Super-resolution stimulated emission depletion imaging of slit diaphragm proteins in optically cleared kidney tissue

David Unnersjö-Jess[1], Lena Scott[2], Hans Blom[1] and Hjalmar Brismar[1,2]

[1]Science for Life Laboratory, Department of Applied Physics, Royal Institute of Technology, Solna, Sweden; and [2]Science for Life Laboratory, Department of Women's and Children's Health, Karolinska Institutet, Solna, Sweden

The glomerular filtration barrier, consisting of podocyte foot processes with bridging slit diaphragm, glomerular basement membrane, and endothelium, is a key component for renal function. Previously, the subtlest elements of the filtration barrier have only been visualized using electron microscopy. However, electron microscopy is mostly restricted to ultrathin two-dimensional samples, and the possibility to simultaneously visualize multiple different proteins is limited. Therefore, we sought to implement a super-resolution immunofluorescence microscopy protocol for the study of the filtration barrier in the kidney. Recently, several optical clearing methods have been developed making it possible to image through large volumes of tissue and even whole organs using light microscopy. Here we found that hydrogel-based optical clearing is a beneficial tool to study intact renal tissue at the nanometer scale. When imaging samples using super-resolution STED microscopy, the staining quality was critical in order to assess correct nanoscale information. The signal-to-noise ratio and immunosignal homogeneity were both improved in optically cleared tissue. Thus, STED of slit diaphragms in fluorescently labeled, optically cleared, intact kidney samples is a new tool for studying the glomerular filtration barrier in health and disease.

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Correspondence: *Hjalmar Brismar, SciLifeLab, Box 1031, Solna 171 21, Sweden. E-mail: brismar@kth.se*

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Traditionally, electron microscopy has been the only method available to visualize the glomerular filtration barrier, as the dimensions of the podocyte foot processes and the slit diaphragm are on the nanometer scale.[1] In terms of spatial resolution, electron microscopy is superior to light microscopy, due to the short effective wavelengths of electrons used to reconstruct images.[2] Even if the morphology of renal foot processes has been dissected with electron microscopy, many questions remain regarding the functional distribution of proteins.[1] The possibility to stain for proteins using immunostaining or by expression of fluorescent proteins would here be beneficial, if high enough spatial resolution using light microscopy could be generated. With the use of super-resolution stimulated emission depletion (STED) microscopy,[3] we demonstrate that the nanoscale localization of proteins at the slit diaphragm is possible to study. Our results show that super-resolution imaging in intact tissue can be difficult due to unspecific/inhomogeneous staining and autofluorescence background seen in paraformaldehyde (PFA) fixed samples. These issues are probably reflected in the limited number of high-resolution studies performed in renal tissue to date. To the best of our knowledge, only two superresolution studies have been carried out,[4,5] both performed on ultrathin sections using a combination of transmission electron microscopy and super-resolution single molecule localization microscopy. High-resolution confocal microscopy has been used in one study of podocyte foot processes, where genetic labeling was used to label a sparse subset of podocytes.[6] On this background, we have identified the need for improved sample preparation protocols for superresolution microscopy in order to study functional structures in kidneys using immunofluorescence at the nanoscale. By applying an optical clearing protocol based on the CLARITY[7–9] and SeeDB[10] methods, we show that background as well as staining problems can be significantly decreased, making it possible to study the spatial distribution of proteins at the slit diaphragm in intact tissue.

RESULTS

Rat kidneys were optically cleared using a hydrogel-based protocol,[9] followed by immunostaining and mounting in fructose solution.[10] The optical transparency (Figure 1a) and antibody penetration depth (Figure 1b and c) were sufficient for imaging samples on the mm-scale using confocal

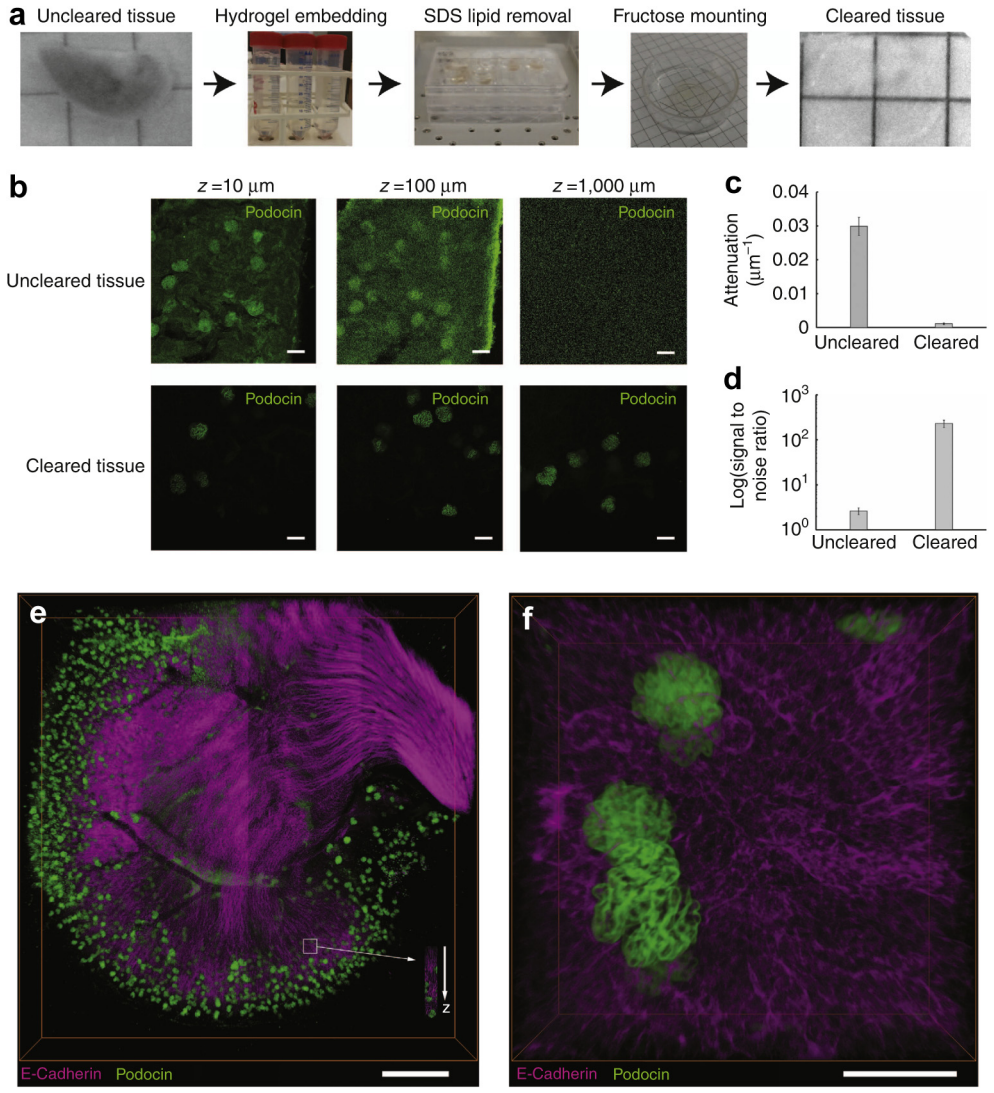

Figure 1 | Optical clearing of rat kidneys and millimeter-scale imaging. For confocal imaging, a Zeiss LSM780 system was used. **(a)** Overview of the clearing process, with before and after images of 1 mm thick slices of a P20 rat kidney. **(b)** Confocal images acquired at 3 different depths in cleared/uncleared 1 mm thick P20 rat kidney slices stained for podocin with Abberior STAR635P. Uncleared samples were paraformaldehyde fixed, stained following the same immunostaining protocol as cleared kidneys, and mounted in phosphate-buffered saline (PBS). Scale bars = 100 µm. **(c)** Attenuation coefficients in uncleared and cleared tissue. Cleared and uncleared kidney slices were stained for E-cadherin with Alexa-647, and confocal z-stacks were acquired with an excitation laser of constant intensity. Mean fluorescence for each z-slice was then plotted as a function of depth. Exponentials were fitted to the plots using MATLAB, and attenuation coefficients could then be extracted. Error bars showing SD ($n = 10$). **(d)** Signal-to-noise ratios (SNRs) in cleared and uncleared kidney tissue stained for podocin, which is expressed only in the glomerulus. The SNRs were measured as the ratio between mean fluorescence signal in the glomerulus and mean fluorescence signal in an area with no glomerulus. Error bars showing SD ($n = 7$). **(e)** Three-dimensional (3D) rendering of a 1 mm thick P20 kidney slice stained for E-cadherin with Alexa-647 and podocin with Alexa-546. Inset showing an xz-view of the marked area. Confocal stacks (tile scanning) acquired using a 5× NA 0.25 air objective. Scale bar = 1 mm. **(f)** Substack of the same sample as in **(e)** acquired at a depth of 400–600 µm. Confocal stack acquired using a 10× NA 0.45 air objective. Scale bar = 100 µm. SDS, sodium dodecyl sulfate.

microscopy, allowing for a global view of kidney morphology and protein expression (Figure 1e and f). Further, we show a substantial increase in fluorescence contrast or signal-to-noise ratio by a factor of ~100 (2.6 ± 0.44 to 230 ± 43) in cleared samples by applying standard immunostaining protocols to both PFA fixed and cleared samples (Figure 1d). For super-resolution STED microscopy, samples were prepared with the same protocol as for millimeter-scale imaging. Immunostaining in cleared samples stained for podocin was specific and highly localized (Figure 2d and e), which allows elucidating the localization on both sides of the slit diaphragm (Figure 2f). Comparison with non-cleared control samples show that optical clearing was crucial to reveal the spatial distribution of podocin on the nanometer scale. Control samples were stained following the same immunoprotocol as cleared samples, but here the immunosignal was incomplete (Figure 2g and h) and the nanoscale distribution of podocin could not be revealed (Figure 2i). Furthermore, the resolution as a function of depth is kept constant up to at least 30 µm using oil immersion objectives (Figure 2j). This