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Three-dimensional structured illumination microscopy of liver sinusoidal endothelial cell fenestrations

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ABSTRACT

Fenestrations are pores in liver sinusoidal endothelial cells that filter substrates and debris between the blood and hepatocytes. Fenestrations have significant roles in aging and the regulation of lipoproteins. However their small size (<200 nm) has prohibited any functional analysis by light microscopy. We employed structured illumination light microscopy to observe fenestrations in isolated rat liver sinusoidal endothelial cells with great clarity and spatial resolution. With this method, the three-dimensional structure of fenestrations (diameter 123 ± 24 nm) and sieve plates was elucidated and it was shown that fenestrations occur in areas of abrupt cytoplasmic thinning (165 ± 54 nm vs. 292 ± 103 nm in non-fenestrated regions, $P < 0.0001$). Sieve plates were not preferentially co-localized with fluorescently labeled F-actin stress fibers and endothelial nitric oxide synthase but appeared to occur in primarily attenuated non-raft regions of the cell membrane. Labyrinthine structures were not seen and all fenestrations were short cylindrical pores. In conclusion, three-dimensional structured illumination microscopy has enabled the unlimited power of fluorescent immunostaining and co-localization to reveal new structural and functional information about fenestrations and sieve plates.

1. Introduction

Liver sinusoidal endothelial cells (LSECs)[1] separate blood in the hepatic sinusoid from the extracellular space of Disse and surrounding sheets of hepatocytes. In order to maximize the transfer of substrates between blood and hepatocytes, LSECs have a unique morphology with cytoplasmic extensions that are very thin and perforated with pores called fenestrations. Fenestrations are fully patent, non-diaphragmed pores that connect the luminal membrane with the extracellular membrane of LSECs. Most fenestrations are clustered together in groups of 10–100 called liver sieve plates, which occupy 2–20% of the LSEC surface (Cogger and Le Couteur, 2009; Fraser et al., 1995; Wisse et al., 1985).

Fenestrations are smaller than the optical diffraction limit (~250 nm) and there are no known cell surface markers available to specifically label them, therefore observation of fenestrations has been limited to electron microscopy (EM), and recently, atomic force microscopy (AFM). While both techniques can yield ultrastructural morphology, they also have significant limitations. EM requires fixatives and treatment of tissue with resins and dehydration, resulting in a lack of temporal information, and the inability to utilize versatile and specialized probes including genetically encoded fluorescent proteins and concentration-sensitive ion indicators (Braet et al., 2007; Cogger and Le Couteur, 2009). AFM yields formidable topographic physical information, but again without specific markers. In addition, the cytoplasmic extensions of the LSECs are technically difficult to assess by AFM because they are extremely thin (Braet et al., 2007). Furthermore, both imaging techniques are relatively low-throughput which handicaps exhaustive studies (Cogger and Le Couteur, 2009).

Here we have used ultra-high resolution light microscopy (Huser, 2008; Lippincott-Schwartz and Manley, 2009) to overcome these limitations. Three-dimensional structured illumination microscopy (SIM) is a relatively fast, easy-to-use multi-color ultra-resolution light microscopy technique (Schermelleh et al.,

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[1] *Abbreviations:* LSEC, liver sinusoidal endothelial cell; SIM, structured illumination microscopy; EM, electron microscopy; eNOS, endothelial nitric oxide synthase; AFM, atomic force microscopy.

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2008). It uses patterned illumination from a coherent light source to convert otherwise unobservable structures below the resolution limit of light microscopy into observable ones by generating difference/beat frequencies called Moiré fringes. By varying the light pattern and observing a sufficient number of Moiré fringes, a higher resolution image can be reconstructed resulting in a resolution two times better than conventional light microscopy, which is well within the average diameter of fenestrations. Importantly, in comparison with electron microscopy, SIM can be used with any conventional fluorophore and cell preparation is identical to other established light microscopy methods (Carlton, 2008). We investigated the three-dimensional structure of fenestrations and sieve plates as well as determining their co-localization with two potential markers of fenestrations, F-actin (Braet et al., 1996) and endothelial nitric oxide synthase (eNOS) (Yokomori et al., 2001). SIM has the potential to revolutionize research into LSECs and their fenestrations.

2. Materials and methods

2.1. Materials

Reagents included Type 1A Collagenase (Sigma Chemical, St. Louis, MO #C9891), RPMI (Gibco Invitrogen #11875-093), CellMask Orange (Invitrogen, #C10045), Prolong Gold (Invitrogen #P36930), eNOS antibodies (BD Biosciences, #610298), Alexa Fluor 488 Phalloidin (Invitrogen, #A12379), and Alexa Fluor 532 Phalloidin (Invitrogen, #A22282).

2.2. Cell culture

Experiments were performed in adherence with the guidelines outlined in the National Institutes of Health "Guide for the Care and Use of Laboratory Animals" (Revised 1985) prepared by the National Academy of Sciences as described previously (DeLeve et al., 2006). The experiments followed protocols approved by the Animal Care and Use Committee of the University of Southern California. Male Sprague Dawley rats (body wt. 250–280 g) were anesthetized with pentobarbital and then treated with 200 μL porcine intestine heparin (1000 U/ml). The liver was perfused for 10 min with calcium-free Geys buffered saline at 37 °C at 10 ml/min, followed by perfusion in a recirculating fashion for 20 min with GBS containing 0.05% type 1a collagenase (Sigma Chemicals, St. Louis, MO). The livers were mechanically dissociated, pressed through polypropylene mesh, and centrifuged, and the digest was resuspended in 50 ml GBS. The resulting digest was used to isolate LSECs by gradient centrifugation and centrifugal elutriation. Purity was 98% as determined by positive staining for fluorescent acetylated low-density lipoprotein and a peroxidase stain to reveal contaminating Kupffer cells. Viability was 95% and yield averaged >100 million cells. Cells were plated on #1.5 coverslips and cultured overnight in serum-free media. The following day the LSECs were fixed with 4% fresh paraformaldehyde in PBS. Following fixation the cells were prepared for visualization using SIM.

2.3. Fluorescence studies

To visualize the filamentous actin within LSECs the fixed cells were permeabilized with 0.05% saponin in blocking solution and then incubated with Alexa Fluor 488 Phalloidin (Invitrogen, #A12379) or Alexa Fluor 532 Phalloidin (Invitrogen, #A22282) for 10 min at room temperature. For cell membrane visualization, 0.5 ug/ml of Cell Mask Orange (Invitrogen, #C10045) was applied to the fixed cells for 10 min at room temperature. Cells were mounted with Prolong Gold (Invitrogen, #P36930) prior to examination. To visualize eNOS distribution, fixed cells were permeabilized with 0.05% saponin in blocking solution for 1 h prior to an overnight incubation (4 °C) with rabbit polyclonal anti-eNOS (BD Biosciences, #610298) (1:50). The following day cells then were treated with goat anti-rabbit antibody conjugated to Alexa Fluor 532 (Invitrogen, #A11009) or goat anti-rabbit antibody conjugated to Alexa Fluor 488 (Invitrogen, #A11034) for 45 min at room temperature. Cells were mounted with Prolong Gold (Invitrogen, #P36930) and examined with SIM.

2.4. Structured illumination microscopy

The structured illumination microscope is based on a prototype of the commercial model Deltavision|OMX V2.0 (Applied Precision Inc, Issaquah, WA). It delivers 488 and 532 nm lasers with variable power to the environmentally isolated microscope system using a multimode optical fiber and fiber shaker. The laser light passes through a movable phase grating where the grating's image plane is projected onto the sample using a collimation lens, a 100× 1.40 NA oil objective (UpanSApo, Olympus, Japan), and 1.514 index immersion oil. Fluorescence emission is collected by the same objective, split by channel, and filtered using a FF01-512/25-25 fluorescence filter (Semrock, Rochester, NY) for the 488 excitation channel or a FF01-593/40-25 (Semrock, Rochester, NY) fluorescence filter for the 532 excitation channel. Each channel was imaged by a dedicated Cascade II 512 EM-CCD camera (Photometrics, Tuscon AZ), which used 10–70 ms exposures, 1 pre-amp gain, and variable EM gain. Acquisition and all mechanics were controlled by the OMXN controller software (Applied Precision Inc, Issaquah, WA) while reconstructions were made with the OMX specific SoftWoRx v4.5.0 software package (Applied Precision Inc., Issaquah, WA). The reconstruction algorithms takes into account the specific optical transform functions for each channel, which are wavelength-dependent. Each channel required 5 exposures for a given angle (five lateral translations of the interference pattern by 72°), and three different angular grating positions (in steps of 60°) for a total of $3 \times 5 = 15$ exposures per optical slice. As each channel uses a different EM-CCD camera, there were slight differences in the observed grating angles between channels. To obtain 3D images, the sample is translated vertically in steps of 125 nm, resulting in 16 vertical slices for a 2 μm thick sample.

2.5. Image analysis

Image analysis was performed using Volocity (Perkin–Elmer) and ImageJ software (http://rsb.info.nih.gov/ij/).

2.6. Statistics

All data are presented as mean ± standard deviation and comparisons of two groups performed using a Students *t*-test.

3. Results

3.1. SIM of LSECs and sieve plates

Conventional deconvolution fluorescence microscopy of LSECs stained with the lipophilic fluorophore Cell Mask Orange showed that freshly isolated LSECs plated on glass substrates were intact and growing in a near confluent monolayer (Fig. 1A). Further examination of the cells by fluorescence microscopy showed many areas of interest indicating the potential presence of sieve plates and these areas were further examined at higher magnification by both conventional confocal fluorescence deconvolution