Single-molecule Localization Expansion Microscopy

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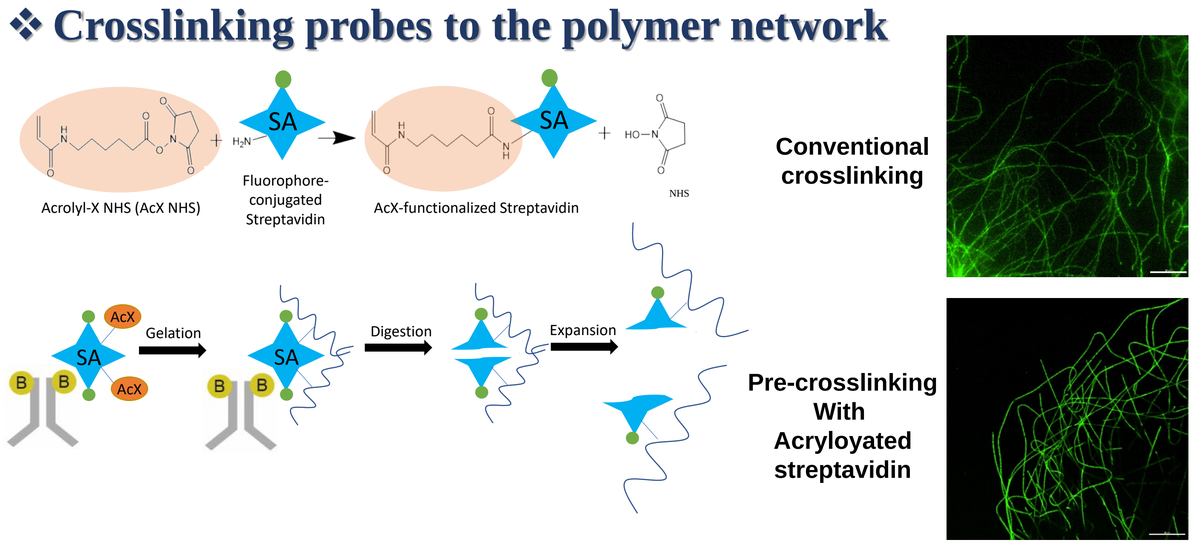
ABSTRACT

Expansion Microscopy (ExM) is a method to improve the spatial resolution by physically expanding a sample linked to swellable gel. An advantage of the expansion microscopy is that it allows observers to overcome the diffraction limit using the conventional fluorescence microscopy. On the other hand, single-molecule localization microscopy (SMLM) is a super-resolution microscopy offering 10-20 nm resolution. This work combined ExM and SMLM by labeling the enlarged sample with photoswitchable fluorophores and separately localizing the centroid positions of the single molecules. Since ExM expands the sample volume by 4 times and SMLM improves by 10 folds, the combined method has potential to achieve ~5 nm resolution, reaching the molecular scales. In order to achieve the goal, we developed a method with three major components: (1) We re-embedded the expanded gels in non-swellable polymer to prevent substantial shrinkage of the gel under high salt concentrations; (2) We utilized short peptide tags to reduce the distance between the target molecule and fluorophores; (3) We functionalized streptavidin for minimizing loss of fluorescent labels during digestion and expansion. Reducing the target-fluorophore distance is important in locating the position of the target molecule more precisely. These combined efforts open windows for resolving the molecular structures of biomolecules inside cells.

KEYWORDS: Expansion Microscopy, single-molecule localization microscopy (SMLM), Stochastic Optical Reconstruction Microscopy (STORM), DNA-PAINT (points accumulation for Imaging in Nanoscale Topography)

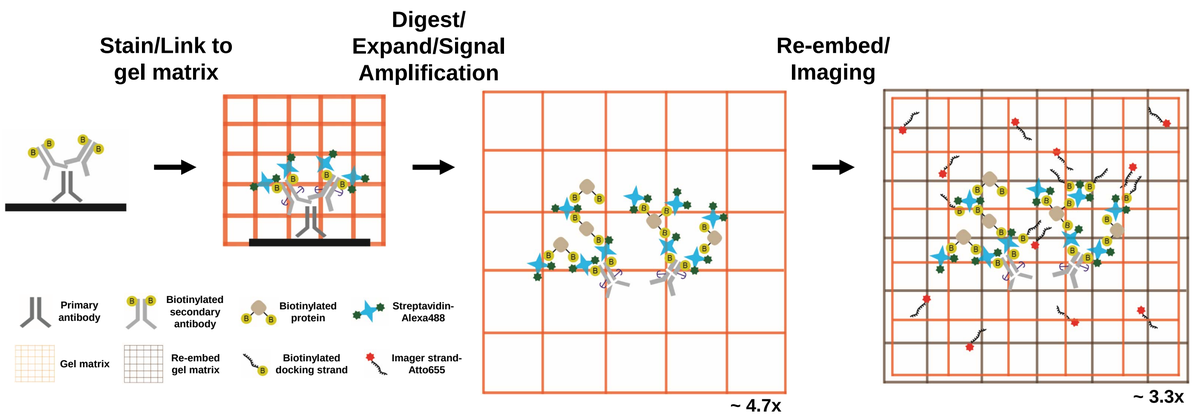
Chozinsky et al. *Nature Method* (2016)
Schnitzbauer et al. *Nature Protocol* (2017)

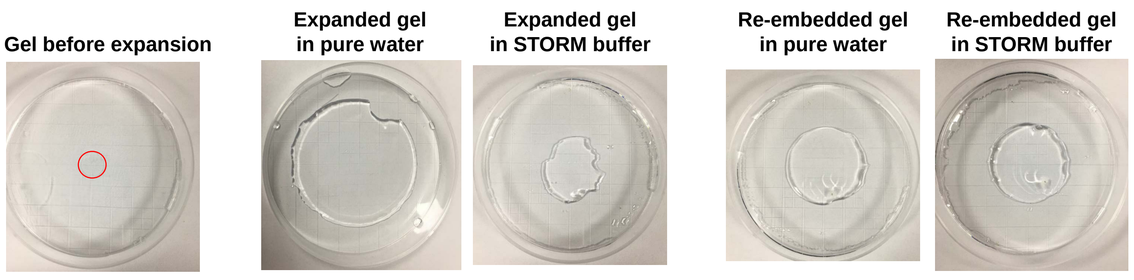
❖ Crosslinking probes to the polymer network



- Since a significant portion of streptavidin is lost during digestion step, we devised a way to directly conjugate streptavidin to the polymer network.
- Streptavidin is functionalized with Acryloyl-X crosslinker prior to labeling so that it can improve chance to link to polymer gel resulting in improvement of density of fluorophores.

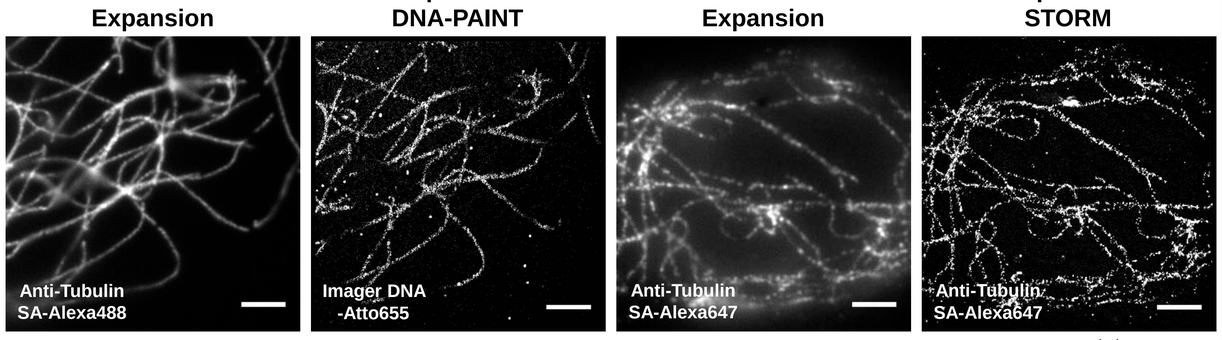
❖ Re-embedding for using high-salt buffer





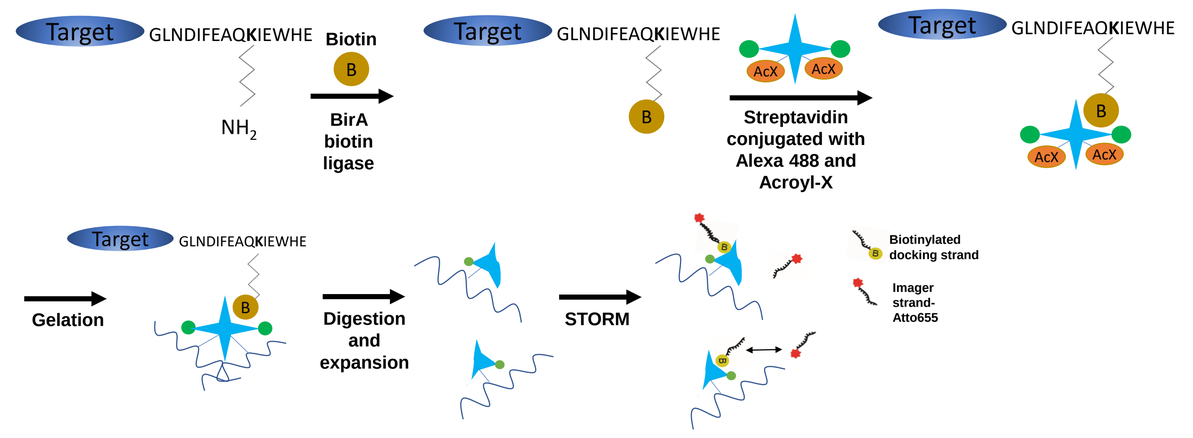
- The switching chemistries in single-molecule localization microscopy (SMLM) require imaging buffer with high salt concentration that result in shrinkage of the expanded gel.
- To prevent the gel shrinkage, prior to SMLM imaging, we re-embedded expanded gels with non-swellable polymer.
- After re-embedding, the gel shrinks slightly by ~30%, yielding final expansion factor of ~3.3.
- The re-embedded gels do not shrink noticeably in STORM buffer with 100 mM mercaptoethyl amine for photoswitching of cyanine dyes.

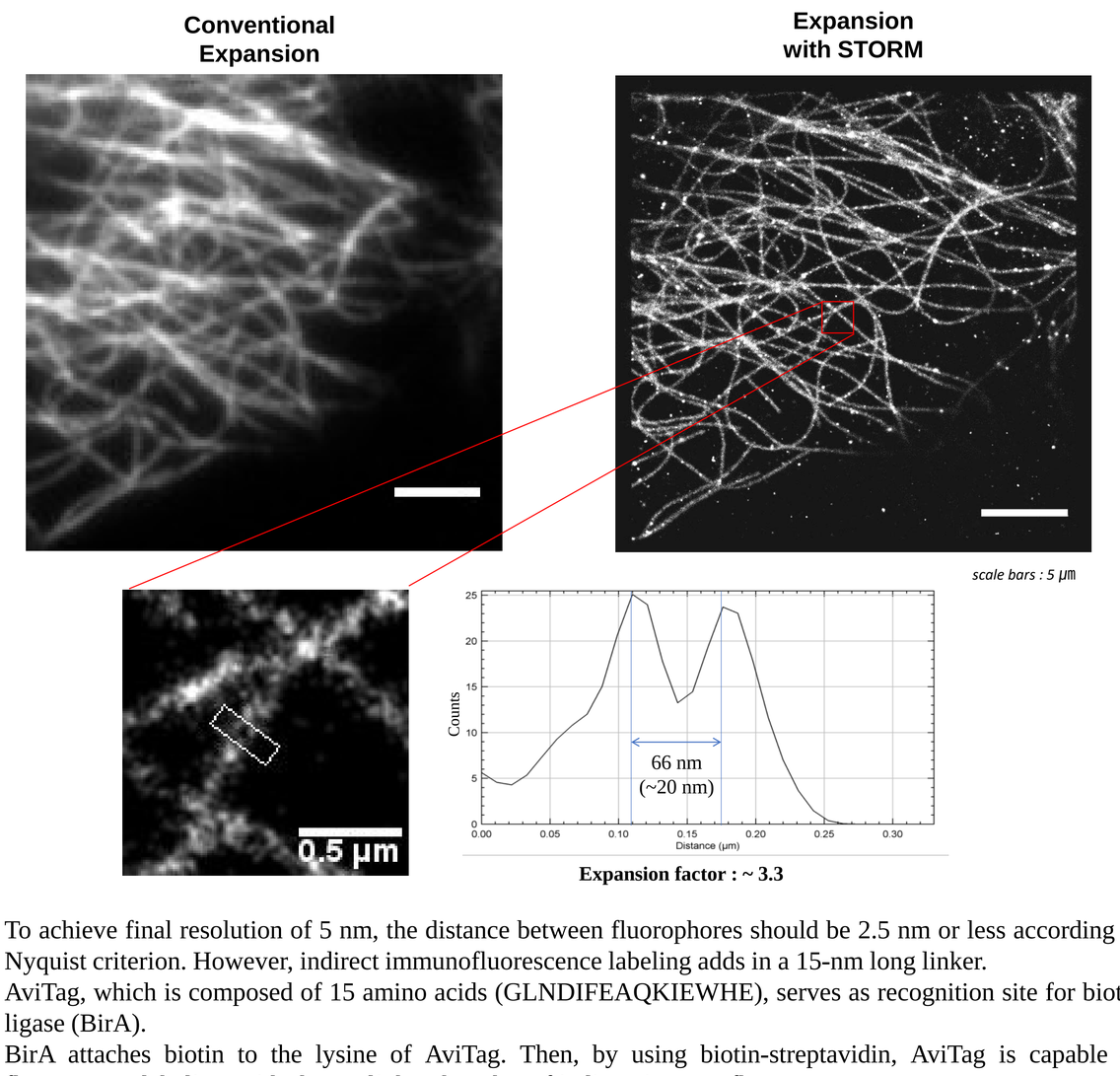
❖ DNA-PAINT and STORM imaging of expanded samples



- The re-embedded gel can be imaged with various SMLM modalities such as DNA-PAINT and STORM.
- Microtubules stained with anti-tubulin, biotinylated secondary antibody and streptavidin-Alexa488 are physically expanded. Then, the biotinylated docking strand binds to streptavidin.
- DNA-PAINT images of expanded samples were obtained by using transient binding between docking strands and their complementary imager strands conjugated to Atto655.
- STORM images of the expanded samples were obtained using streptavidin conjugated with Alexa647 that undergoes photoswitching in the presence of 100mM mercaptoethyl amine.
- We can theoretically achieve improvement of resolution to sub-10-nanometer with SMLM and ExM.
- Challenges remained
  - To achieve final resolution of 5 nm, the distance between fluorophores should be 2.5 nm or less according to Nyquist criterion. However, indirect immunofluorescence labeling technique makes target of interest too large to observe with ExM with DNA-PAINT or STORM.
  - DNA-PAINT imaging of expanded samples are limited in application due to the requirement of TIRF illumination for reducing the background from unbound imager strands.
  - STORM imaging suffer from photobleaching and require labeling method with higher labeling density.

❖ *Labeling the target protein with minimal linker with AviTag*





- To achieve final resolution of 5 nm, the distance between fluorophores should be 2.5 nm or less according to Nyquist criterion. However, indirect immunofluorescence labeling adds in a 15-nm long linker.
- AviTag, which is composed of 15 amino acids (GLNDIFEAQKIEWHE), serves as recognition site for biotin ligase (BirA).
- BirA attaches biotin to the lysine of AviTag. Then, by using biotin-streptavidin, AviTag is capable of fluorescence labeling with shorter linker than that of indirect immunofluorescence.
- We transfected cells with vimentin fused with AviTag and enzymatically biotinylated and then fluorescently labeled it with fluorophore-conjugated streptavidin. Then, a biotinylated DNA strand is added to bind to the streptavidin. Finally, a complementary strand conjugated with fluorophore (imager strand) is used for DNA-PAINT imaging.

❖ Conclusion

- Expanded gels in expansion microscopy shrink in high-salt buffers for single-molecule localization microscopy. We re-embedded the expanded gels with unswellable polymer matrix that allowed for both DNA-PAINT and STORM imaging.
- Expansion microscopy improves the spatial resolution with physically expanded samples, however suffers from the reduction of fluorescence intensity due to elongated distances between fluorophores. We used short peptide tag for labeling biotin to a protein of interest and acryloyl-functionalized streptavidin to prevent loss of fluorophores after digestion.
- We found imaging condition compatible both for expansion microscopy, DNA-PAINT and STORM.
- Since ExM expands the sample volume by ~4 times and STORM resolution is ~20 nm, we expect to achieve final resolution of ~5 nm.
- We used short peptide tag (AviTag) to reduce distance between target and fluorophore.
- We resolved the double-walled cross-sectional profile of vimentin fibers whose peaks are separated by ~20 nm.