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A Review of Expansion Microscopy

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Abstract. Expansion microscopy is a type of powerful and simple super-resolution microscopy that provides sub-diffraction images of biological samples by physically expanding them anchored in hydrogels. It has been combined with other existing methods to better visualize the microscopic structures of the samples. We will review some very important papers in this area about the early developments and explorations of general procedures and fundamental mechanisms of expansion microscopy. We will also review the recent developments and applications as well as their advantages and insufficiencies. Through our review, it is clear that the current expansion microscopy applications can help researchers to identify many sub-diffraction structures of the samples with many different fluorescence staining strategies, but there is a tradeoff between those strategies. Based on these points, we anticipate expansion microscopy will achieve a higher expansion factor and more powerful staining techniques.

Keywords: imaging, expansion microscopy, expansion factor, fluorescence staining

1. Introduction

Since the invention of optical microscopy, the resolution of optical microscopy has increased dramatically until the reaching diffraction limit. While the diffraction limit refers to the minimal distance that can be distinguished under the microscope, and it is proportional to the wavelength of the incident light, for visible light, this value is about 200 nm [1]. In the past decades, the scientists have come out a lot of methods to overcome the diffraction limit, and they are well known as super-resolution microscopy. Super-resolution microscopy protocols overcame the diffraction limit by different optical methods. These methods have achieved a higher resolution: for example, the resolution of Stimulated Emission Depletion (STED) could reach 30 nm [2]. However, these methods have some defections due to some optical problems, such as photobleaching of the fluorophores and the lower axial resolution, that limit their applications in many areas. Under these circumstances, Chen et al. proposed a new type of super-resolution microscopy in 2015, which enables the researchers to expand the samples before taking images, thus bypassing the optical limitations. Even though the resolution of the microscopes remains the same, the length measured in the post-expansion images is in fact several times larger than the corresponding length in the original sample. Therefore, the resolution can go below 100 nm, which enables the researchers to discover much more details in the images than before. This paper mainly reviews the technique of expansion microscopy and its biological applications. And at the end of this review, we also provide an outlook on this field based on the recent breakthrough of expansion microscopy.

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2. Technique

2.1. General procedure

The general procedure of expansion microscopy includes fluorescent staining, monomer solution incubation, gelation, digestion, and expansion [3]. In the first step, the samples are incubated in the fluorescent dyes or antibodies. Then, they are incubated in a monomer solution that usually contains monomer acrylamide (AA), co-monomer sodium acrylate (SA), crosslinker N-N'-methylenebisacrylamide (MBAA). This step is very important for expansion; it allows the monomer solution to diffuse uniformly within the sample, so the gelation can also be uniform in the later step. The time for incubation can vary from several minutes to overnight [4-5]. Then, the researchers prepare a gelation solution containing the monomer solution, an accelerator N,N,N,N-tetramethyl-ethane-1,2-diamine (TEMED). Samples are spread on a piece of coverslip or a gelation chamber, and gelation solution with the polymerization initiator ammonium persulfate (APS) is added to sample. The gelation chamber is set in an incubator for an hour at 37 degrees to form the gel. During the gelation, APS and TEMED react with the monomers and crosslinkers to form free radicals in the solution. These radicals, after radical propagation, react with each other to form a crosslinked polymer. After this step, samples are covered in a piece of hydrogel. The operators gently remove the gel from the gelation chamber and measure the diameter of the gels. The gel is placed in protease to digest the protein in samples so that the gels become homogeneous. This homogeneous hydrogel can absorb water, so it can absorb the liquid and expand itself three-dimensionally in deionized water. Therefore, to expand the gel with samples, researchers can simply left it in deionized water [3].

2.2. Expansion factor and improvement

The goal of expansion microscopy is to overcome the diffraction limit by expanding the sample in a hydrogel to reveal more details than conventional optical microscopy. Therefore, one of the goals of researches on expansion microscopy is to increase the expansion factor while avoiding distortion. After seven years of development, researchers have developed many different types of expansion microscopy methods to image different samples. The fundamental difference between them is the composition of the monomer solution they used. Hu et al. had tried expansion microscopy with different concentrations of the crosslinkers [6]. Their research shows that lowering the concentration of crosslinker increases both the expansion factor and distortion, while the latter is unexpected. Therefore, to retain the shape and structure of the samples, researchers cannot only pursue larger expansion factor and resolution of the images unscrupulously. The upper limit of the expansion factor of this method is thus limited at about 5x, so that the root-mean-square error of length as below 1% to 3% [4]-[5]. To solve this problem, Trukenbrodt et al. developed a new protocol with a very different monomer solution. They used N,N-dimethylacryamide (DMAA) as the monomer, sodium acrylate (SA) as the crosslinker, potassium persulfate (KPS) and TEMED as the catalyzer to prepare a gel. During the polymerization, KPS reacts with DMAA to from free radicals while TEMED reacts with SA to from free radicals. These radicals then react with SA and DMAA to form a crosslinked polymer. This new method can expand the hydrogel up to ten folds, which is much larger than the previous expansion microscopy [7].

Although scientists have achieved a great progress in expansion microscopy, there are still some problems of those methods. First, the hydrogel is very fragile. Although researchers have done many experiments and attempts to increase its hardness, the hydrogel is still easily broken, especially after the expansion. Second, the digestion process of those early expansion microscopy protocols have to break done most proteins in the samples in order to expand the sample freely in DI water. Thus, researchers can no longer use expansion microscopy to study protein distribution in the samples. This significantly limits the application of this technique and disappoints many scientists. To solve this problem, in 2016, Taeyun Ku et al. came out a new type of expansion microscopy protocol, magnified analysis of the proteome (MAP), that avoided digestion in the procedure. This protocol starts with monomer solution incubation, gelation, denaturation, and expansion. The monomer solution also uses AA, SA, and MBAA, as monomers and crosslinker. Instead of the traditional persulfate initiator APS or KPS, they used

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VA-44 as initiator, which is a non-nitrile, cationic water-soluble azo polymerization initiator [8]. Furthermore, MAP gelation no longer needs the toxic accelerator TEMED. The conditions for gelation are a little different from other expansion microscopy protocols. MAP sets the reaction time at 45 degrees, and the time is about 2 to 2.5 hours. The hydrogel of MAP is very resilient and does not become fragile while being transferred. It is put in the denaturation solution made of sodium dodecyl sulfate (SDS), a detergent, sodium chloride, and Tris. The sample in the solution is heated at 70 degrees overnight and then at 95 overnight. At the end, the hydrogel is washed and expanded in DI water. MAP enables researchers to perform the staining process at the last step of MAP, which provides them opportunities to do the post-expansion labeling such as biotin-streptavidin interaction.

3. Application

Breaking the diffraction limit, expansion microscopy becomes a very powerful tool in many areas. It has been applied in many areas and proved that this technique could be combined with different fluorophore staining techniques.

The process of how DNA is transcribed into RNA and then translated into peptide chains is always a very popular field of study in biochemistry. However, studying this process under microscopes is not an easy thing due to the tiny distances between those molecules. Expansion microscopy allows the researchers to expand the tissues to 10x in one dimension, which makes the distance between those molecules large enough to be distinguished [9]. The researchers also solved the problem of labeling those bio-molecules with fluorophores. Nowadays, there are innumerable methods to stain molecules that participate in this process.

The stanning of DNA and RNA, even though both of them are nucleic acid, can be very different. There are various stains that can label DNA readily. One of them is called Hoechst 33342. This is a commercial fluorophore that has been widely used in many labs to stain DNA and nuclei in the samples. There are many advantages: it is a small molecule, so researchers do not need to permeabilize the samples in advance, and it only takes less than an hour to stain a large piece of sample. The signal from it is very strong and clean under microscope. Unlike many other stains that may stain many other analogues, it exclusively creates signals from the locations of DNA. Another advantage is that this molecule is soluble in most solvents used in expansion microscopy, such as phosphate buffered saline (PBS) and DI water. Therefore, researchers can label DNA anytime they need. However, this type of small molecules labeling nucleic acid is not specific, so it cannot be used to visualize a specific DNA sequence. Thus, fluorescence in situ hybridization (FISH) comes out to stain specific nucleic acid chain with single strand RNA with about 50 nucleotides which is linked to a fluorophore. Thus, the RNA chains in the samples are visualized under the microscope [10].

How to visualize protein is another very popular topic in this area. Similar to the staining of nucleic acid, some techniques label all the proteins by labeling the amino groups in the protein, while others only label specific proteins with antibodies in the samples. In 2017, Aaron Halpern et al. used fluorophore-conjugated antibodies to stain tubulin, a type of protein microtubules in cells. This strategy is called immunofluorescence (IF). It takes the advantage of antibodies, that they can selectively bind to the target proteins in the sample. They first used Triton-100 to permeabilize the samples. Then they used antibodies that target exclusively tubulin, which is called primary antibodies. Then, they used secondary antibodies which is connected to fluorophores that can target primary antibodies to label the primary antibodies. After this step, the cells is gelled and expanded with expansion microscopy [11]. Although IF came out before the invention of expansion microscopy, expansion microscopy, with its high resolution, makes IF capable in staining more delicate structures and molecules in the samples, such as proteins. Later, in 2020, Chenyi Mao et al. proposed a new staining strategy that can label all the proteins in the sample with simple small molecules bound to fluorophores. This method utilized the reaction between NHS and the amino groups in the peptide to form covalent bonds between NHS and NH2 in the proteins [12]. Both of these two strategies have some advantages: IF can label only a specific type of protein, so it can visualize the distribution of that single kind of protein, such as tubulin or actin. However, the second strategy, as it only uses small molecules, does not require permeabilization of the

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samples, and it can visualize the general distribution of all the protein in the sample. For some instances, especially when different components of a sample have different patterns of protein distribution, this method can visualize these differences with a microscope as well. However, there are some disadvantages and limitations of them. IF cannot use post-expansion staining, while the other method can. Nonetheless, the signal from the NHS staining can have a large noise and undesired background that have to be identified and removed. Generally, these two ways of labeling protein applicable with expansion microscopy have their own advantages and drawbacks, and should be selected carefully by the researchers.

In 2021, to better visualize the distribution of mRNAs and proteins in the samples, In Cho et al. combined IF and FISH, and established a method that can visualize mRNA and protein simultaneously. They used a FISH probe that targeted GAPDH mRNA and antibodies that stained GM130 and vimentin, two types of proteins, in the samples. Then, they used the traditional version of gelation solution and digested the samples with the protease [13]. This method helps them visualize the process of translation, especially the approximate ratio of mRNA that is translated into protein. Their result revealed that after expansion, most of the noise in the images was cleared after expansion, which again proved the advantages of expansion microscopy, that it can better display samples' structure with much higher resolution.

4. Conclusion

In conclusion, we have reviewed the history of expansion microscopy since its first appearance in 2015. This technique, although it has a very short history compared to other super-resolution microscopy, has shown its unique uses. It effectively avoids rapid photobleaching of the fluorophores; it does not require elaborate hardware but only a diffraction-limited optical fluorescent microscope. Since its invention in 2015, expansion microscopy has developed dramatically and applied widely in this area. It has cooperated with many other microscopic techniques, such as IF. Many other staining strategies, such as small molecule labeling, are also combined with expansion microscopy. Based on the general idea, by exploring the effects of different compositions, scientists also introduced protocols like MAP which made up some deficiencies of the original protocol. After that, in recent years, scientists have also tried to combine many other techniques in this field, such as Stimulated Raman Spectroscopy (SRS) [14], STED (Ex-STED) [15], and still approaching higher resolution by iterative expansion microscopy.

From the development of expansion microscopy reviewed in the paper, we believe that researchers are still approaching larger expansion factor and higher resolution from many perspectives. For example, based on iterative expansion microscopy, researchers could perform the expansion for third or even forth times, so that the total expansion factor can be 15x and even larger, and the resolution can be up to nanometers instead of 30-50 nm. Another promising direction for the development of expansion microscopy is increasing the variety of labeling targets and strategies. There are still some biomolecules that are difficult to be labeled with expansion microscopy currently, such as lipids. Researchers can also explore some new staining strategies to stain them and provide us with more sophisticated images of tissues and cells.

Reference

[1] Lee, S., Li, L., Ben-Aryeh, Y., Wang, Z., & Guo, W. (2013). Overcoming the diffraction limit induced by microsphere optical nanoscopy. Journal of Optics., 15(12). https://doi.org/10.1088%2F2040-8978%2F15%2F12%2F125710

[2] WILDANGER, D., MEDDA, R., KASTRUP, L., & HELL, S. (2009). compact STED microscope providing 3D nanoscale resolution. Journal of Microscopy (Oxford), 236(1), 35–43. https://doi.org/10.1111/j.1365-2818.2009.03188.x

[3] Chen, F., Tillberg, P. W., & Boyden, E. S. (2015). Expansion microscopy. Science, 347(6221), 543–548. https://doi.org/10.1126/science.1260088

[4] Gambarotto, D., Zwettler, F. U., Le Guennec, M., Schmidt-Cernohorska, M., Fortun, D., Borgers, S., Heine, J., Schloetel, J.-G., Reuss, M., Unser, M., Boyden, E. S., Sauer, M., Hamel, V., &

DOI: 10.54254/2753-8818/4/20220674

Guichard, P. (2019). Imaging cellular ultrastructures using expansion microscopy (U-ExM). Nature Methods, 16(1), 71–74. https://doi.org/10.1038/s41592-018-0238-1

[5] Min, K., Cho, I., Choi, M., & Chang, J.-B. (2020). Multiplexed expansion microscopy of the brain through fluorophore screening. Methods (San Diego, Calif.), 174, 3–10. https://doi.org/10.1016/j.ymeth.2019.07.017

[6] Hu, Y., Chu, X., Chen, T., Pan, Q., Liu, C., Yi, J., & Chu, X. (2020). Improving resolving ability of expansion microscopy by varying crosslinker concentration. Chemical Communications (Cambridge, England), 56(3), 4176–4179. https://doi.org/10.1039/d0cc00052c

[7] Truckenbrodt, S., Maidorn, M., Crzan, D., Wildhagen, H., Kabatas, S., & Rizzoli, S. O. (2018). X10 expansion microscopy enables 25‐nm resolution on conventional microscopes. EMBO Reports, 19(9). https://doi.org/10.15252/embr.201845836

[8] Ku, T., Swaney, J., Park, J.-Y., Albanese, A., Murray, E., Cho, J. H., Park, Y.-G., Mangena, V., Chen, J., & Chung, K. (2016). Multiplexed and scalable super-resolution imaging of three-dimensional protein localization in size-adjustable tissues. Nature Biotechnology, 34(9), 973–981. https://doi.org/10.1038/nbt.3641

[9] Damstra, H. G. J., Mohar, B., Eddison, M., Akhmanova, A., Kapitein, L. C., & Tillberg, P. W. (2022). Visualizing cellular and tissue ultrastructure using Ten-fold Robust Expansion Microscopy (TREx). eLife, 11. https://doi.org/10.7554/eLife.73775

[10] Raj, A., Tyagi, S., van den Bogaard, P., Rifkin, S. A., & van Oudenaarden, A. (2008). Imaging individual mRNA molecules using multiple singly labeled probes. Nature Methods, 5(10), 877–879. https://doi.org/10.1038/nmeth.1253

[11] Halpern, A. R., Alas, G. C. M., Chozinski, T. J., Paredez, A. R., & Vaughan, J. C. (2017). Hybrid Structured Illumination Expansion Microscopy Reveals Microbial Cytoskeleton Organization. ACS Nano, 11(12), 12677–12686. https://doi.org/10.1021/acsnano.7b07200

[12] Mao, C., Lee, M. Y., Jhan, J.-R., Halpern, A. R., Woodworth, M. A., Glaser, A. K., Chozinski, T. J., Shin, L., Pippin, J. W., Shankland, S. J., Liu, J. T. C., & Vaughan, J. C. (2020). Feature-rich covalent stains for super-resolution and cleared tissue fluorescence microscopy. Science Advances, 6(22), eaba4542–eaba4542. https://doi.org/10.1126/sciadv.aba4542

[13] Cho, I., & Chang, J.-B. (2022). Simultaneous expansion microscopy imaging of proteins and mRNAs via dual-ExM. Scientific Reports, 12(1), 3360–3360. https://doi.org/10.1038/s41598-022-06903-3

[14] Shi, L., Klimas, A., Gallagher, B., Cheng, Z., Fu, F., Wijesekara, P., Miao, Y., Ren, X., Zhao, Y., & Min, W. (2022). Super‐Resolution Vibrational Imaging Using Expansion Stimulated Raman Scattering Microscopy. Advanced Science, 9(20), e2200315–n/a. https://doi.org/10.1002/advs.202200315

[15] Gao, M., Maraspini, R., Beutel, O., Zehtabian, A., Eickholt, B., Honigmann, A., & Ewers, H. (2018). Expansion Stimulated Emission Depletion Microscopy (ExSTED). ACS Nano, 12(5), 4178–4185. https://doi.org/10.1021/acsnano.8b00776