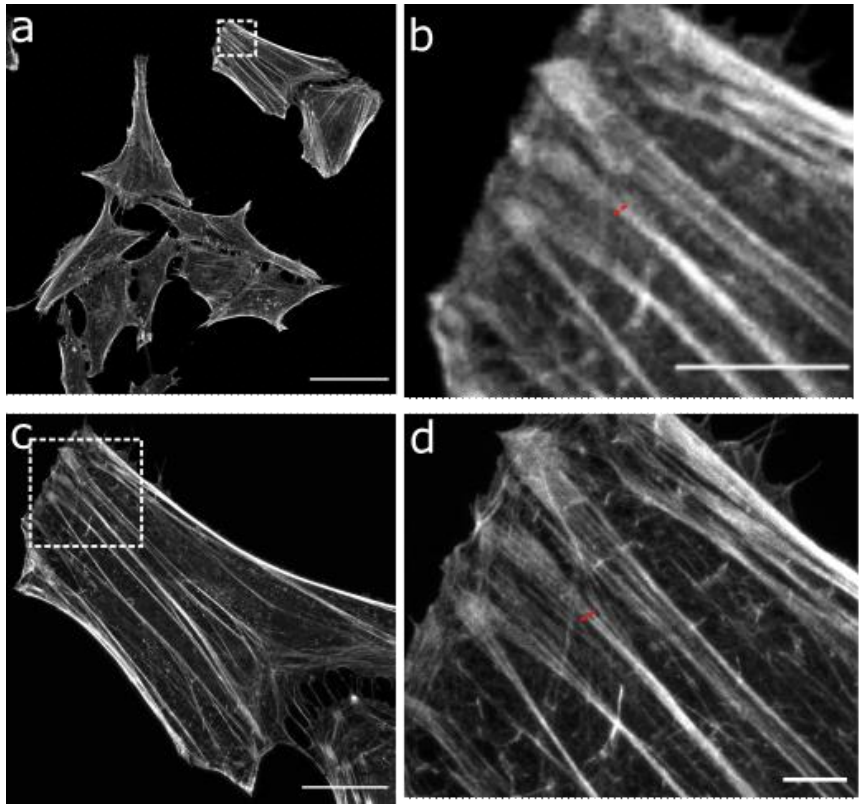
1+AcX: 0.004 ± 0.001 (n= 137)
2: 0.024 ± 0.004
3: 0.023 ± 0.005
4: 0.019 ± 0.003

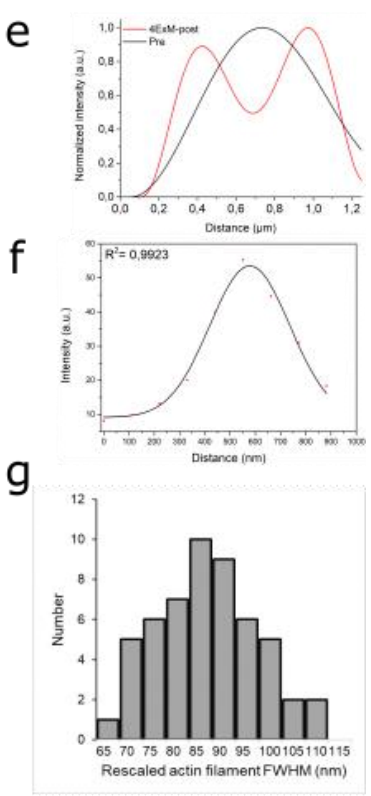
ACS Nano, 2023, 17 (20): 20589-20600.

Small-molecule ligands for structural elements/cytoskeleton

- Labeling is reliable, even in view of large variety of ExM protocols
  - ➢ So far, reagents work across all ExM protocols tested (radical based)

4-fold expansion

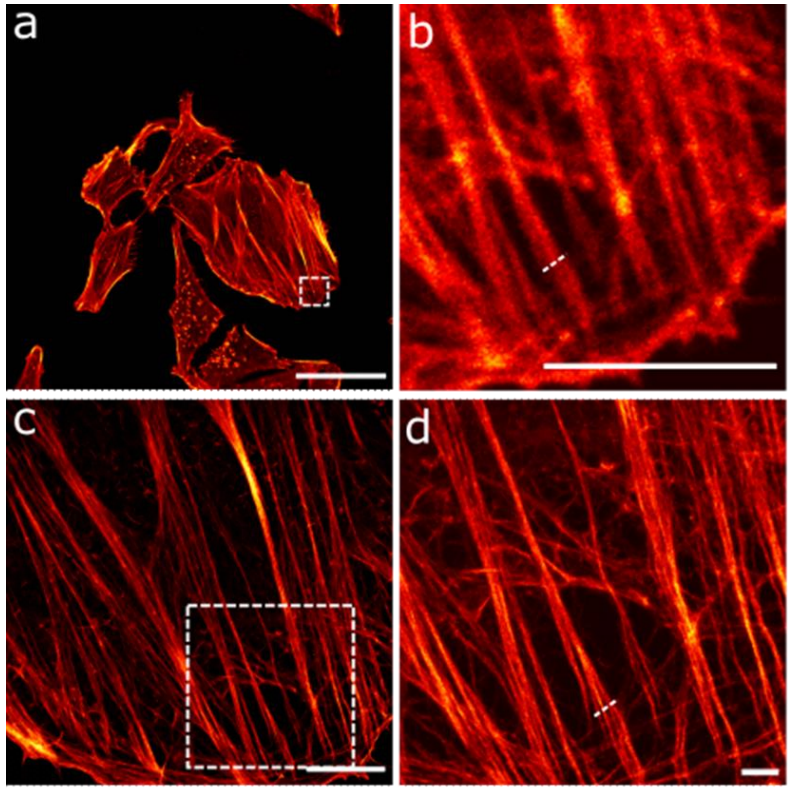


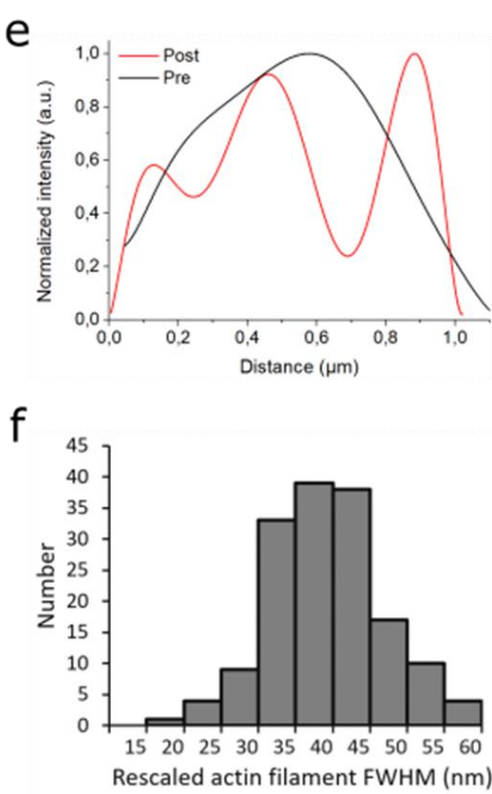


Scale bars: 50 µm (a, c), 10 µm (b, d).

89 ± 11 nm (n= 53)

TREx: 10-fold expansion



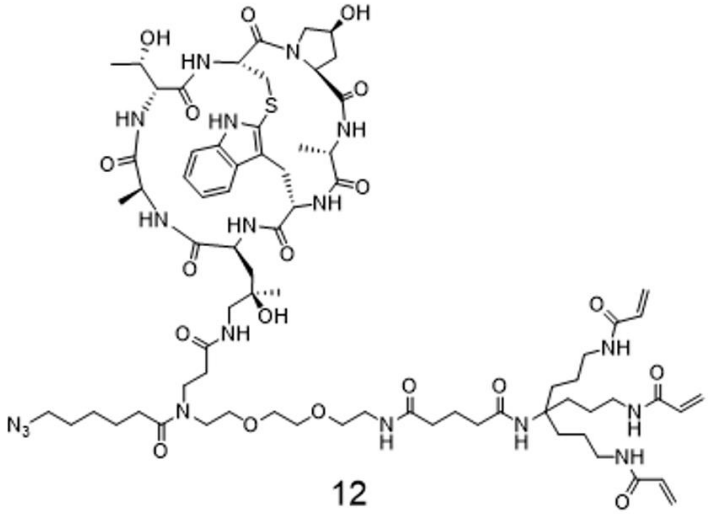


Scale bars: 50 µm (a, c), 10 µm (b, d).

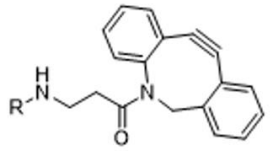
39 ± 7 nm (n= 152)

Post-digestion labeling offers signal flexibility

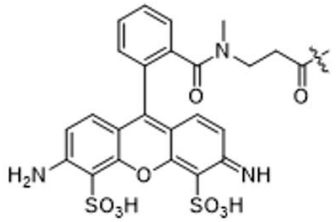
TREx: 10-fold expansion



12



13



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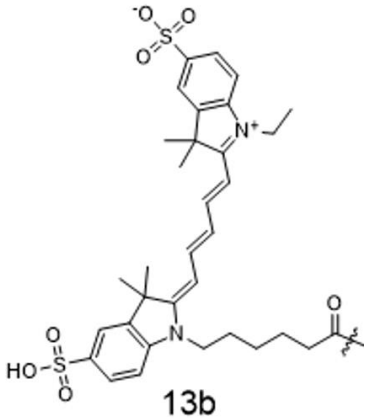
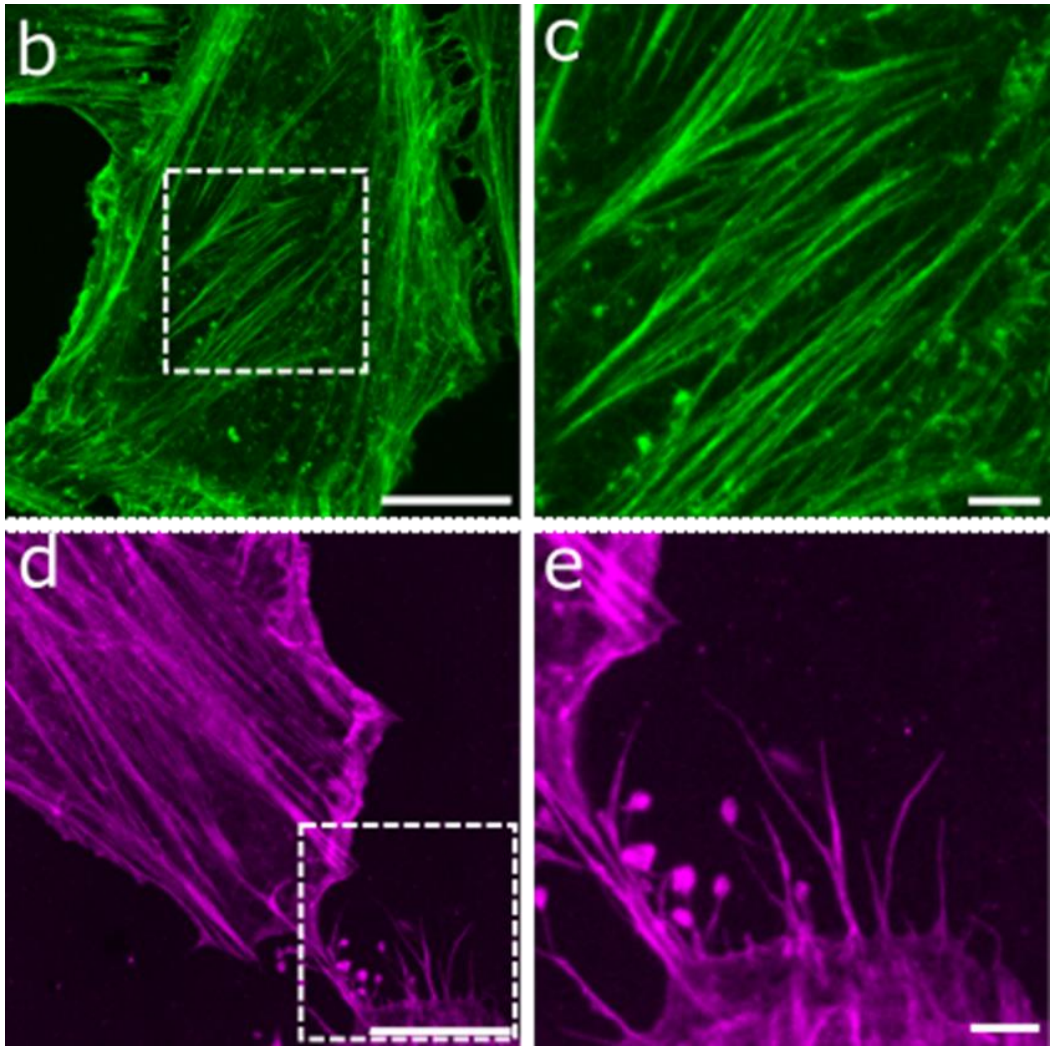


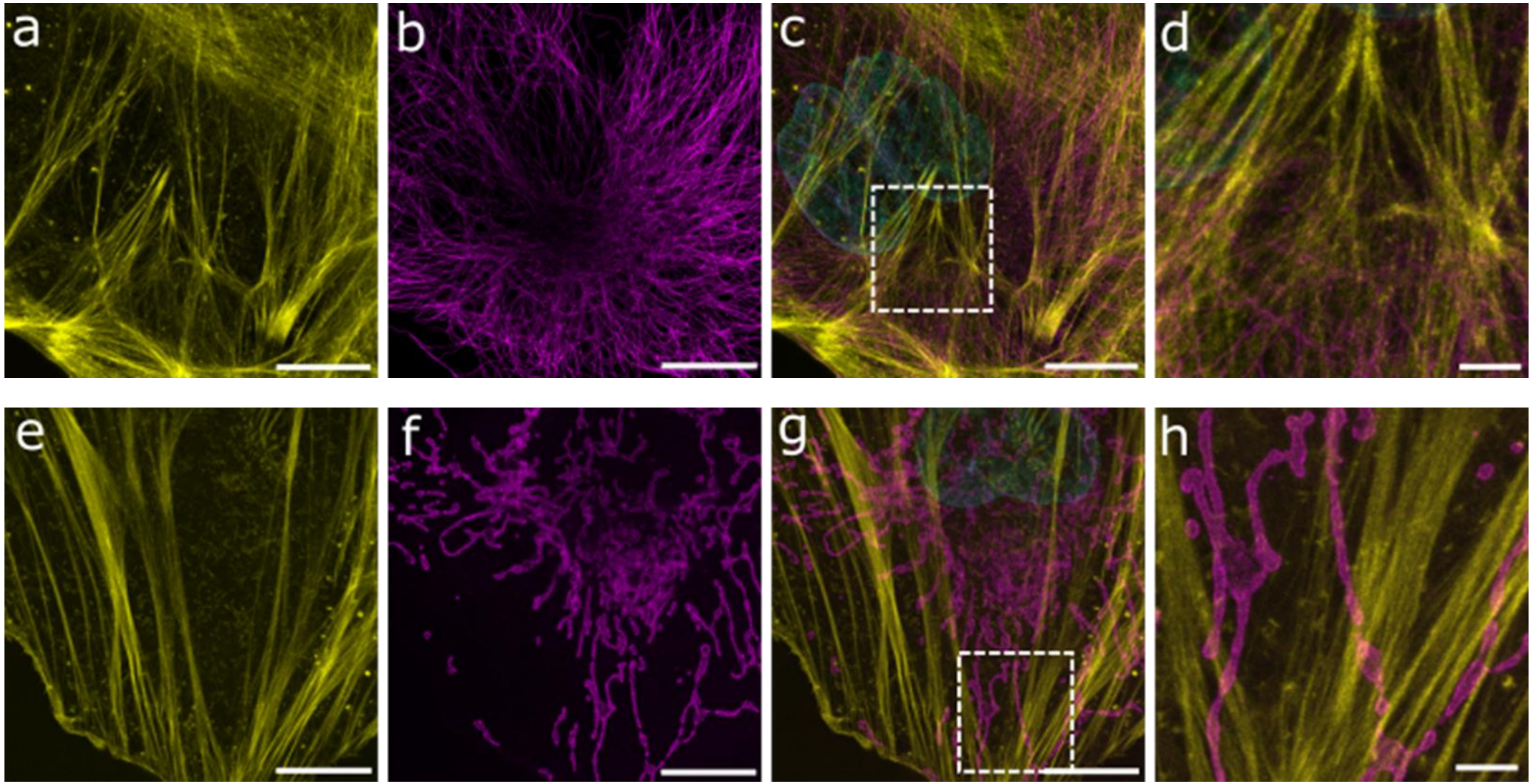
13b



Actin Staining in ExM combined with Immunostaining

Actin + Microtubules

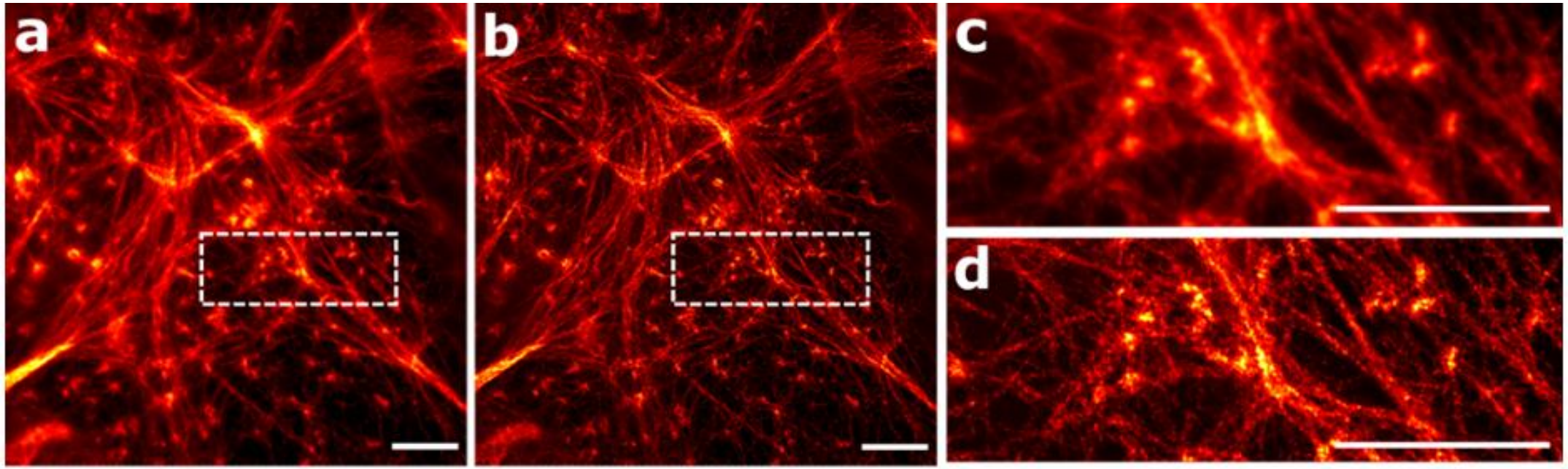
Actin + Mitochondria



Scale bars: 50 um (a, b, c, e, f, g), 10 um (d, h).

Actin Staining: Pushing resolution with Microscopy

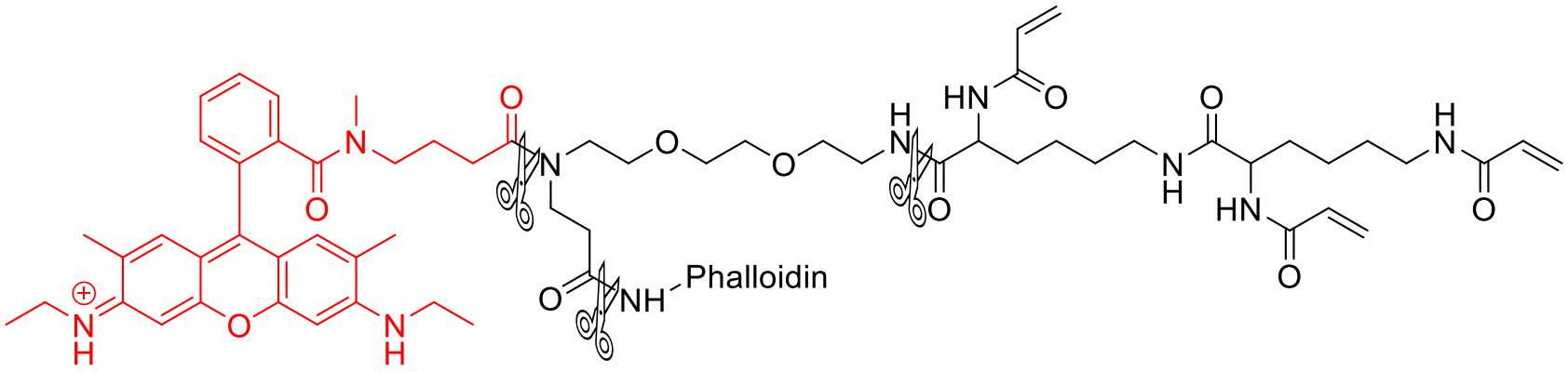
4x ExM-SIM



Widefield image

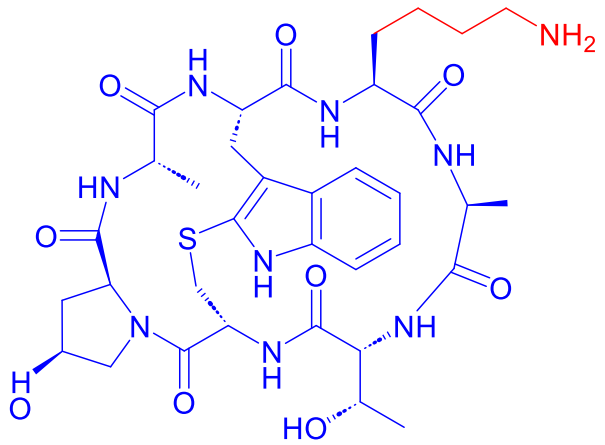
SIM image

Scale bars: 10 um (a-d).



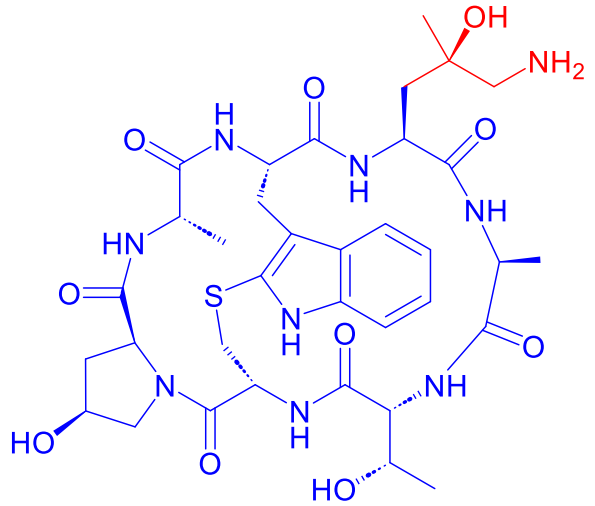
Chain Extended Rho 6G

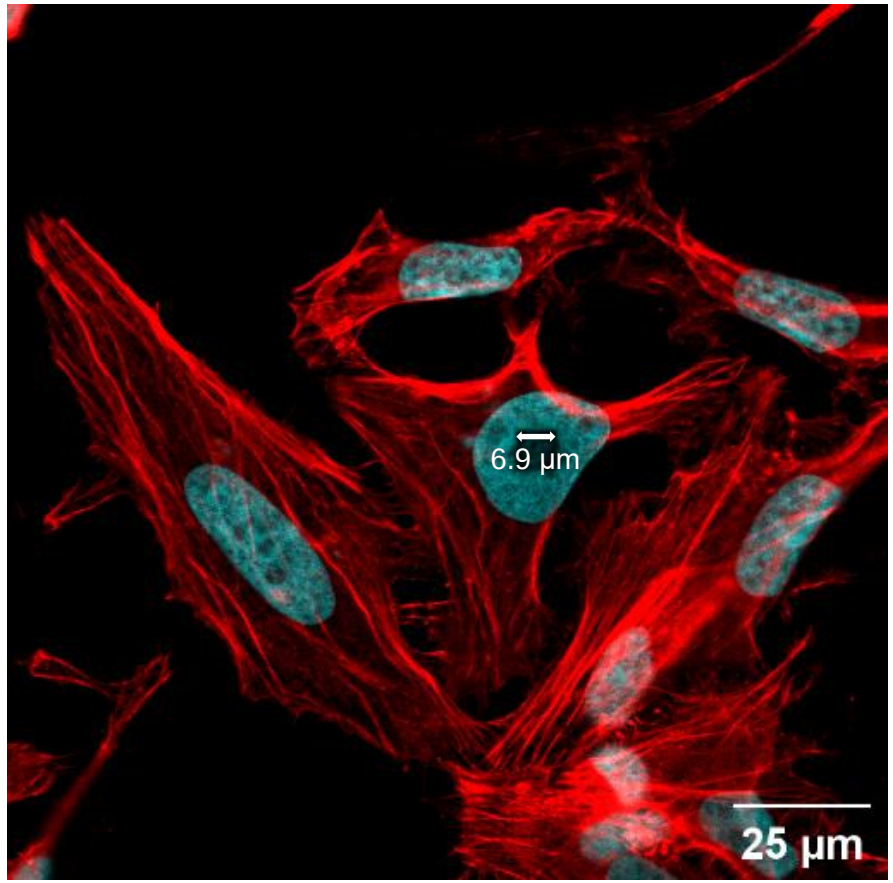
Phalloidin lysin

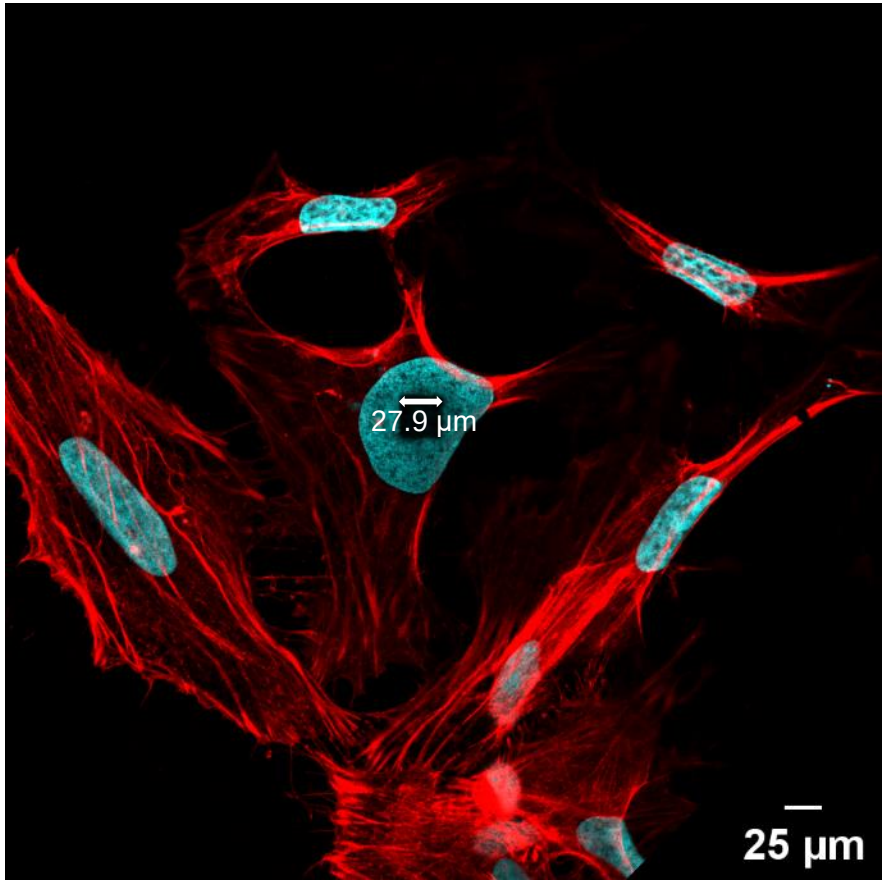


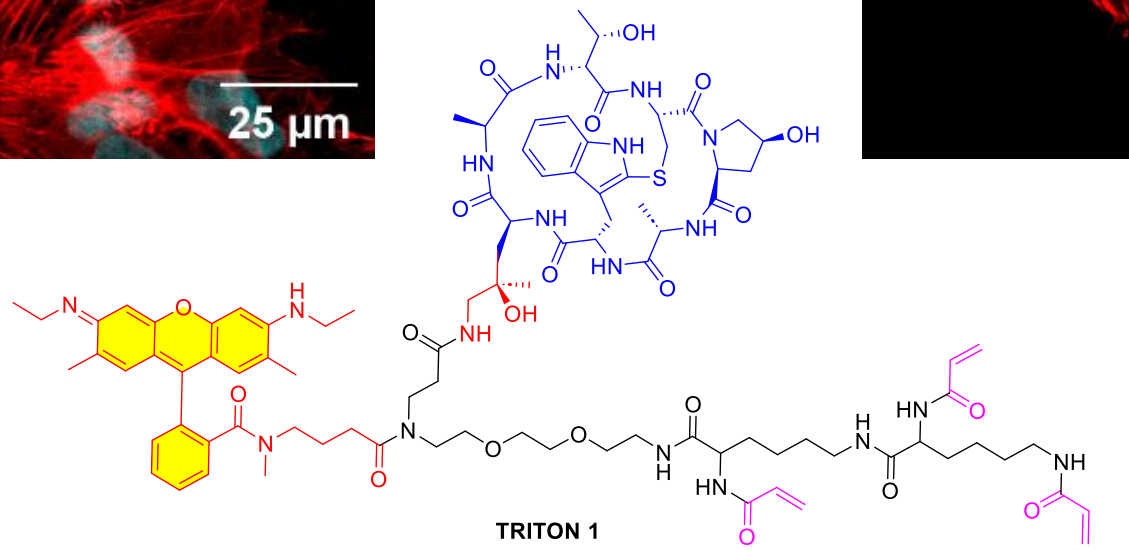
- Strong absorption ε_{max} 1.15×10^{5} $M^{-1}\,cm^{-1}$
- Extraordinarily high fluorescence quantum yield 94%
- High thermal and photo-stability
- Moderately hydrophilic
- Carries a net electric charge of +1
- Excited efficiently in the range 515 - 545 nm, λ_{abs} 533 nm / λ_{fl} 557 nm
- A suitable excitation source for **Chain extended Rho6G** is the 532 nm line of the frequency-doubled laser.

Phalloidin

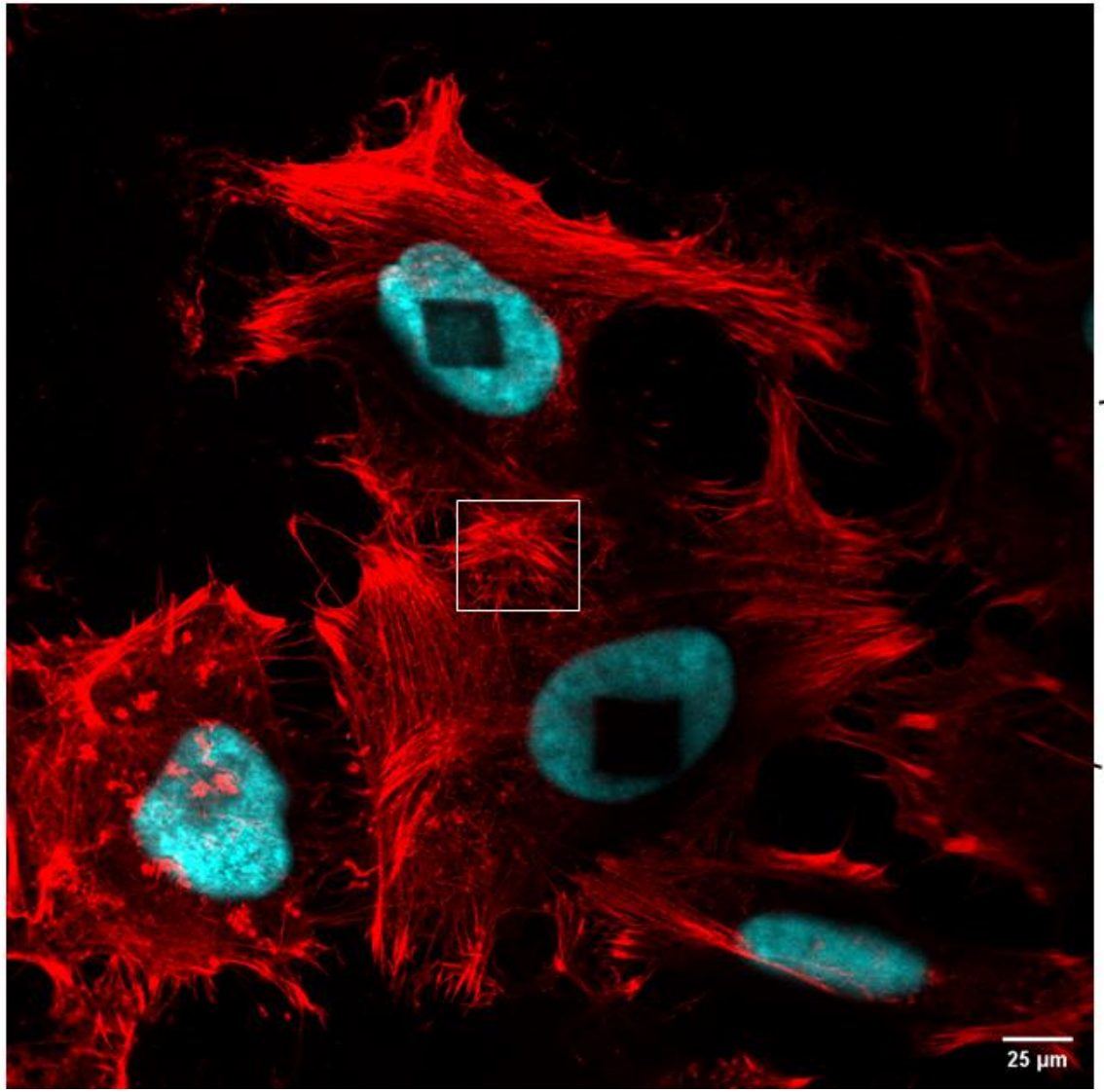


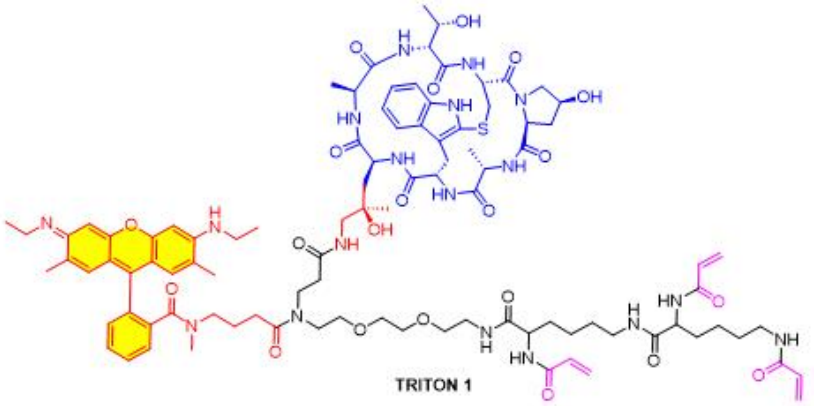


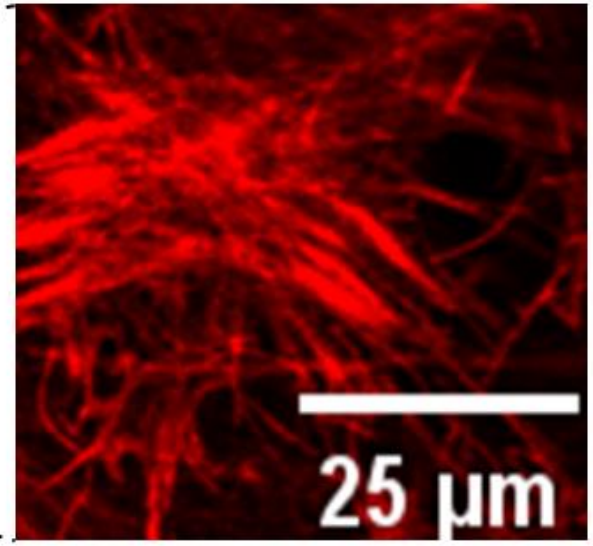


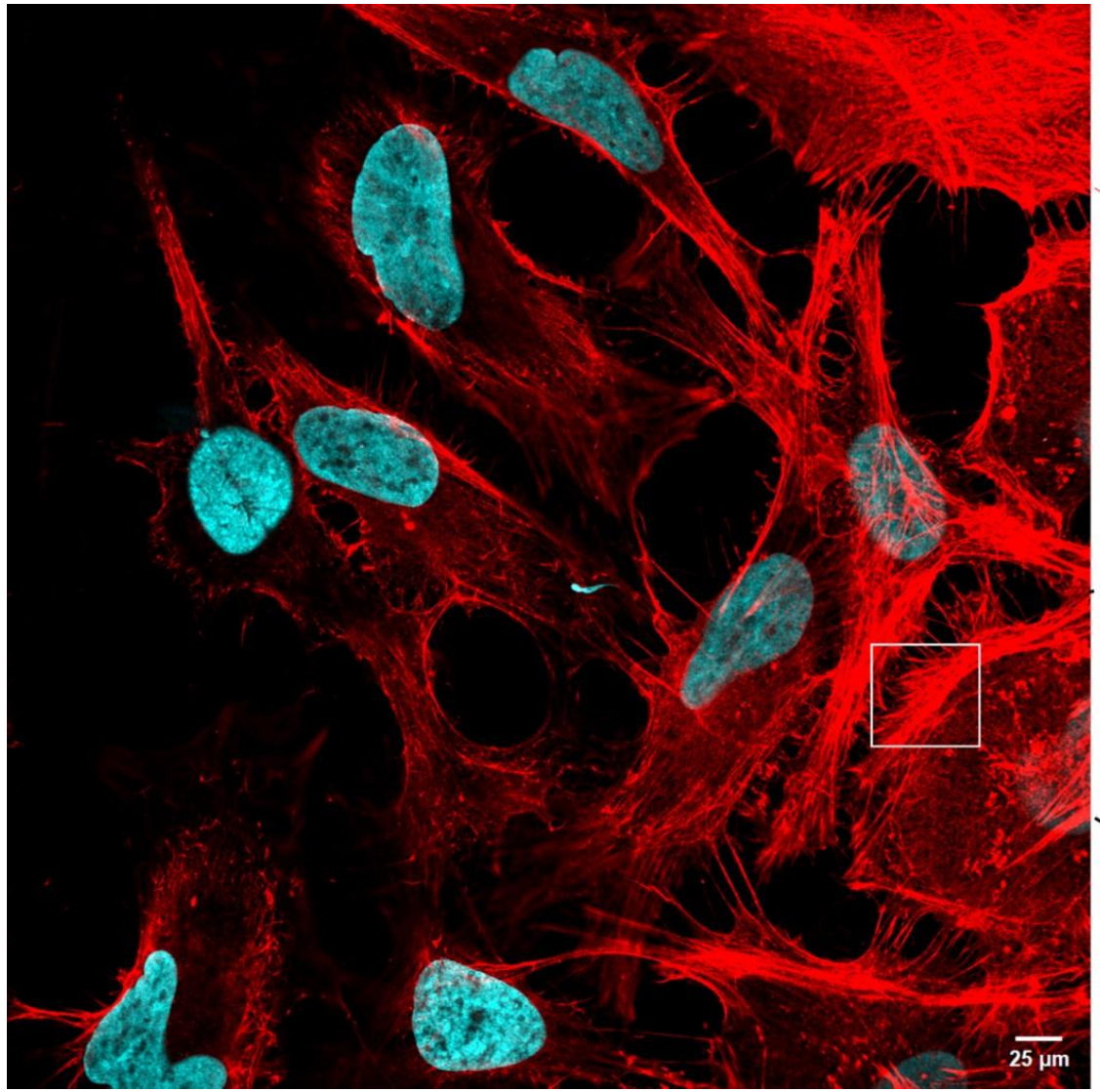


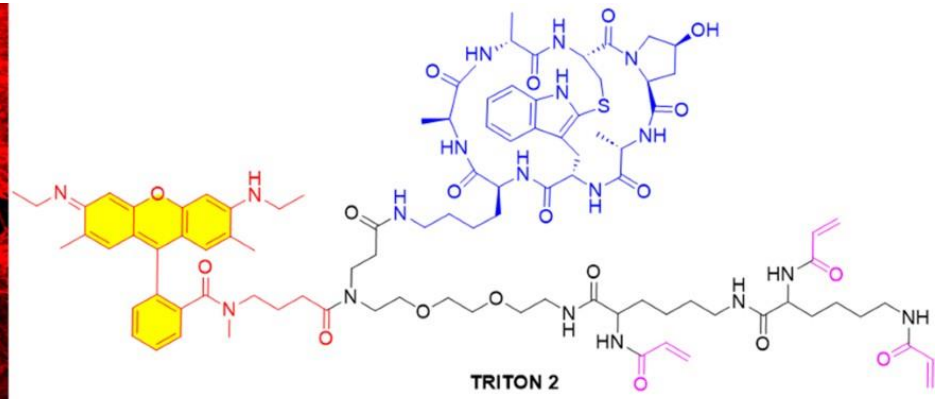
TRITON 1

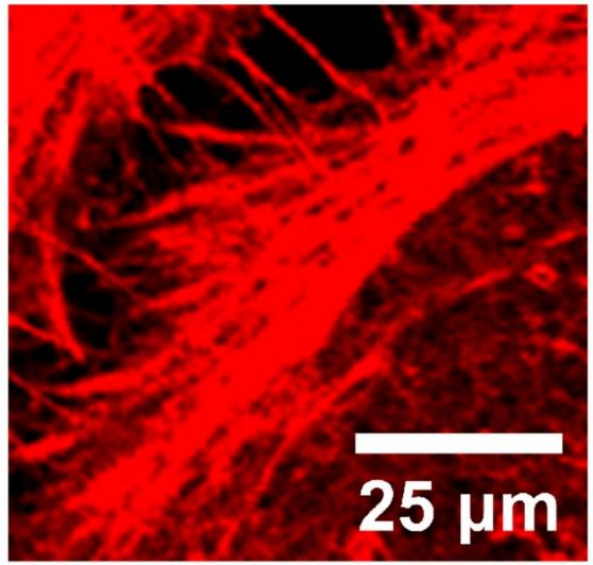

25 µm


TRITON 1


25 µm


25 µm


TRITON 2


25 µm

Conclusions and Outlook

Conclusions:

Approach of multifunctional linkers for ExM is now well accepted, with several groups iterating on the concept.

- Various types of biological information addressable.
- Improved signal retention
- Simple protocols
- Compatible with different ExM modalities

What next?

- Single approach for oligo-tagged read out of multiple targets at different levels of expression
- Use of Multifunctional linkers a scaffold in error-corrected read-outs
- Extension into non-radical based gel formulas
- Further mechanistic understanding on the issue of “AcX always helps!”

Key References:

- Wen, *ACS Nano*, 2020, 14(7), 7860
- Wen, *J. Am. Chem. Soc*, 2021, 143(34),13782
- Wen, *Chemical Reviews*, 2023, 123(6), 3299
- Wen, *ACS Nano*, 2023, 17(20), 20589

Acknowledgements & Contacts

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