Confocal super-resolution imaging of the glomerular filtration barrier enabled by tissue expansion

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The glomerular filtration barrier, has historically only been spatially resolved using electron microscopy due to the nanometer-scale dimensions of these structures. Recently, it was shown that the nanoscale distribution of proteins in the slit diaphragm can be resolved by fluorescence based stimulated emission depletion microscopy, in combination with optical clearing. Fluorescence microscopy has advantages over electron microscopy in terms of multiplex imaging of different epitopes, and also the amount of volumetric data that can be extracted from thicker samples. However, stimulated emission depletion microscopy is still a costly technique commonly not available to most life science researchers. An imaging technique with which the glomerular filtration barrier can be visualized using more standard fluorescence imaging techniques is thus desirable. Recent studies have shown that biological tissue samples can be isotropically expanded, revealing nanoscale localizations of multiple epitopes using confocal microscopy. Here we show that kidney samples can be expanded sufficiently to study the finest elements of the filtration barrier using confocal microscopy. Thus, our result opens up the possibility to study protein distributions and foot process morphology on the effective nanometer-scale.

Kidney International (2017) ■, ■–■; https://doi.org/10.1016/j.kint.2017.09.019

KEYWORDS: glomerulus; imaging; podocyte; renal pathology

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Received 8 June 2017; revised 17 August 2017; accepted 7 September 2017

An important marker for diagnosis of glomerular diseases is the effacement of the podocyte foot processes. However, such structures are separated by less than 100 nm, making them impossible to resolve using diffraction-limited confocal microscopy. With the advent of super-resolution microscopy techniques,[1] the resolution has been pushed into the sub–100-nm scale also for light microscopy, making glomerular filtration barrier (GFB) structures resolvable.[2] However, to prepare tissue samples of sufficiently high quality to extract the desired information at the nanometer scale is challenging. Recently, our group showed that by applying an optical clearing protocol, such challenges can be overcome when imaging with stimulated emission depletion (STED) fluorescence microscopy.[3] Although STED microscopy can improve the resolution of a fluorescence microscope up to 10 times,[4] it has some practical drawbacks. First, it is a relatively expensive piece of equipment, not accessible to all researchers. Also, because STED microscopy is dependent on high laser powers, high-end photostable fluorescent reporters have to be used for optimal results.[1] Further, even with the best synthetic dyes, bleaching makes the acquisition of 3-dimensional (3D) stacks for volumetric nanoscale representations challenging. Such limitations motivate method developments to make it possible to view the same structures using more conventional microscopy techniques.

Recently, protocols to expand biological tissue by a factor of approximately 5 have been published,[5,6] allowing for 5 times higher effective imaging resolution. By applying one of these protocols[6] to kidney tissue, we show that proteins in the slit diaphragm can be studied with confocal microscopy at comparable resolution as when using super-resolution STED microscopy in nonexpanded tissue. As shown, STED can, when combined with expansion microscopy, further increase the effective resolution inside the GFB to below 20 nm.

RESULTS

Adult rat kidneys were expanded using a protocol similar to the previously described magnified analysis of the proteome protocol,[6] resulting in spatial expansion of kidney samples by a factor of approximately 5 (Figure 1a and b, e), as well as

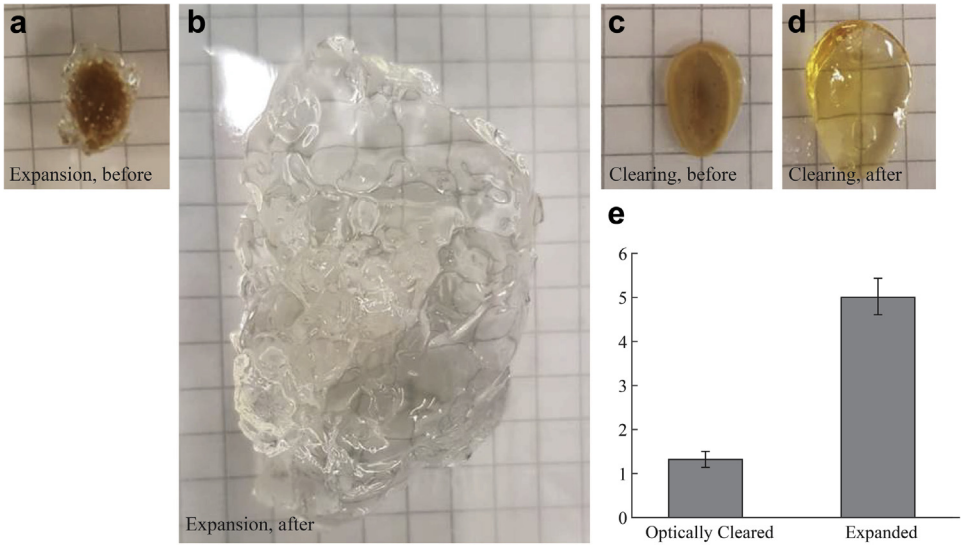

Figure 1 | Expansion of kidney samples using the magnified analysis of the proteome protocol. (**a**) A 1-mm-thick piece of adult rat kidney tissue, after polymerization and removal of the excess hydrogel, but before expansion. (**b**) The same piece of tissue after full expansion protocol. (**c**) A 1-mm-thick piece of adult rat kidney tissue, after polymerization and removal of the excess hydrogel, but before optical clearing. (**d**) The same piece of tissue after full clearing protocol. (**e**) Expansion factors for both cleared and expanded samples. The expansion factors were calculated as the mean linear expansion (width after expansion divided by the same width before expansion) of 3 different samples measured in 2 orthogonal directions. Error bars show SD.

a high degree of optical transparency (Figure 1b). As a comparison, optical clearing of kidney samples was performed, resulting in an expansion factor of approximately 1.3 (Figure 1c and d). Optically cleared (nonexpanded) and expanded rat kidney samples were immunostained for podocin, a component of the slit diaphragm, and imaged with confocal microscopy and STED microscopy (Figure 2a–c). The widths of the slit diaphragms (measured as podocin-podocin distance) and foot processes were resolved to be between 70 and 90 and 220 and 260 nm, respectively, for the 2 approaches (expanded confocal and nonexpanded STED, Figure 2d–f). The ratio between the podocin-podocin distance and the foot process width is constant for the 2 approaches (Figure 2g), showing that relative distances in the tissue are preserved after expansion. In addition, we show that expansion microscopy can be used to perform volumetric and multiplex confocal imaging of different proteins located in the GFB (see Figure 3a–f). Moreover, 3D data can be acquired in thick expanded samples in the form of z-stacks, which gives the opportunity to study foot processes on a full volumetric perspective with sub–100-nm effective resolution (Figure 3a and Movie S1). The complete architecture of podocyte foot processes can be resolved confocally, while simultaneously staining for other constituents of the GFB, such as collagen IV and podocin (Figure 3b–e). Further, using dual-color confocal microscopy, the nanoscale localizations of the slit diaphragm proteins podocin and nephrin can be visualized simultaneously (Figure 3f). We also demonstrate that the technique can be used to image nanoscale pathological alterations in foot process morphology, both on the 3D and the 2D perspective (Figure 4a–h), obtaining quantitative data of foot process effacement in mice with induced glomerulonephritis (Figure 4i and j). Further, we show that expanded samples can be imaged with super-resolution STED microscopy, achieving an improved effective resolution below 20 nm (Supplementary Figure S1). This gives the opportunity to localize single podocin and nephrin molecules in the filtration slit (Supplementary Figure S2).

DISCUSSION

The fine structures of the podocyte foot processes were previously resolvable using only electron microscopy (EM) or super-resolution fluorescence microscopy. In this work, we present evidence that by physically expanding kidney samples, standard diffraction-limited fluorescence microscopy can be used to localize proteins in the slit diaphragm. Importantly, we show that foot process effacement, a widely used diagnostic sign for proteinuric disorders, can be detected using this method. A comparison between confocal microscopy in expanded samples, and super-resolution STED microscopy in nonexpanded cleared samples, shows similar results for both approaches. The small difference in mean slit diaphragm (podocin-podocin) width and foot process width between the 2 methods most likely reflects the difficulty in measuring the expansion factors in an accurate way. This is supported by the fact that the ratio between the podocin-podocin width and the foot process width is kept constant, showing that the expansion is isotropic (relative distances are preserved). However, for measuring absolute distances, this could be a potential pitfall that must be considered. Worth noting is that our measurement of the slit diaphragm width (measured as podocin-podocin distance) is not a direct