Expansion Microscopy: Recent Developments in Gelation, Labeling, and Imaging Strategies

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Abstract

Light microscopy is a powerful approach for studying the dynamic spatiotemporal organization of the molecular processes in cellular life. However, the resolution that can be obtained using conventional light microscopy is insufficient to resolve many macromolecular structures. While various super-resolution imaging approaches have been developed that overcome this resolution limit, they require specialized equipment that is largely inaccessible to most scientists. Recently, an alternative method for super-resolution imaging was developed, called expansion microscopy, which bypasses the resolution limit by spatially separating biomolecules through expansion of the sample. Unlike previous super-resolution imaging approaches, expansion microscopy can be performed using commercially available compounds and widely-available conventional microscopes, making super-resolution imaging more accessible for non-expert researchers. In this review, the original expansion microscopy concept and followed by early developments in its workflow will first be described. Next, this review will focus on challenges within the field of expansion microscopy, including labeling density, expansion factor, and expansion isotropy, and critically review recent developments aimed at addressing these challenges. Finally, recently developed alternative labeling strategies for expansion microscopy and the possibility to combine expansion microscopy with other super-resolution microscopy approaches will be discussed.

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Introduction

Light microscopy is arguably the most powerful and versatile technique available in our quest to unravel the intricate molecular mechanisms behind the cellular processes governing life and disease. It allows researchers to better understand the dynamic, non-random spatiotemporal organization of specific biomolecules of interest, which is specific information that many other molecular cell biology approaches fail to provide. However, the resolution (i.e. the minimum distance between two points that can still be distinguished as separate entities) that can be obtained with light microscopy systems is fundamentally limited to approximately 200 nm, due to the physical phenomenon of diffraction[1,2]. While this limit seems negligible in relation to the size of most biological cells, it is insufficient for detailed visualization of the composition and structure of macromolecular assemblies, such as nuclear pore complexes (107 nm)[3] or microtubules (25 nm)[4], as they are smaller than the diffraction limit. Therefore, scientists aiming to research cellular structures smaller than the diffraction limit have historically relied on the superior resolving power offered by electron microscopes. However, electron microscopy has many practical difficulties that limit its utility in biological research in comparison to light microscopy, including elaborate sample preparation protocols, limited labeling options, low sample penetration depth, and the requirement for samples to be imaged in a vacuum[5]. Consequently, research efforts over the last three decades have been aimed at developing novel light microscopy-based approaches that are able to circumvent this diffraction limit, so-called super-resolution microscopy. Super-resolution microscopy approaches can generally be split into two categories: (1) structured-light-based super-resolution techniques like STED[6] and SIM[7], which improve the resolution of the overall sample structure by shaping the excitation light beam, and (2) single-molecule localization-based super-resolution techniques like STORM[8,9], PALM[10,11] and DNA-PAINT[12,13], which reconstruct a high resolution composite image by sequentially imaging individual fluorophores within the sample[14,15]. The development of such super-resolution imaging techniques have enabled researchers to create super-resolved images with a resolution down to 20 nm, a big improvement over the diffraction limit[16].

Despite these powerful developments, super-resolution microscopy remains largely inaccessible to non-expert users, as the majority of methods require expensive, specialized hardware, software, fluorophores and reagents, as well as complicated data analysis pipelines. Additionally, super-resolution imaging approaches suffer from slow image acquisition speeds and remain largely incompatible with imaging of tissue samples[1,2,16]. As such, there is a need for more readily accessible and broadly applicable methods to circumvent the diffraction limit. Recently, one such super-resolution microscopy approach was developed, referred to as Expansion Microscopy (ExM)[17]. In ExM, the diffraction limit is circumvented by increasing the distance between molecules in a diffraction-limited region through physical isotropic expansion of the biological sample in a swellable hydrogel, making those molecules easier to resolve (Figure 1). ExM can be performed using easily adaptable protocols that utilize reagents that are readily accessible for most laboratories, and imaging of expanded samples can be performed on widely available conventional systems, such as confocal microscopes. Furthermore, ExM benefits from fast image acquisition through compatibility with conventional confocal or light sheet imaging, low background noise due to expanded samples almost fully consisting of water molecules, compatibility with thick tissue samples, and can be used in conjunction with other super-resolution methods[1,2,16].

In this review, the original ExM methodology and its benefits and limitations will be highlighted first, after which the development of protein retention, which is now considered standard workflow practice, will be discussed. Next, this review will critically review recent developments aimed at overcoming inherent limitations of ExM, including efforts to improve the labeling density and reduce linkage error associated with immunolabeling, further increasing the resolution by improving the expansion factor, and minimizing local heterogeneity introduced by expansion. Furthermore, this review will discuss recently developed alternative labeling strategies that allow for visualization of the cellular context and facilitate super-resolution imaging of various classes of biomolecules. Finally, the possibilities of performing ExM in conjunction with optical super-resolution microscopy methods, including STORM, STED and SIM, will be discussed.

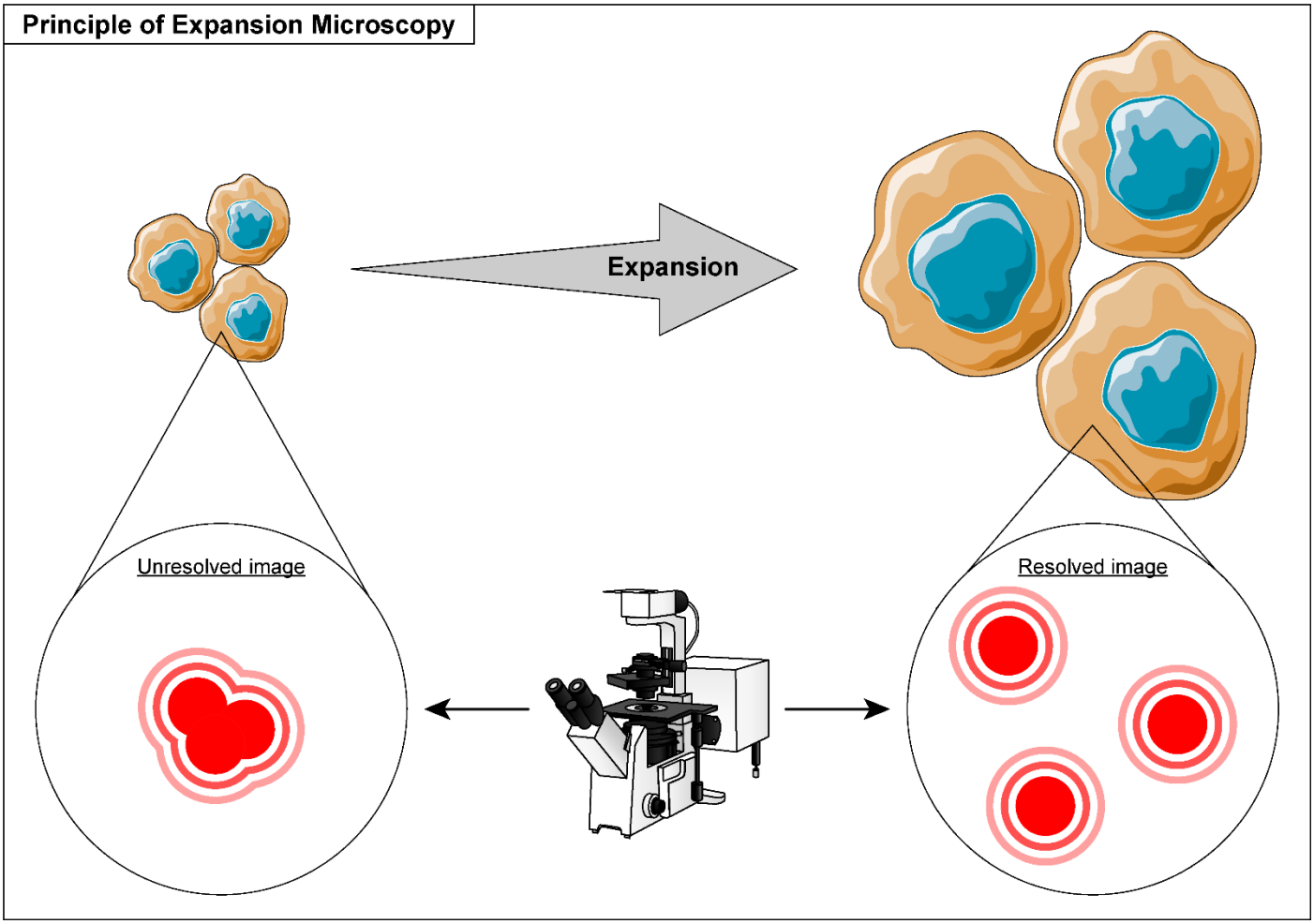

Figure 1: An illustration showing the principle of achieving super-resolution images using Expansion Microscopy. In Expansion Microscopy, samples are physically expanded in a swellable hydrogel, which increases the spatial distance between the biomolecules within the sample. As a result, better resolved fluorescent images can be obtained in expanded samples compared to non-expanded samples. Cell and microscope icons are adapted from bioicons.com.

Initial Concept and Early Workflow Improvements

The earliest iteration of expansion microscopy (ExM 1.0) was first developed in 2015 by the research group of Edward Boyden[17]. In this original method, samples are fixed, permeabilized and labeled prior to gel embedding and expansion[17]. Samples are then first immersed in a monomer solution containing acrylamide (AA) monomers, charged sodium acrylate (SA) monomers, and crosslinking agent N,N'-methylenebisacrylamide (MBAA) (Figure 2A)[17]. This solution has the ability to form a dense, crosslinked polyelectrolyte hydrogel through free-radical polymerization between monomers, which is started through addition of ammonium persulfate (APS) radical initiator and tetramethylethylenediamine (TEMED) polymerization accelerator[1,17]. After complete gelation, the mechanical properties of the sample are homogenized by proteolytically disrupting protein-protein interactions using nonspecific proteinase K treatment, in order to ensure expansion of the polyelectrolyte hydrogel is as isotropic as possible[1,2,17]. Finally, the sample-hydrogel composite is immersed in pure water, which diffuses into the polyelectrolyte hydrogel driven by osmotic force, causing the sample to expand[1,17]. With this ExM 1.0 protocol, samples are expanded 4.5-fold in each linear dimension, or rather by about 100x in volume, without any large distortions in the gross structure of the sample[17]. This level of expansion allows researchers to greatly improve in the resolving power of a microscope system. For example, a conventional confocal microscope system with a Numerical Aperture (NA) of 1 imaging a green

fluorescent protein (GFP) with a maximum emission at 500 nm in a non-expanded sample would have a final resolution of about half that wavelength, around 250 nm. However, assuming perfect remaining labeling density, the effective resolution would be improved by the 4.5-fold expansion factor if this sample were to be expanded, achieving an effective resolution of around 60 to 70 nm[1,2,17].

The impressive effective resolution improvements achieved by ExM 1.0 combined with its strong performance in thick tissue slices, the simplicity of hydrogel preparation and its independence from expensive, specialized imaging equipment, made it an attractive super-resolution imaging option for cell biology laboratories. However, the earliest ExM method suffers from a technical limitation in relation to biomolecule labeling, which prevents the method from being easily adoptable for many laboratories. Specifically, the earliest protocol lacks a method to universally anchor biomolecules to the hydrogel network, which is required to prevent proteins from displacing from their native position relative the hydrogel during expansion, making commonly-used labeling protocols using genetic tags or antibodies highly unreliable[18]. Instead, labeling in ExM is dependent on usage of expensive, custom-designed fluorescent labels that covalently incorporate themselves into the hydrogel mesh, in order to retain the spatial information of each specific protein of interest after expansion (Figure 2A)[2,17–19]. These labels consist of an oligonucleotide sequence conjugated to a chemical fluorophore, and to a methacryloyl group that is capable of incorporating itself into the hydrogel network through free-radical polymerization during gelation[2,17–19]. The oligonucleotide sequences are complementary to oligonucleotides conjugated to secondary antibodies, which are not widely available[1,2,18,19]. Thus, further developments in ExM methodology to support the use of standard labeling techniques were required to lower the barrier for laboratories to adopt the method.

Protein retention

Only a year and a half after ExM had first been developed, three different independent research groups had tackled this labeling limitation by developing variations of ExM in which proteins themselves are covalently anchored to the swellable hydrogel, omitting the requirement for custom-designed fluorescent labels[18–20]. The first two methods are quite similar to each other, and are collectively referred to as Protein retention Expansion Microscopy (ProExM) (Figure 2B). In these two ProExM methods, fixed samples are treated with either acryloyl-X (AcX), methacrylic acid N-hydroxy succinimidyl ester (MA-NHS), or glutaraldehyde (GA), all of which are commercially available compounds[1,2,18,19]. These compounds react with amines on proteins, labeling them with functional groups that can participate in free-radical polymerization reactions during gelation, allowing native proteins and antibodies to be incorporated within the swellable hydrogel[1,2,18,19]. For pre-stained samples, ProExM follows the same gelation, proteolytic homogenization and expansion steps as seen in the ExM 1.0 protocol, and have been validated to be compatible with a wide range of β-barrel-structured fluorescent proteins and antibody-conjugated fluorescent dyes (both retaining >50% fluorescence)[1,2,18,19]. However, ProExM cannot only be performed on pre-stained samples, but also with antibody labeling after the sample has been expanded, which comes with major benefits that will be discussed in more detail in the next chapter (Figure 2C)[18]. In order to facilitate post-expansion labeling, the epitopes that are to be recognized by antibodies need to be preserved during the homogenization step. Therefore, post-expansion labeling protocols use a more gentle homogenization method compared to the nonspecific proteinase K treatment used by pre-expansion labeling ExM. This is most commonly done by incubation in high temperature detergent solutions to denature and disrupt protein aggregates, or by lysine-specific LysC protease treatment, which leaves relatively large peptides[1,2,18,20].

The third method, called Magnified Analysis of the Proteome (MAP), is not actually directly based on the ExM 1.0 protocol. Instead, it is based on a hydrogel-based tissue clearing method in which, inspired by ExM 1.0, an isotropic expansion step is incorporated[20–22]. MAP avoids conventional paraformaldehyde (PFA) fixation, but utilizes a solution containing PFA and a surplus of acrylamide monomers to anchor proteins to the swellable hydrogel (Figure 2D)[1,2,20,23]. PFA forms reactive methylol groups on proteins, which would react with amide groups to form intra- and interprotein crosslinks during conventional PFA fixation[20]. However, the high concentration of acrylamide monomers quenches the formation of these crosslinks by reacting with the methylols themselves[1,2,22]. This not only prevents intra- and interprotein crosslinks, allowing for efficient dissociation of protein complexes during isotropic expansion, but also allows native proteins to be tethered into the hydrogel mesh by the bound acrylamide groups that can participate in

free-radical polymerization during the subsequent gelation step[1,2,20]. MAP solely utilizes post-expansion antibody labeling, of which the benefits will be explained in the next chapter, and sample homogenization is mediated by incubation in detergent solution at high temperature[1,2,20]. MAP has been demonstrated to be compatible with a large library of antibodies, with an >80% success rate over the 122 antibodies that were tested[1,2,20]. Due to the success of both ProExM (mostly the AcX variant) and MAP, they are now considered standard practice for ExM, and have been the foundation for many subsequently developed methods.

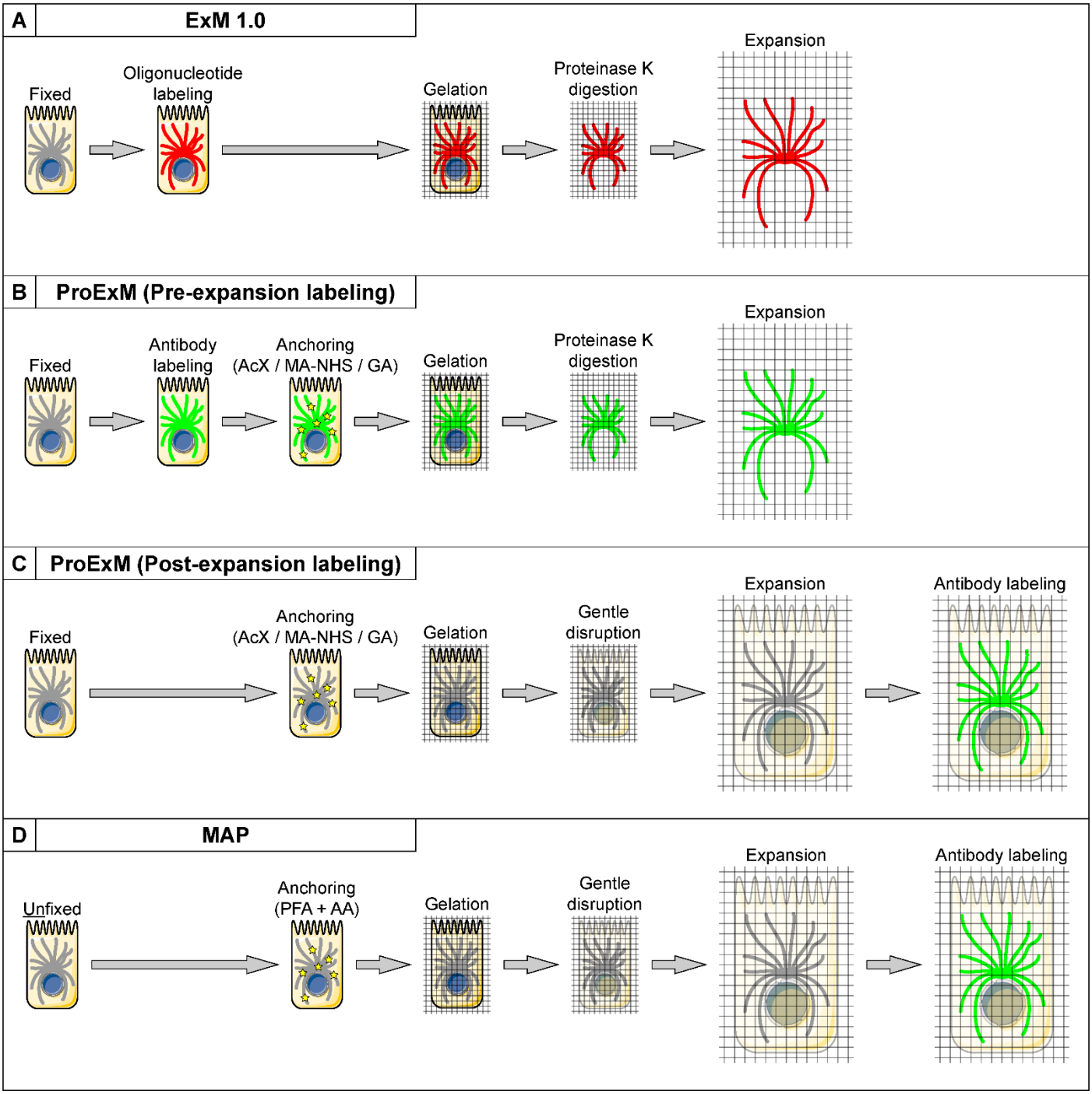

Figure 2: An illustration showing the general workflow for commonly used Expansion Microscopy modalities. (A) The general protocol for ExM 1.0. Fixed samples are first labeled using specialized antibodies conjugated to an oligonucleotide sequence, which are subsequently bound by complementary fluorescent oligonucleotides that allow for incorporation into the hydrogel. The hydrogel is formed using a combination of polymerization monomers, a crosslinking agent, and reaction initiator and accelerator. The mechanical properties of the sample are

homogenized by digestion using proteinase K. Finally, the sample is expanded by immersing it in water. (B) The general protocol for pre-expansion labeling ProExM. Fixed samples are first labeled using primary and secondary antibodies, after which samples are treated with either AcX, MA-NHS or glutaraldehyde (GA), to covalently label proteins and antibodies with chemical anchors that allow for incorporation into the hydrogel. The gelation, mechanical homogenization and expansion steps are the same as for ExM 1.0. (C) The general protocol for post-expansion labeling ProExM. Fixed samples are first treated with either AcX, MA-NHS or glutaraldehyde (GA), to covalently label proteins with chemical anchors that allow for incorporation into the hydrogel. The hydrogel is then formed using a combination of polymerization monomers, a crosslinking agent, and reaction initiator and accelerator. The mechanical properties of the sample are homogenized using gentle disruption methods, such as incubation in detergent solutions at high temperature or LysC treatment. Next, the sample is expanded by immersing it in water. Finally, the sample is labeled using primary and secondary antibodies. (D) The general protocol for MAP. Unfixed samples are first treated with a combination of paraformaldehyde (PFA) and a surplus of acrylamide (AA) monomers, to covalently label proteins with chemical anchor that allow for incorporation into the hydrogel. The gelation, mechanical homogenization and expansion steps are the same as for post-expansion labeling ProExM. Cell icons are adapted from bioicons.com.

Post-Expansion Labeling

The development of protein retention methods in ProExM and MAP not only resulted in ExM becoming a much more accessible and widely adopted super-resolution imaging method, it offers additional benefits in enabling the sample to be labeled after expansion. This post-expansion labeling helps to reduce the linkage error associated with antibody labeling and to improve the antibody labeling efficiency, ultimately resulting in improved structural resolution over pre-expansion labeling ExM, as well as over other super-resolution imaging approaches[1,2,4,18]. Both of these concepts will be explained using primary and secondary antibody labeling of microtubules as an example.

Reduced linkage error

Microtubules are hollow tubes with an outer diameter of 25 nm, composed of 13 linear protofilaments in a cylindrical arrangement[4]. Conventional antibody labeling protocols most commonly use antibodies that belong to the IgG class. These antibodies are typically about 14.5 nm x 4.5 nm x 8.5 nm in size, but for the simplicity of this example, they will be regarded as 10 nm in each dimension[4,24]. Since both primary and secondary antibodies are used, the total height of their complex is about 20 nm. Thus, by conventionally labeling the microtubule surface with primary and secondary antibodies, you end up with a linkage error of 20 nm between the fluorophore conjugated to the secondary antibody, and the microtubule epitope recognized by the primary antibody[4]. Since the microtubule is coated with antibodies all around its cylindrical surface, the total outer diameter of the microtubule-antibody complex will appear to be about 65 nm (Figure 3A)[4]. While most super-resolution imaging setups will have the optical resolution to resolve the fluorescent labels on the outer edge of this 65 nm diameter structure, the structural resolution of the microtubule-antibody complex makes it impossible to obtain a true image of the 25 nm diameter microtubule structure[4]. Expansion microscopy approaches that utilize pre-expansion labeling suffer from this same phenomenon as well. The expansion of the cylindrical structure of the microtubule does make it easier to resolve, thus increasing the optical resolution, but the size of the linkage error between the fluorescent antibody and the microtubule surface is also being expanded by the same relative amount, resulting in unchanged structural resolution (Figure 3A)[4].

The extent to which post-expansion labeling ExM is affected by this problem is much less. As antibodies are added only after hydrogel expansion, the linkage error caused by the size of the antibody complex is never expanded, thus remaining 20 nm[4]. Translating this to real-world examples, it means that the true size of the microtubule outer diameter after 4-fold expansion would be 100 nm, but would appear to be 140 nm on a super-resolution image (Figure 3B). In the case of 10-fold expansion, the true microtubule outer diameter would be 250 nm, but on an image would appear to be 290 nm. In other words, the size of the linkage error virtually decreases by the expansion factor, becoming 5 nm in the case of 4-fold expansion, and only 2 nm in the case of 10-fold expansion[1,2,4]. Thus, post-expansion labeling ExM increases not only the optical resolution, but also greatly increases the structural resolution by reducing the antibody linkage error, making it an attractive super-resolution imaging approach for structural biology applications[1,2,4].

Increased labeling density

Another issue that pre-expansion ExM methods suffer from is poor labeling efficiency, due to two main limitations. First, the hydrogel polymerization and proteolytic homogenization steps after antibody labeling severely reduce the fluorescence signal[1,2,4,18,19]. While most β-barrel-structured fluorescent proteins and fluorescent dyes commonly used with antibodies are resilient enough to be compatible with proteolytic homogenization and free-radical polymerization, with most retaining over 50% of their original fluorescence, this does mean there is a significant drop in fluorescence signal[1,2,4,18,19]. The second limitation again stems from the use of antibody labeling in non-expanded samples, which will again be explained using microtubules as an example. As described earlier, microtubules consist of 13 linear protofilaments arranged cylindrically. These protofilaments are essentially polymers made out of periodically repeating dimers of α- and β-tubulin. α- and β-tubulin each are about 4 nm in size, so the length of each repeating dimer in the protofilament is about 8 nm (Figure 3C)[4]. This 8 nm distance is shorter than the approximate 10 nm size of the IgG antibodies used to label a specific tubulin monomer, resulting in a single antibody overlapping with and blocking another antibody from binding to the second dimer[4]. This so-called steric hindrance reduces the labeling density that can be achieved in non-expanded samples[4].

Post-expansion labeling ExM manages to improve upon these listed flaws, most obviously on the first: loss of fluorescence signal during proteolytic homogenization and free-radical polymerization are entirely avoided by performing antibody labeling after these steps have been performed[4]. Furthermore, proteins of interest are more accessible for antibodies after sample expansion, because decrowding of the sample largely eliminates steric hindrance between antibodies competing for overlapping adjacent epitopes, and reveals new epitopes that would otherwise be masked inside molecular complexes pre-expansion, such as epitopes facing the inner side of microtubules (Figure 3D)[1,2,4,18]. As such, post-expansion labeling ExM is able to obtain a higher antibody labeling density compared to pre-expansion labeling approaches, which results in an improved fluorescence signal, as well as improved resolution by visualizing more proteins of interest[4]. On a final note, the process of expansion inherently dilutes the fluorescence signal proportionally to the volumetric expansion factor, i.e. 4-fold expansion results in 64x fluorescent signal dilution, and 10-fold expansion results in 1000x signal reduction[4]. While this remains true for both pre- and post-expansion labeling, the fact that post-expansion labeling has a better labeling efficiency due to higher epitope accessibility, decreased steric hindrance, and no loss of fluorescence signal during homogenization and polymerization, means post-expansion labeling ExM has the ability to deliver a stronger fluorescent signal[4]. In conclusion, post-expansion labeling ExM is not only a highly accessible super-resolution imaging approach, but it also has the ability to create images that are much closer to true molecular resolution than obtained with pre-expansion labeling ExM or classical super-resolution microscopy approaches, by both virtually decreasing the linkage error and increasing the antibody labeling efficiency.

Improving The Expansion Factor

Assuming high labeling density and perfect isotropic expansion, the effective resolution that can be achieved using expansion microscopy is dependent on the expansion factor of the hydrogel, as higher expansion results in higher spatial separation between labeled biomolecules and thus higher resolving power[2,25]. The three main baseline ExM strategies (ExM 1.0, ProExM and MAP) that have been discussed so far all typically expand up to 4.5-fold before the gels become too fragile, achieving imaging resolutions of just under 70 nm[1,2,17–20,26]. While this is a significant improvement over the diffraction limit of around 200 nm in non-expanded imaging, it is not yet sufficient to resolve macromolecular structures like microtubules (25 nm) and is generally outperformed in imaging resolution by other super-resolution imaging approaches[4,16]. Therefore, research efforts within the ExM field have been dedicated to developing novel modalities that improve upon the expansion factor.

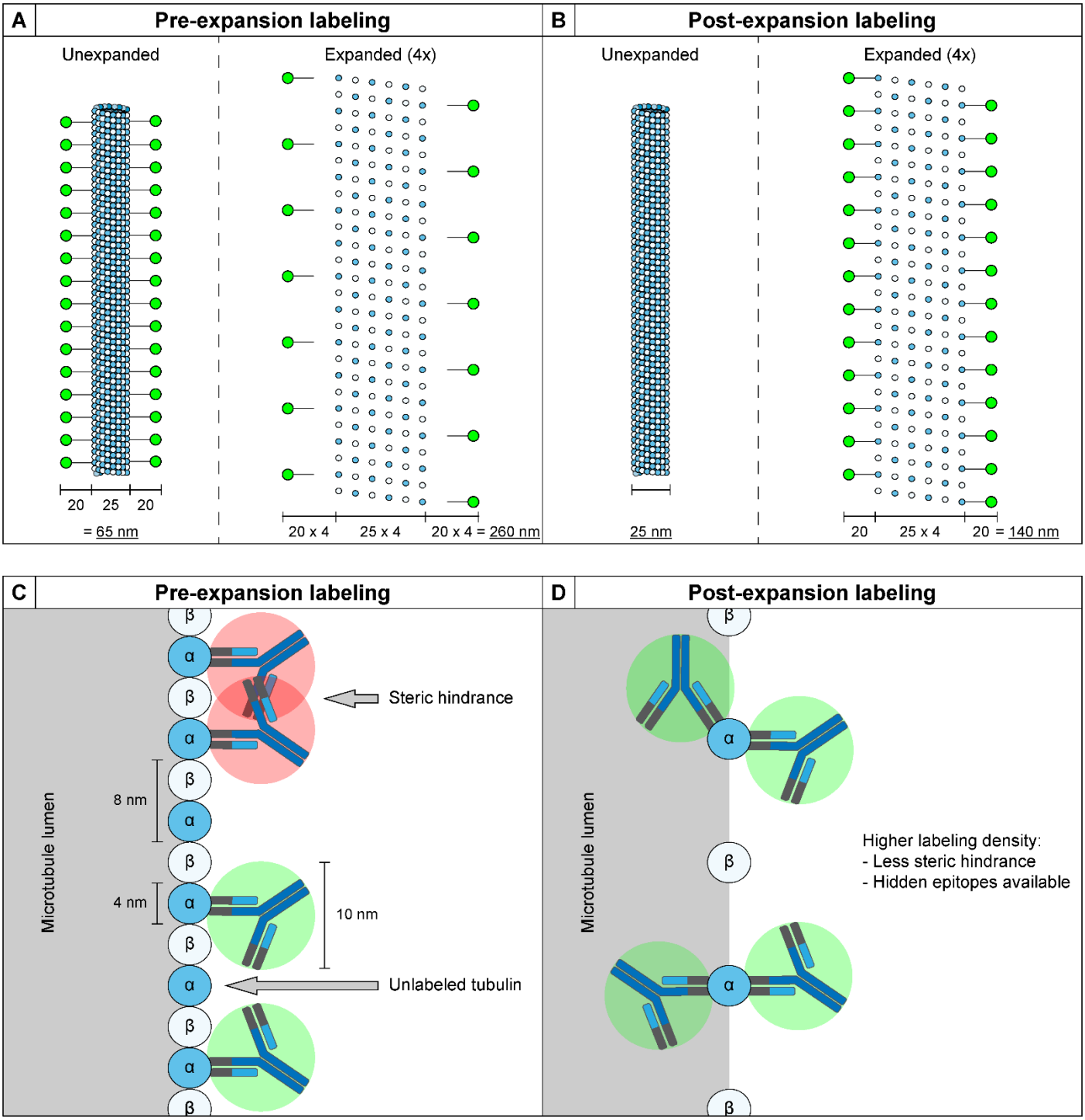

Figure 3: An illustration showing the positive effect post-expansion labeling has on the linkage error and antibody labeling density. (A) A labeled unexpanded microtubule and a pre-expansion labeled expanded microtubule. Labeling using primary and secondary antibodies inherently introduces a linkage error of about 20 nm on both sides of the labeled structure. This linkage error limits the structural resolution of the image, which in this case would be an image of a 65 nm labeled structure instead of the true 25 nm microtubule. Due to pre-expansion labeling, the linkage error itself is also expanded, but remains the same size relative to the expanded sample. For 4-fold expansion, this results in a labeled structure with a 260 nm diameter, while the true diameter of the microtubule is 100 nm. (B) An unlabeled unexpanded microtubule and a post-expansion labeled expanded microtubule. Due to post-expansion labeling, the linkage error itself is not expanded. Therefore, the relative size of the linkage error compared to the expanded sample is smaller in post-expansion labeling compared to pre-expansion labeling. For 4-fold expansion, this results in a labeled structure with a 140 nm diameter, which is much closer to the true 100 nm diameter of the microtubule compared to the 260 nm labeled structure in pre-expansion labeling. (C) A schematic cross-section along the length of an unexpanded microtubule, in which α-tubulin monomers are labeled by antibodies. Since antibodies are larger than the spatial separation between adjacent α-tubulin monomers, steric hindrance between antibodies prevents every single α-tubulin monomer from being labeled. Instead, only every second α-tubulin monomer is labeled. (D) A schematic cross-section along the length of an expanded microtubule, in which

α-tubulin monomers are post-expansion labeled by antibodies. Since the spatial separation between adjacent α-tubulin monomers has increased due to expansion, there is less steric hindrance between antibodies, resulting in more densely labeled α-tubulin monomers. Furthermore, α-tubulin epitopes that are normally hidden away inside the microtubule lumen or interacting with adjacent β-tubulin monomers are now accessible for polyclonal antibodies, again enhancing the labeling density. Microtubule and antibody icons are adapted from bioicons.com.

Iterative expansion microscopy

The first modality with an increased expansion factor that was developed is referred to as Iterative Expansion Microscopy (iExM)[26]. In iExM, samples are expanded in two successive rounds of expansion[1,2,25,26]. To achieve this, the method utilizes a special crosslinking compound called N,N'-(1,2-dihydroxyethylene) bisacrylamide (DHEBA), which is cleavable at high pH[1,25,26]. In more detail, the sample is first embedded in a hydrogel that consists of AA monomers, charged SA monomers as well as the cleavable DHEBA crosslinker[25,26]. After homogenization and expansion, the sample-hydrogel composite is then embedded in a second, noncharged hydrogel that consists of solely AA monomers and DHEBA crosslinker[25,26]. The main function of this second hydrogel is to retain the first hydrogel in its expanded state during subsequent steps[25,26]. Finally, a third hydrogel consisting of AA, charged SA monomers and commonly used MBAA crosslinker is synthesized within the sample-hydrogel composite, which can be expanded after dissolving the first and second hydrogels by cleaving their crosslinks[25,26]. Both successive rounds of expansion are in the range of 4 to 4.5-fold expansion, for a total 20-fold expansion, and achieve an imaging resolution of around 25 nm, and the technique has been shown to be compatible with adherent cells as well as thick tissue slices (>100 μm)[1,2,25,26]. For adherent cells, it was shown to be possible to extend the iExM method even further by performing a third round of expansion, using another cleavable crosslinker whose cleaving chemistry is orthogonal to the cleaving chemistry of DHEBA, for imaging resolutions of up to 5 nm[26].

While this resolution is impressive in comparison to other ExM modalities and even to other super-resolution imaging methods, the iExM method has never been widely adopted[27]. The most obvious reason for this is the complexity of the protocol: it adds two additional hydrogel embedding steps, a step to transfer fluorescent labels between the two expanding hydrogels, as well as a hydrogel dissolving step that requires a special crosslinker, over existing ExM modalities[27]. iExM is also not compatible with commonly used fluorescent labeling methods. It is instead reliant on using the same custom oligonucleotide-conjugated antibodies and fluorescent complementary oligonucleotides as seen in ExM 1.0, further limiting its adoptability[1,2,25,26]. Furthermore, while the imaging resolution is impressive, the structural accuracy achieved with the technique remains limited. Not only is iExM incompatible with post-expansion labeling in order to reduce the linkage error, the linkage error of oligonucleotide-conjugated antibodies is also much larger compared to regular fluorescent antibody pairs, severely limiting the structural resolution achieved with the method[28]. Finally, due to iExM using multiple rounds of hydrogel embedding and expansion, it can be speculated that the ultrastructural accuracy achieved in iExM is more limited compared to ExM modalities that use only a single hydrogel, due to the inherent spatial distortions that arise in these hydrogels due to heterogeneity during free-radical polymerization and expansion between different cellular compartments and macromolecular assemblies[23,29,30].

While iExM itself was never widely adopted, the idea of using iterative rounds of expansion to improve the expansion factor has not been dismissed by the scientific community. A number of studies, even published as recently as this year, make further developments in utilizing iterative rounds of expansion to improve the expansion factor[31,32]. Possibly the most promising example is a method referred to as Expansion Revealing (ExR), which utilizes the entanglement between polymer chains of the first and second swellable hydrogels to strengthen and further expand the existing first hydrogel, beyond where the first hydrogel would become too fragile on its own[25,31,32]. Since the first hydrogel is never dissolved, it enables protein retention within the first hydrogel using AcX anchoring, and thus the use of regular immunofluorescence staining and post-expansion labeling. This makes the method more adoptable and enables higher structural accuracy, while offering the same expansion factor and compatibility with thick tissue slices as seen with iExM[25,32]. Another research group has developed similar principles, which instead of entanglement relies on transferring anchored proteins from the first hydrogel to the second swellable hydrogel by

acrylamide/formaldehyde treatment such as seen in MAP[31]. However, this method again introduces the additional anchoring and labeling transfer and hydrogel cleavage steps in the protocol, which are eliminated in ExR.

Single-round expansion strategies

In contrast to iExM, a number of different modalities that aim to achieve high expansion factors using only a single round of expansion have been developed. The first of these methods is called X10 Expansion Microscopy (X10)[33]. X10 uses a different hydrogel monomer composition compared to most ExM protocols. The hydrogel consists of the commonly used SA monomers, but also uses a special monomer named N,N-dimethylacrylamide acid (DMAA), which functions in forming both polymer chains and crosslinks within the hydrogel[1,2,25,33,34]. This hydrogel composition has the ability to expand samples by 10-fold, as its name had already suggested, achieving imaging resolutions of around 25 nm with just a single round of expansion[1,2,25,33,34]. However, this method also comes with some major limitations. First of all, it has not been shown to be compatible with thick tissue slices, only with tissues that are thin (20-30 μm) relative to what has been shown to be compatible with other ExM modalities (>100 μm)[27,33]. Second, the gelation protocol in X10 is significantly more challenging than that of existing 4.5-fold expansion methods. The initial gel polymerization reaction is extremely fast, hardening within minutes, which therefore requires the reaction to occur at low temperature and requires well-developed handling skills from researchers performing the protocol[33,34]. Additionally, the polymerization reaction is inhibited by molecular oxygen (O_2), and thus requires rigorous nitrogen bubbling prior to and during gelation[27,33,34]. Finally, while the method has been shown to be compatible with protein retention using AcX anchoring, the method requires extremely extensive proteinase K treatment in order to achieve 10-fold expansion, likely due to the density of the hydrogel network[1,2,33,34]. This treatment results in the loss of epitopes on proteins of interest, limiting the technique to solely being able to use pre-expansion immunolabeling.

As with iterative expansion methods, single-round high expansion factor modalities have seen continued developments beyond the first emergence its first emergence in X10. One such recent development, referred to as Ninefold Swelling (NIFS), applies a similar strategy to X10 by utilizing a different crosslinking agent in N,N-ethylenebisacrylamide (EBIS) to form a hydrogel with enhanced expansion properties, in this case 9-fold[25,35]. The advantage of NIFS is that its protocol bears more resemblance to the existing 4.5-fold expansion modalities, lacking the challenging steps seen in X10[35]. However, the EBIS crosslinking agent is not commonly used in the ExM research field, limiting its adoptability. Furthermore, the method has also not yet been shown to be compatible with tissue slices, nor with post-expansion labeling[35]. Though it can be speculated that, since the NIFS protocol uses the same parameters for proteinase K-mediated mechanical homogenization as in pre-expansion labeling ProExM, this protocol too might be able to adopt more gentle mechanical homogenization methods to enable post-expansion labeling without compromising on the 9-fold expansion factor.

Two different strategies in order to achieve higher expansion factors have been discussed up until now: using multiple rounds of hydrogel embedding and expansion, or using special monomers or crosslinkers that result in hydrogels with enhanced single-step expansion properties. However, the mentioned strategies all come with compromises in comparison to established 4.5-fold expansion modalities, for example in the form of highly complex protocols, uncommonly used hydrogel components, or not being validated with thick tissue samples. Therefore, there is still a niche for the development of an easily adoptable, robustly validated and widely applicable ExM method that offers high, single-step expansion. One example of such a well-optimized method was published earlier this year, which is referred to as Ten-fold Robust Expansion Microscopy (TREx)[27]. In order to be as easily adoptable as possible, TREx uses the same hydrogel components (AA, SA, MBAA, etc.) and general protocol as used in the original, most widely adopted ProExM method. The study systematically optimizes the concentration of each component and parameter in the gelation recipe, in order develop hydrogels that achieve 10-fold single-round expansion while retaining optimal mechanical properties for gel integrity and easy handling[25,27]. The authors demonstrate that the method is compatible with both adherent cells and thick tissue slices (100 μm), as well as different labeling strategies like pre-expansion antibody labeling and small molecule stains for proteins and membranes[27]. While compatibility with post-expansion labeling itself has not been validated, the study optimized protein retention by looking at the signal intensity of the general protein stain, suggesting post-expansion labeling should be possible with minor additional optimization[27].

Finally, it is reported the optimal crosslinker concentration for 10-fold expansion using TREx varies somewhat between different laboratories[25,27]. While this may be a downside of the TREx methodology specifically, one can speculate it is more likely for this is a common but underreported occurrence in ExM, that stems from the differences in custom gelation chamber design between different laboratories, suggesting a need for a more unified ExM gelation chamber design[25,27].

In conclusion, improving the expansion factor of ExM hydrogels has been a hot topic within the ExM research field. While older strategies like iExM and X10 have never become the standard practice for ExM, it will be interesting to see how recently reported improvements and new developments like ExR, NIFS and TREx will affect the widespread adoption of high expansion factor ExM over the original 4.5-fold expansion methods in future.

Preserving Ultrastructural Integrity

A major challenge for super-resolution imaging the native ultrastructure of protein complexes is the fact that, while isotropic in the gross sample anatomy, expansion inherently introduces nanoscale distortions in the native structure of samples, potentially on the scale of tens of nanometers. All thus far discussed methodologies report deformations between 1-4% in microtubule alignment pre- and post-expansion images from the same samples[17–20,26,27,31–33,35]. Additionally, numerous studies have found inhomogeneous expansion between the global sample and local regions such as organelles or macromolecular protein structures, including nuclear pore complexes or centrioles[3,23,27,29,30,36]. As an example, one study reports a 29% difference between the global expansion factor (4.5-fold) and the local expansion factor in nuclear pore complexes (3.2-fold)[3]. These distortions are thought to originate from a few different sources during hydrogel embedding and expansion. For example, spatial fluctuations in monomer and crosslinker concentration during free-radical polymerization are known to introduce heterogeneity in the hydrogel network[25,30]. Additionally, the most commonly used hydrogel recipe (AA, SA, MBAA) is known to suffer from local structural defects, such as polymer chain loops, dangling polymer ends, or entangled polymer chains[25,30]. Furthermore, differences in biomolecule density between different cellular compartments can be speculated to affect local polymer chain penetration and local expansion capacity. Finally, use of fixatives that introduce crosslinks between proteins (such as PFA) limits the ability to spatially separate those crosslinked proteins during hydrogel expansion[20]. Research efforts within the field have therefore been focused on developing novel strategies that aim to minimize these local distortions, as would be required in order for ExM to become a viable method for accurate super-resolution imaging of macromolecular assembly ultrastructure and composition.

Ultrastructure expansion microscopy

The first major development in retaining native molecular ultrastructures is Ultrastructure Expansion Microscopy (U-ExM)[23]. In an effort to characterize the native ultrastructure expansion performance of well-established protocols such as MAP, the authors of this study observed that MAP-treated centrioles displayed non-isotropic expansion, showing a 1.6-fold lower expansion factor compared to the global hydrogel[23]. While MAP attempts to prevent sample fixation to facilitate expansion, its protocol does in fact use an anchoring treatment that uses a high concentration of PFA (4%) with a surplus of AA monomers (30%), for a relatively long incubation period of 4-5 hours at 37°C[20,23]. The authors hypothesized that this PFA concentration and incubation time is too extensive, resulting in unintended crosslinking between proteins that prevent isotropic expansion of macromolecular assemblies despite the crosslink quenching activity by AA monomers[20,23,37]. Subsequent systematic optimization of the MAP protocol performed in this study showed that an anchoring treatment using a comparatively lower concentration of PFA (0.3-1%) and AA monomers (0.15-1%) for 4-5 hours at 37°C more efficiently prevents protein crosslinking, facilitating optimal isotropic expansion in which the native centriolar ultrastructure is unambiguously retained[23]. This optimized approach was termed U-ExM. Its ultrastructural performance in combination with post-expansion labeling and exceptionally high labeling density,

since no epitopes are lost to crosslinking, has resulted in this method has become a preferred approach for super-resolution imaging of the ultrastructure and composition of macromolecular assemblies using ExM[23,28,37].

While U-ExM performs well on stable structures like centrioles or flagellar axonemes, the lack of fixation results in loss of the ultrastructure of more dynamic cellular structures, such as cytoplasmic microtubules, are lost due to depolymerization[37,38]. As such, some dynamic cellular structures require the use of fixatives in order to retain their native ultrastructure. An interesting recent development in order to address this issue is the combination of U-ExM with cryofixation, a method in which the sample is fixed by ultra-rapid cooling to the temperature of liquid nitrogen, referred to as Cryo-ExM[38]. Cryofixation perfectly preserves native ultrastructures, and far outclasses chemical fixatives due to the lack of structural artifacts and loss of antigenicity[38]. Cryofixation is a well-validated method, as it has been used in electron microscopy for decades[38]. The cryofixation protocol used in Cryo-ExM closely resembles that of cryo-electron microscopy, with the main difference being that the vitrified sample is embedded in a swellable hydrogel instead of the resin polymer used in cryo-electron microscopy[38]. This familiarity combined with the relative ease of the cryofixation protocol and the availability of the required chemicals and equipment should make Cryo-ExM an attractive and easily adoptable ultrastructural super-resolution imaging approach for many laboratories.

Tetrahedron-line monomers

Another promising recent development aimed at improving the structural integrity achieved by ExM is the use of tetra-hydrogels[30]. Tetra-hydrogels are assembled using tetrahedron-like monomers through small chemical reactions referred to as click-chemistry, instead of the free-radical polymerization chemistry seen in typical ExM hydrogels[30]. These tetrahedron-like monomers form a highly repetitive, rigid diamond-shaped polymer network that is almost entirely void of structural abnormalities, both during synthesis and expansion[30]. Tetra-hydrogels achieve expansion factors of around 3-fold, however, since one variant of the tetrahedral-like monomers supports post-expansion cleavage of the polymer network, higher expansion factors can be achieved by combining it with iterative expansion microscopy[30]. iExM using tetra-hydrogels and typical hydrogels (AA, SA, MBAA) performed on herpes simplex type 1 (HSV-1) virions revealed that the median spatial error in expanded HSV-1 virions using tetra-hydrogels is significantly smaller compared to expanded HSV1 virions using typical hydrogels (9.2 nm vs. 14.3 nm, normalized for expansion factor), meaning tetra-hydrogels perform substantially better at retaining native ultrastructure of macromolecular assemblies[30].

However, the use of tetrahedron-like monomers is very much in its first iteration, and therefore currently comes with a lot of downsides. First of all, these tetrahedron-like monomers are not yet commercially available and currently have to be custom-designed[30]. Furthermore, since the cleavable tetrahedral-like monomers that enable combination with iExM is not compatible with high-temperature alkaline treatment as a gentle homogenization method, the method is currently incompatible with post-expansion labeling. This would however be the most desirable labeling strategy, in order to be able to fully benefit from the enhanced native ultrastructure retention offered by tetra-hydrogels[30]. Since the method is thus limited to pre-expansion labeling, it should be mentioned that because tetra-hydrogel assembly occurs through non-radical polymerization, this method does not suffer from loss of fluorescence due to chemical fluorophores being destroyed during free radical polymerization, which is observed using typical hydrogels[1,4,18,30]. Next, the current generation of tetrahedral-like monomers are much larger than typically used monomers, meaning these tetrahedral-like monomers are likely to form a less dense polymer network and are likely worse at penetrating samples[30]. Finally, protein retention in tetra-hydrogels is currently mediated by anchoring to the ends of monomers through click-chemistry. As a result, every anchored protein results in the termination of a polymer chain, introducing a defect in the polymer network[30]. This is in contrast to anchoring strategies in typical hydrogels, where AcX, for example, incorporates proteins in the hydrogel as a side chain of the main polymer backbone[18]. In conclusion, while this first iteration of tetra-hydrogels has shown to be promising in terms of minimizing local anisotropy, numerous developments are required for a future second-generation tetrahedral-like monomer-based hydrogel to be able to become a widely adopted super-resolution imaging approach.

Alternative Labeling Strategies

Thus far this thesis has been mostly limited to discussing ExM strategies in the context of super-resolution imaging of specific proteins of interest, labeled using typical strategies using fluorescent antibodies or genetically encoded fluorescent proteins. However, recent developments have enabled compatibility with a variety of alternative labeling strategies, including approaches that enable labeling other types of biomolecules, nonspecific labeling, and labeling using small tags[31,39–42]. These alternative labeling approaches may offer a number of advantages over classical immunolabeling and fluorescent protein expression, depending on the application.

Small protein tags

The first alternative labeling approach is the use of genetically-encoded small protein tags, such as seen in a recently developed method called Label Retention Expansion Microscopy (LR-ExM)[39]. LR-ExM uses SNAP and CLIP tags, which are subsequently marked by trifunctional anchors that contain three molecular arms with a different functional group on each: (1) benzynguanine for SNAP tag labeling or benzylcytosine for CLIP tag labeling, (2) methoctramine for anchoring to the hydrogel, and (3) biotin or digoxigenin for recognition by fluorescent reporters using fluorescent streptavidin or fluorescent anti-digoxigenin primary antibodies respectively, which can be used orthogonally for two-color imaging[39]. This labeling method mainly offers alternative advantages over expressed fluorescent proteins. Both methods use genetically-encoded fusion proteins. However, genetically-encoded fluorescent proteins inherently suffer from loss of fluorescence signal during the polymerization and digestion steps in the ExM protocol, which LR-ExM is able to circumvent by adding the fluorescent reporters to the trifunctional anchors post-expansion[1,2,18,39]. This results in higher labeling density and severalfold higher fluorescence intensity, ultimately enhancing the achievable molecular resolution[39].

Nucleic acid anchoring and labeling

The second alternative labeling approach that will be discussed focusses on universally anchoring all nucleic acids to the hydrogel network, and subsequently expanding the sample and labeling specific RNA sequences by Fluorescence *In Situ* Hybridization (FISH). Logically referred to as ExFISH, this method allows for imaging of specific RNA sequences in cells and tissues with nanoscale resolution, which had previously remained difficult[1,2,40]. RNA is anchored to the hydrogel using a compound called LabelX, which is synthesized from commercially available compounds Label-IT and AcX[1,2,40]. The Label-IT-side of the LabelX compound is anchored to guanines through an alkylation reaction, while the AcX-side of the compound functions as an acrylamide group for incorporation into the hydrogel during free-radical polymerization, similar to its function in protein retention[1,2,40]. ExFISH in cells can be performed with single-molecule FISH (smFISH), a method in which single RNA molecules are labeled by multiple fluorescent oligonucleotide probes[1,40,43]. However, since the signal obtained by smFISH in thick tissue slices is too dim, signal amplification strategies such as Hybridization Chain Reactions (HCR), in which RNA molecules of interest are targeted by complementary RNA probes that contain fluorophore-labeled self-assembling hairpins, are required in thick tissue slices[1,40,44]. Multiplexing by multiple wash and labeling cycles is also possible with this method, although it does require re-embedding the expanded sample in a second noncharged hydrogel. ExFISH can be performed simultaneously with ProExM for simultaneous visualization of both RNA and proteins[40,45]. However, *in situ* hybridization requires special buffers that reduce the achievable expansion factor of the hydrogel to 3-fold, compared to the 4.5-fold achieved by ProExM alone[40,45]. Finally, RNA retention using LabelX has resulted in the recent development of *in situ* Expansion Sequencing (ExSeq) methods[46]. In ExSeq, the standard next-generation sequencing chemistry is performed within expanded cells or tissues, and imaged using standard fluorescence microscopes. This allows researchers to obtain highly detailed datasets on *in situ* RNA localization, in both specially targeted and untargeted bulk approaches[46].

Imaging of membranes

Biological membranes are highly interesting structures relevant to a wide range of research applications, including research on synaptic function, organelles, and cell-cell interactions[47,48]. However, super-resolution imaging of membranes with ExM has remained challenging, as lipids are removed by detergent-based permeabilization prior to hydrogel formation and anchoring in conventional ExM protocols[49]. As such, the development of alternative lipid anchoring and labeling strategies has been a focus within the expansion microscopy field. These strategies typically use only mild detergent-based permeabilization, or avoid detergent-based permeabilization altogether, in order to preserve membrane structural integrity[27,47–50]. Early membrane labeling and anchoring approaches use custom-synthesized lipids that intercalate in target membranes, for example, palmitoyls modified with a peptide backbone and biotin tag for anchoring and labeling respectively, or sphingolipids functionalized with amino and azide groups for anchoring and fluorescence labeling by click chemistry respectively[48,50]. More recent studies show a trend towards commercially available modified lipids is observed, facilitating the adoptability of the method. Most of these compounds follow a similar design and working mechanism to the custom-synthesized lipids mentioned above. Examples include mCLING, a fluorescent amino-modified palmitoyl, and papSph, a sphingosine that can be crosslinked to adjacent proteins by UV exposure and fluorescently labeled by click chemistry[27,47,51]. Finally, a different approach to lipid anchoring and labeling emerged in a recently published study. Instead of incubating cells with exogenous modified lipids that intercalate into the target membrane, endogenous phosphatidylcholines are metabolically modified with an alkynyl group[49]. These metabolically modified phosphatidylcholines are subsequently covalently bound to trifunctional anchors through azide-alkyne click chemistry, ultimately labeling endogenous phosphatidylcholines with fluorophores and functional methacrylamides for anchoring to the swellable hydrogel[49].

Nonspecific bulk labeling

A number of bulk fluorescent labeling methods for various classes of biomolecules have recently been emerging within the field of expansion microscopy, because they specifically take advantage of biomolecule decrowding in expanded samples[27,31]. An example of this is illustrated by a novel bulk labeling method called Pan-ExM[31]. In Pan-ExM, the entire proteome is labeled by small fluorescent dyes conjugated to NHS-esters that react with primary amines on proteins[31]. Since non-expanded samples are so densely crowded by proteins, such bulk protein labeling results in an essentially uniform staining, in which very little structural information can be resolved due to a lack of contrast[27,31]. On the other hand, proteins in expanded samples are sufficiently decrowded to the point the images become well-contrasted and that details too small to resolve using conventional fluorescence microscopy, such as mitochondrial cristae, can now be easily be distinguished[31]. The main advantage of using nonspecific staining is that it reveals the entire cellular context, similar to what is typically seen in electron microscopy, but has historically been challenging in fluorescence microscopy[27,31]. Pan-ExM stainings are compatible with staining of both cells and thick tissue slices (100 μm)[27,31]. Furthermore, Pan-ExM can be combined with conventional immunolabeling, allowing for super-resolution imaging of specific proteins in their cellular context, and can also be used simultaneously with well-established DNA dyes such as SYTOX Green[31]. Finally, bulk staining of proteins is not restricted to using NHS-esters to label the entire proteome. For example, it is possible to bulk stain palmitoylated proteins specifically, by metabolically incorporating palmitic acid into the swellable hydrogel[31].

In addition to bulk staining of proteins, a number of methods for bulk staining and super-resolution imaging of other biomolecule classes have recently been developed. One of these methods is called Click-ExM, which utilizes various small chemical reactions to nonspecifically attach orthogonal azide or alkyne labels to glycans, lipids, proteins, or nucleic acids[41]. These azide or alkyne functional groups are subsequently conjugated to fluorescent reporters, such as a biotin/streptavidin, digoxigenin/anti-digoxigenin antibody, FLAG/anti-FLAG antibody, or TAMRA, and are finally anchored to the hydrogel[41]. Click-ExM can be applied to both cells and thick tissue slices (150 μm)[41]. Furthermore, Click-ExM is shown to be compatible with multi-color imaging, which includes compatibility with immunolabeling of specific proteins, as well as two-color imaging of two different classes of biomolecules[41]. Another similar bulk labeling ExM method is called Fluorescent Labeling of Abundant Reactive Entities (FLARE)[42]. FLARE sequentially uses small hydrazide-reactive dyes to label oxidized carbohydrates, amine-reactive dyes to label proteins (similar to Pan-ExM),

and well-established DNA dyes such as Hoechst. These nonspecifically labeled biomolecules can then be imaged in three colors simultaneously, which can be further extended using conventional immunolabeling[42]. FLARE has mostly been applied to thick tissue slices (100 μm)[42].

Combining Super-Resolution Methods

The unique property of ExM is that it bypasses the diffraction limit on a sample preparation level, which is in contrast to other super-resolution microscopy methods that typically do so on a microscope optics level[25]. As such, it is possible to combine ExM with optical super-resolution microscopy methods, allowing expert researchers to image their samples with unprecedented resolution in terms of light microscopy (<10 nm)[25,28,52]. ExM offers a number of additional benefits to optical super-resolution imaging approaches, such as low background noise due to the transparency of expanded samples. Additionally, the possibility of using post-expansion labeling to improve structural resolution and increase labeling density is a major benefit, as the linkage error and high labeling density requirement are major challenges for optical super-resolution methods[28,52]. However, combining these super-resolution approaches comes also with a number of additional challenges and limitations[25]. In general, the large imaging depth of expanded samples requires the use of epi-illumination, as well as long working-distance water-immersion objectives to prevent spherical aberrations, instead of Total Internal Reflection Fluorescence (TIRF) illumination and the short working-distance, high NA oil-immersion objectives commonly used to increase resolution and signal-to-noise-ratio in some optical super-resolution imaging approaches[25,28,52,53]. As such, the effective improvement in resolution when combining ExM with another super-resolution imaging approach is not as significant as would theoretically be expected. Furthermore, performing ExM in combination with another super-resolution microscopy approaches significantly increases the acquisition time of those optical methods, due to the fact that the effective imaging Field Of View (FOV) is much smaller in expanded samples compared to non-expanded samples[54]. Examples of how ExM has been used in conjunction with various optical super-resolution methods, and how those strategies have overcome their respective limitations, will be discussed in the remainder of this section.

ExSIM

Structured Illumination Microscopy (SIM) was the first optical super-resolution imaging approach to be used in conjunction with ProExM, called ExSIM[53,55]. While SIM on its own achieves only a relatively modest 2-fold resolution increase (110 nm) over the diffraction limit, ExSIM with an expansion factor of 3.5-fold achieves imaging resolutions of around 30 nm, which is comparable to methods that typically outperform SIM in terms of resolution, such as STED and SMLM[53]. The advantages that ExSIM offers over those methods include its compatibility with a wide range of fluorophores, multicolor imaging, and its ability to image relatively thick specimen[53]. In an effort to facilitate super-resolution imaging of thick tissue specimen using short working distance, high NA oil-immersion objectives, researchers have combined ExSIM with cryo-sectioning, which allows them to separately image reduced thickness tissue sections and subsequently recreate a 3D reconstruction of the entire specimen[55]. Finally, an especially interesting recent development is the emergence of a method that uses large FOV imaging and FOV stitching to enable high-speed ExSIM. Using this method, authors were able to image hundreds of centrioles in human cells and thousands of purified centrioles, with a resolution of 35 nm, per hour[54]. This enabled them to reconstruct 3D maps of the positions of various post-translational modifications on centrioles, adding an unprecedented amount of statistical relevance to super-resolution imaging[54].

ExSTED

The next combination to emerge was the combination between ProExM and Stimulated Emission Depletion Microscopy (STED), referred to as ExSTED, which enables imaging with a resolution below 10 nm at 4-fold expansion[52]. Because STED inherently is a confocal method, it is relatively easy to combine with ExM, only really requiring the use

of long working-distance water-immersion objectives for deep tissue imaging instead of typically used short working-distance oil-immersion objectives[25,52]. The most significant challenge in STED is obtaining a sufficiently high labeling density, which is inherently necessary due to high fluorophore bleaching by the high power excitation and STED beams[52]. Researchers who originally developed ExSTED found that this necessity was further amplified by the loss of fluorescence during the gelation, mechanical homogenization and expansion steps in pre-expansion ExM[4,52]. As such, they adopted an elaborate brute force labeling strategy in which they achieved sufficiently high labeling density by using four fluorescent labels for pre-expansion labeling of microtubules: GFP-tagged α-tubulin, immunofluorescence labeling of α-tubulin, β-tubulin, as well as additional immunofluorescence labeling of GFP[52]. However, it can be argued that post-expansion labeling would be a superior method, as post-expansion labeling inherently achieves a higher labeling density and circumvents loss of fluorescence during the ExM protocol[4]. Other researchers have since successfully performed STED post-expansion labeled U-ExM treated samples, which should be adopted as standard practice in ExSTED going forward.

ExSMLM

Stochastic Optical Reconstruction Microscopy (STORM) is an optical super-resolution imaging approach that was most recently combined with U-ExM, in the form of ExSTORM[28]. However, ExSTORM has some additional limitations relative to ExSTED and ExSIM[25]. First of all, STORM relies on the stochastic blinking of individual antibody-conjugated dyes in order to reconstruct a diffraction-unlimited composite image. However, the fluorescent dyes with the most suitable blinking properties, Alexa Fluor 647 and Cy5, are destroyed during free-radical polymerization[1,4,18,25,28]. Therefore, ExSTORM is forced to use less optimal blinking dyes, or otherwise limited to using post-expansion labeling, which may anyway be the superior approach due to its enhanced structural resolution aided by a reduced linkage error and increased labeling density. Furthermore, STORM utilizes special buffers to optimize the photoswitching rate of the fluorescent dyes[25,28]. Use of these buffers results in undesired shrinkage of the polyelectrolyte hydrogels used in ExM[25,28]. Therefore, combined use of ExM and STORM requires re-embedding the sample in a second, noncharged hydrogel to retain the sample in its expanded state, which complicates the protocol[25,28]. Finally, STORM typically uses TIRF illumination in combination with short working-distance oil-immersion objectives, but is limited to using epi-illumination and long working-distance water-immersion objectives instead when combined with ExM[25,28]. Despite these additional limitations, ExSTORM may currently be the option for imaging the native ultrastructure of macromolecular assemblies using light microscopy, as it is arguably the best performer in terms of resolution, achieving impressive imaging resolutions below 10 nm at expansion factors of around 3-fold, while specifically utilizing U-ExM, a well-validated method for preservation and post-expansion labeling of those native ultrastructures[28,37].

ONE

A newly emerging class of optical super-resolution approaches use algorithms to generate super-resolution images based on higher-order statistical analysis of images. One such method is called Super-Resolution Radical Fluctuations (SRRF), which performs statistical analysis on temporal fluctuations in point spread function radial symmetry, data which is otherwise discarded by single-molecule localization-based super-resolution methods[56,57]. The resolution of images generated using SRRF is positively influenced by the distance between fluorophores, and is affected less by dim fluorescent signals compared to other optical super-resolution microscopy approaches[56,57]. Furthermore, the method does not require special buffers such as seen in ExSTORM, and can be performed using fast and accessible open-source software on images obtained using readily-available confocal microscopes[57]. These benefits make SRRF an especially interesting candidate for combined use with ExM, both from a technical as well as a usability perspective[57]. Using this combination, researchers were able to achieve unprecedented resolutions down to below 1 nm on conventional confocal microscopes, fittingly referred to as One-Nanometer Expansion (ONE) microscopy[57]. While still in preprinting phase, it will be interesting to see how this method will help bridge the gap between structural biology and light microscopy in the near future.

Discussion

Expansion microscopy is a versatile super-resolution imaging approach that, contrary to other such approaches, uses commercially available compounds and widely available optical systems, making super-resolution imaging more accessible to the average researcher. Furthermore, its compatibility with both cellular samples and thick tissue sections, a wide variety of available labeling strategies, as well as simultaneous usability with optical super-resolution approaches, make the method applicable to a wide range of biological studies. The expansion microscopy field has seen rapid developments since its first conceptualization, ranging from protein retention to post-expansion labeling, modalities that increase the expansion factor to achieve better resolutions, and modalities that minimize expansion isotropy to faithfully preserve native ultrastructures. However, many of these developments come with significant downsides, for example, the highly elaborate re-embedding and crosslinker cleaving protocols seen in iExM, or use of commercially unavailable compounds such as tetrahedral-like monomers, limiting the widespread adoptability of those methods. Other methods perform exceptionally well in some areas, such as native ultrastructure retention performance of U-ExM, but perform subpar in others, such as in this case the expansion factor achieved by U-ExM.

As such, the field of expansion microscopy needs a unified protocol that combines the best properties from all major expansion microscopy modalities, including using an easily adoptable protocol, high single-round expansion factor, minimal post-expansion isotropy, compatibility with a variety of sample types, labeling strategies and optical super-resolution imaging approaches. The recently published TREx method shows a lot of potential in this regard, achieving high resolution through 10-fold single-round hydrogel expansion, being easily adoptable by using the most widely accepted general protocol and hydrogel components, and proven compatibility with a variety of sample types and labeling methods, and the method can therefore be expected to be adopted by many laboratories. While TREx will work perfectly fine for the majority of applications, it does not yet offer the same nanoscale integrity required for highly accurate imaging of nanoscale macromolecular structures as offered by methods that aim to maximize it, such as U-ExM. It is therefore expected that the search for a unifying protocol will continue in the future, to which the ultimate answer may be a not yet developed alternative expansion matrix that utilized a different monomer design and polymerization reaction, for example, a future generation of the tetrahedral-like monomers.

Finally, an expected trend to occur within the expansion microscopy field is post-expansion labeling becoming a more standard practice. As it stands, there is a number of expansion microscopy methods that ultimately aim to achieve nanoscale imaging resolution but have not yet adopted post-expansion labeling (iExM, ExSTED, ExSIM), even though their effective resolution ultimately ends up being limited by the structural resolution and density of their labeling approach, in which post-expansion labeling can be such a valuable tool. Adopting epitope conservation and post-expansion labeling into a protocol indeed requires additional optimization of the polymerization, homogenization and labeling steps, which reduces the adoptability of the protocol. However, some of these methods use similarly laborious yet likely less effective measures to tackle their labeling limitations, such as the brute force labeling strategy adopted by the original ExSTED methodology.

Nederlandse samenvatting

Lichtmicroscopie is een van de krachtigste middelen die wetenschappers tot hun beschikking hebben om de moleculaire processen in onze cellen te kunnen onderzoeken. Echter is de resolutie (de minimale afstand tussen twee objecten die nodig is om deze van elkaar te kunnen onderscheiden) waarmee lichtmicroscopen deze moleculaire processen in beeld kunnen brengen fundamenteel gelimiteerd: de natuurkundige eigenschappen van licht zorgen ervoor dat de resolutie die lichtmicroscopen kunnen waarnemen niet kleiner kan zijn dan ongeveer 200 nanometer, een fenomeen dat de diffractielimiet wordt genoemd. Echter zijn veel belangrijke eiwitstructuren nog kleiner dan deze limiet, waardoor we ze niet gedetailleerd in beeld kunnen brengen en niet goed kunnen onderzoeken. Denk bijvoorbeeld eiwitstructuren die poriën in de celkern vormen (diameter van 107 nanometer), of de microtubuli die het skelet van de cellen vormen (diameter van 25 nanometer). Om zulke kleine structuren te onderzoeken wordt normaal gesproken elektronenmicroscopie gebruikt, waarmee een veel hogere resolutie bereikt kan worden. Echter is elektronenmicroscopie erg onhandig in gebruik, door onder andere uitgebreide preparatie protocollen, gebrek aan methoden om specifieke biomoleculen aan te kleuren, en dat het niet toegepast kan worden op dikke weefselpreparaten. Omdat lichtmicroscopie op deze vlakken beter presteert, is het van belang dat er lichtmicroscopie methoden worden ontwikkeld die de diffractielimiet kunnen doorbreken.

De afgelopen twee decennia zijn enkele van zulke technieken ontwikkeld, zogeheten super-resolutie microscopie methoden. Een van deze technieken is hier bijvoorbeeld toe instaat door de laser waarmee cellen belicht worden te omringen met een speciale donut-vormige laser. Deze donut-vormige laser zorgt ervoor dat de fluorescente labels waarmee eiwitten gevisualiseerd worden tijdelijk niet kunnen fluoresceren, waardoor alle fluorescente signalen die de lichtmicroscoop detecteert enkel uit het zeer kleine oppervlak in het centrum van de donut afkomstig kan zijn. Dit maakt het mogelijk om met zeer hoge nauwkeurigheid eiwitstructuren in cellen kaart te brengen, met een resolutie van tot wel 20 nanometer, wat een stuk beter is dan de diffractielimiet. Helaas zitten er enkele grote nadelen aan het gebruik van zulke super-resolutie technieken. Zo gebruiken deze technieken bijvoorbeeld speciale apparatuur en materialen die enorm duur zijn, duren deze technieken vaak lang om uit te voeren, en is het niet mogelijk om deze technieken toe te passen op weefselpreparaten. Hierdoor is het gebruik van super-resolutie microscopie enkel toegankelijk voor een kleine groep experts en kan het niet op een breed spectrum van onderzoek toegepast worden.

Er is echter recent een nieuwe super-resolutie microscopie methode ontwikkeld die hier verandering in kan brengen. Deze methode heet expansiemicroscopie. Met deze techniek wordt er een netwerk van polymeren in de te onderzoeken cellen gevormd. Dit netwerk van polymeren kan veel water op nemen, waardoor het netwerk (en dus ook de cellen in dit netwerk) kan uitzetten. Als gevolg van deze expansie komt er meer ruimte tussen de eiwitten en andere biomoleculen waaruit de cel is opgebouwd, waardoor het gemakkelijker wordt om deze eiwitten van elkaar te onderscheiden met een microscoop. Met andere woorden, de resolutie van het systeem wordt hoger. Het grote voordeel van deze techniek is dat het gebruik maakt van gemakkelijk verkrijgbare en betaalbare materialen, en toegepast kan worden op gebruikelijke microscopen. Daarnaast duurt het uitvoeren van deze techniek over het algemeen minder lang dan andere super-resolutie microscopietechnieken, en kan het relatief gemakkelijk toegepast worden op dikke weefselpreparaten. Door deze voordelen is de techniek een stuk toegankelijker voor de gemiddelde wetenschapper.

In dit verslag worden recente doorontwikkelingen binnen de expansiemicroscopie methode besproken. Zo wordt besproken hoe de haalbare resolutie van deze techniek verder verbeterd kan worden door de mate van expansie te vergroten. Dit kan bijvoorbeeld door twee opeenvolgende rondes van expansie uit te voeren, of door de chemische samenstelling van het netwerk van polymeren aan te passen. Daarnaast worden enkele strategieën besproken om vervormingen in het polymeernetwerk na uitzetting te minimaliseren, zodat de oorspronkelijke structuren van de cellen zo goed mogelijk behouden blijven. Vervolgens worden er enkele recent ontwikkelde methoden om verschillende biomoleculen fluorescent aan te kleuren besproken die gemakkelijk toegepast kunnen worden bij expansie microscopie, maar niet bij andere super-resolutie microscopie methoden. Tenslotte worden enkele mogelijkheden besproken om expansie microscopie te combineren met andere super-resolutie microscopie

methoden. Hoewel dit natuurlijk wel de nadelen van de andere techniek met zich mee brengt, maakt deze combinatie het mogelijk om eiwitstructuren in beeld te kunnen brengen met resoluties die voor licht microscopie ongekend goed zijn.

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