Investigation of Endothelial Surface Glycocalyx Components and Ultrastructure by Single Molecule Localization Microscopy: Stochastic Optical Reconstruction Microscopy (STORM)

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On the luminal surface of our blood vessels, there is a thin layer called endothelial surface glycocalyx (ESG†) which consists of proteoglycans, glycosaminoglycans (GAGs), and glycoproteins. The GAGs in the ESG are heparan sulfate (HS), hyaluronic acid (HA), chondroitin sulfate (CS), and sialic acid (SA). In order to play important roles in regulating vascular functions, such as being a mechanosensor and transducer for the endothelial cells (ECs) to sense the blood flow, a molecular sieve to maintain normal microvessel permeability and a barrier between the circulating cells and endothelial cells forming the vessel wall, the ESG should have an organized structure at the molecular level. Due to the limitations of conventional optical and electrical microscopy, the ultrastructure of ESG, in the order of 10 to 100 nanometers, has not been revealed until recent development of a super resolution fluorescence optical microscope, Stochastic Optical Reconstruction Microscope (STORM), which is one type of single molecule localization microscopy. This short review describes how the STORM can overcome the diffraction barrier in the conventional fluorescence microscopy to identify the chemical components of the ESG at a high spatial resolution. Examples of the organized ultrastructure of the ESG on the in vitro EC monolayer revealed by the Nikon-STORM system are given as well as how its components get lost during the onset of sepsis, a systemic inflammatory syndrome induced by bacterial infection, which demonstrate that this new technique can be applied to discover the structural and molecular mechanisms at nanometer scales in the native cellular environment for the cellular functions under normal and disease conditions.

INTRODUCTION

Microscopy plays a crucial role in the biomedical research as it has opened the door of the mini world to us. Due to the Abbe’s diffraction limit, i.e., the resolution of a given instrument is proportional to the wavelength of the light being observed, and inversely proportional to the size of its objective, the optical or light microscope has the limited spatial resolution, about several hundred nanometers [1]. Although the electron microscope...
(EM) and the scanning probe microscope (SPM) have much better spatial resolution, due to artifacts in sample preparations such as dehydration, fixation, and ultrathin cutting, they cannot provide the required spatial-chemical organization and structure information for the biomedical samples.

The luminal surfaces of endothelial cells (ECs) that line our vasculature are coated with a glycocalyx of membrane-bound macromolecules comprised of proteoglycans and glycosaminoglycans (GAGs) [2]. The GAGs in the endothelial surface glycocalyx (ESG) are heparan sulfate (HS), hyaluronic acid (HA), chondroitin sulfate (CS), and sialic acid (SA) [3]. Due to its unique location, at the interface of the circulating blood and the vessel wall, the ESG has been found to play important roles in maintaining normal vascular functions [4]. The roles of the ESG are determined by its composition, structure, and mechanical property, which have been widely studied since the 1960s by a variety of methods. However, due to the limitations of spatial resolutions or artifacts in sample preparations in the previous techniques, the spatio-chemical organization of the ESG remains unclear.

By winning the Nobel prize in Chemistry in 2014, the unique combination of molecule-specific contrast and live-cell imaging capability led fluorescence optical super-resolution microscopy to come back to the center stage of the microscopes, especially for the biomedical samples [5-11]. The recently developed Stochastic Optical Reconstruction Microscopy (STORM), one of three types of single molecule localization microscopy [7], employs organic dyes and fluorescent proteins as photo-switchable emitters to trade temporal resolution for a super spatial resolution (~20 nm lateral and ~50 nm axial at present), which is an order of magnitude higher than conventional confocal microscopy. STORM can perform 2-D [7], 3-D [12], and multicolor [13] imaging. STORM and other types of super-resolution optical microscopy enable the discovery and investigation of cellular structures at the nanometer scale, from individual proteins to entire organelles in the native cellular environment [14-16]. This review describes the recent application of STORM to visualize the ESG of cultured ECs under normal and disease conditions.

**BEATING THE DIFFRACTION LIMIT OF RESOLUTION BY STORM**

Before the super-resolution microscopy was invented, the optical microscope is limited by the diffraction, caused by the wave nature of the lights. This diffraction limit was first discovered by Ernst Abbe in 1873 [1], which indicates that any two spots (emitters) closer than \( \sim \frac{\lambda}{2NA} \) cannot be distinguished from each other. Here \( \lambda \) is the wavelength of the light and NA is the numerical aperture of the objective lens of a microscope. This limit led the development of microscopy to other directions, either decreasing the wavelength out of the range of visible lights, such as electron microscopy, or getting rid of the lights, such as scanning probe microscopy. Due to this diffraction limit of resolution, the development of the optical microscope has been slowed down for decades until recent development of the single-molecule fluorescence technology [17].

The diffraction of a single fluorescence molecule can be described as the point spread function (PSF). When the light of wavelength \( \lambda \) excites the fluorophore (emitter), the intensity profile of the spot is defined as the PSF with the width approximately as \( 0.6 \frac{\lambda}{NA} \), NA is the numerical aperture of the objective. As a result, two identical emitters separated by a distance less than the width of the PSF would appear as a single object, not distinguished from each other (top plot at the right panel in Figure 1A). The diffraction-limited image resolution, for a high numerical aperture objective lens, is \( \sim 250 \text{ nm} \) in the lateral direction and \( \sim 550 \text{ nm} \) in the axial direction, for a conventional fluorescence microscope.

The key idea of the single-molecule localization microscopy is to light the molecule in turn to achieve the nanometer-level accuracy of their position and reconstruction into a super-resolution image, such as Stochastic Optical Reconstruction Microscopy (STORM). Figure 1A demonstrates the working principle of the single-molecule localization microscopy (STORM) by using photo-switching mechanisms to stochastically activate individual molecules (photo-switchable or photoactivatable fluorophores) within the diffraction-limited region at different times. Then images with sub-diffraction limit resolution are reconstructed from the measured positions of individual fluorophores [7]. To localize a molecule, the emitter at that molecule should not have any overlap with its neighbors, so that it can be isolated and fitted into the PSF (middle and bottom plots in Figure 1A). One way to keep the fluorescence molecule emitting in turn is to make the photo-switchable fluorophore blink. To achieve the photo-blinking of the fluorophore is to switch between light and dark states, which are usually called “on” and “off.” “On” is the state in which the fluorophore is able to be excited and its emission can be detected by the camera. The “off” state is in which the fluorophore cannot be excited by the laser, including the temporarily blinking or permanently bleaching. The most important factor for STORM to accomplish sub-diffraction resolution is that the photo-switchable fluorophores are used to maintain neighboring molecules in different states, “on” or “off,” enabling to be distinguished from each other [18].

The number of emitters and the image collecting time are the determinants of the efficiency and accuracy of any microscopy. The higher population of the emitter would
have a higher image collection efficiency, however, the
distance between adjacent emitters must be greater than
their PSF to distinguish each other for conventional mi-
croscope. To trade the super spatial resolution (accuracy),
STORM sacrifices its temporal resolution (efficiency) by
switching the state and sequentially exciting the emitters
at high density. Rust et al. [7] employed organic dyes and
fluorescent proteins as photo-switchable emitters to trade
temporal resolution for a super spatial resolution (~20 nm
lateral and ~50 nm axial at present, can go down to a cou-
ple of nanometers if using smaller peptides or anti-body
fragments instead of currently used whole anti-bodies),
which is an order of magnitude higher than conventional
confocal microscopy. STORM can perform 2D [7], 3D
[12], and multicolor [13] imaging. In each frame of a
STORM movie, a set of emitters is randomly activated so
that locations of single emitters isolated within their PSFs
can be spatially resolved.

Since it was developed, STORM has been employed
in many biomedical researches. A number of subcellular
structures including microtubules, actin, clathrin-coated
pits, mitochondria, endoplasmic reticulum, and focal
adhesion complexes have been visualized by STORM
[11], as well as protein organization in bacteria [19] and
molecular architecture of nerve synapses [20]. Using
STORM with data processed by the affiliated algorithm,
the super resolution images of ESG components at the
surface of cultured EC monolayers were first revealed
with unprecedented spatio-chemical resolution [21,22]
and will be described in more details below.

**ENDOTHELIAL SURFACE GLYCOCALYX:
COMPOSITION, STRUCTURE, AND
FUNCTION**

Vascular ECs line the inner wall of every blood ves-
sel. In addition to forming a transport barrier between
the blood and vessel wall, vascular ECs play important roles
in regulating circulation functions. Besides biochemical
stimuli, blood flow induced (hemodynamic) mechanical
stimuli, modulate EC morphology and functions by ac-
tivating mechanosensors, signaling pathways, and gene
and protein expressions [23]. EC responses to the hemo-
dynamic forces (mechano-sensing and transduction) are
critical to maintaining normal vascular functions [24,25].
Failure in mechano-sensing and transduction contributes
to serious vascular diseases including hypertension, ath-
ersclerosis, aneurysms, stroke, thrombosis, and cancer,
to name a few [4,26-28].

**Function**

The functions of vascular ECs are determined by
their structural components. The luminal surface of the
ECs is coated with a glyocalyx of membrane-bound
macromolecules comprised of sulfated proteoglycans and
GAGs, glycoproteins and plasma proteins that adhere to
this surface matrix. Due to its unique location, at the in-

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**Figure 1. Schematics of the working principle and the setup of the stochastic optical reconstruction microscopy (STORM).** (A) The top row shows the two fluorophores (located along the two dashed lines) activated and excited simultaneously by conventional microscopy. Due to the diffraction limit, the two fluorophores cannot be separated, resulting in a blurring image. The middle and bottom rows show the principle of STORM for the localization of a single molecule to a nanometer accuracy. The fluorophores are activated and excited not simultaneously, but sequentially to be localized and separated with each other. This technique can overcome the diffraction barrier for the conventional fluorescence microscopy. (B) A representative optical set up of STORM for the fluorophore Auto 488. (a) The optical path in activation process. The 405nm wavelength laser activates the photo-switchable fluorophore Auto 488, enabling the excitation. (b) The optical path in excitation and imaging processes. The 488nm laser excites the activated “on” fluorophore and its emission is recorded by a camera.
It is widely believed that the negatively charged GAGs in the ESG capture circulating plasma proteins and form an interconnected gel-like structure in an aqueous environment [32-34], and that the ESG would collapse if a GAG component was significantly reduced. However, a recent study by Zeng et al. [30] observed that specific enzymatic removal of HS or HA did not result in cleavage or collapse of any of the remaining components. Simultaneous removal of CS and HA by chondroitinase did not affect HS. Their results suggest that all GAGs and adsorbed proteins are well inter-mixed within the structure of the ESG, but the GAG components do not interact with one another.

**Thickness and Structure**

In addition to its biochemical composition, the thickness and ultrastructure of the ESG determine its functions. The first visualization of the ESG by EM used the cationic dye ruthenium red that binds to acidic mucopolysaccharides and generates electron density in the presence of osmium tetroxide [35]. Subsequent studies [36,37] used gold colloids and immunoperoxidase labeling. Adamson and Clough [38] then demonstrated using a large charged marker protein (unable to penetrate the ESG), cationized ferritin (molecular weight ~450 kDa), that in the absence of plasma proteins, the ESG would collapse, presumably due to elimination of intramolecular interactions with plasma proteins, and that its undisturbed thickness was several times greater than the 20 nm observed with ruthenium red [35]. All of these methods may suffer from dehydration artifacts associated with aqueous fixatives that likely dissolve all but the protein cores of proteoglycans.
This estimate of the in vivo thickness of the ESG is four to five times greater than previous estimates derived from EM studies. This discrepancy was a catalyst for much of the work that has followed on the estimation of ESG thickness and its function as a barrier in cellular interactions as well as a mechno-sensor and transducer of ECs.

The poor spatial resolution of an intravital optical microscope limits the accurate measurement of the ESG thickness [44]. New imaging methods have thus been developed by employing laser scanning confocal microscopy and multi-photon microscopy, and fluorescently tagged antibodies to HS or HA binding protein, or wheat germ agglutinin (WGA) to label major components of the ESG. Application of these new methods has revealed a much thicker ESG in large blood vessels: 4.3-4.5 µm in the mouse common carotid artery [45], 2.2 µm in the mouse internal carotid artery [46], and 2.5 µm in the external carotid artery [47]. Ebong et al. [48] presented the first cryo-EM images of in vitro ESG that avoided the dehydration artifacts of conventional EM and observed structures greater than 5 µm in thickness (up to ~11 µm). Most recently, using high sensitivity and resolution confocal microscopy and in situ/in vivo single microvessel and ex vivo aorta immunostaining, Yen et al. [49] revealed that the thickness of the ESG on rat mesenteric and mouse cremaster capillaries and post-capillary venules is 1-1.5 µm (Figure 3C, D, E). The ESG thickness is 2-2.5 µm on rat and mouse aorta. They also observed that the
ESG is continuously and evenly distributed on the aorta wall but not on the microvessel wall if looking at a vessel segment of length ~100 µm. By comparing the distance between the plasma membrane labeling and the labeling of ESG (SA residues) by WGA in a single microvessel \textit{in vivo}, Betteridge \textit{et al.} [50] found that the ESG thickness is 0.17-3.02 µm in the same type of microvessels as in Yen \textit{et al.} [49], depending on the labeling and analyzing methods. However, the thickness of ESG at the same portion of the vessel is only ~0.08 µm observed by the EM through Alcian Blue labeling.

The ultrastructural organization of the ESG and its relation to the cytoskeleton components (e.g., F-actin) of ECs was first investigated by Squire \textit{et al.} [51]. Using computed autocorrelation functions and Fourier transforms of EM images of frog mesenteric microvessels, they estimated a quasi-periodic substructure in the ESG, which is a 3D fibrous meshwork with characteristic

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**Figure 4. ESG components and ultrastructure visualized by STORM and by confocal microscopy** (A) STORM images of the anti-heparan sulfate (HS) (green, middle panel) and anti-hyaluronic acid (HA) (red, right panel) of the ESG focusing at the bottom of a bEnd3 cell. The left panel is the overlay [21]. The color bars in the middle and right panels at the bottom row (3D side views) are the scale bars in nanometer. (B) Confocal images of the same anti-HS labeled ESG of the bEnd3 cells (mouse brain microvascular endothelial cell) [55]. Yellow line indicates the region for the STORM images.
ial at present due to the size of antibodies used to identify the ESG components), which is an order of magnitude greater than conventional confocal microscopy [7].

**Spatio-Chemical Organization of Intact ESG**

The spatio-chemical organization of the intact ESG at EC (bEnd3, mouse brain microvascular endothelial cell) monolayers was first observed by using Nikon-STORM [21]. After confluent, the bEnd3 cells were immunolabeled with anti-HS, followed by an ATTO488 conjugated goat anti-mouse IgG, and with biotinylated HA binding protein, followed by an AF647 conjugated anti-biotin. The ESG was then imaged by the STORM with a 100×/1.49 oil immersed lens. Multiple Reporters of ATTO488 and AF647 with alternating illumination were used to acquire the 3D images of HS and HA. The field of 256 × 256 (40 × 40 µm$^2$) of HS and HA at the surface of ECs was obtained based on total 40,000 of EM-CCD captured images for each reporter at a capturing speed of 19 ms/frame. Figure 4A demonstrates the STORM images for the HA (top view, red, top row on the right) and HS (green, top row in the middle) and overlay (top row on the left) of the ESG focused at the bottom surface of the bEnd3 cells (yellow line indicated region in Figure 4B). Figure 4B is a confocal microscopic view of the same ESG of bEnd3 cells labeled with anti-HS [55]. The EC plasma membrane is located at the bottom plane of the 3D images (bottom row). The color bars in the 3D side views are the scale bars in nanometer. The

**COMPONENTS AND ULTRASTRUCTURE OF THE ESG REVEALED BY STORM**

The recent development of ultra-resolution STORM has made it possible to visualize the spatio-chemical organization of the intact ESG at high resolution [22,54]. STORM employs organic dyes and fluorescent proteins as photo-switchable emitters to trade temporal resolution for a super spatial resolution (20 nm lateral and 50 nm ax-
figure shows that HA is a long molecule weaving into a network, which is horizontal to the EC cell surface. In contrast, HS is a shorter molecule, which is perpendicular to the cell surface. The height of the HS is ~800 nm. HA and HS seem to overlap with each other at the EC surface. The revealed ultrastructure of ESG by STORM suggests that HS plays a major role in mechanosensing and HA plays a major role in forming the molecular sieve.

**Damage of ESG Observed by STORM at the Onset of Sepsis**

Damage to and modification of the ESG have been observed in many diseases including diabetes, ischemia, myocardial edema, chronic infectious diseases, atherosclerosis, and tumor metastasis [27,28,40,56-62,63]. Sepsis is a systemic inflammatory syndrome induced by bacterial infection that can lead to multiorgan failure. Recently, Zullo et al. [22] employed the STORM to investigate the molecular mechanism by which lipo-polysaccharide (LPS, an endotoxin secreted by bacteria during sepsis) induces a patchy loss of ESG at the onset of sepsis. They found that the loss of ESG is the result of exocytosis of endothelial lysosome-related organelles including Weibel-Palade bodies (WPB) and secretory lysosomes. Figure 5 demonstrates that under control (Figure 5A, top row), ESG was richly presented by fluorescently labeled anti-HS antibodies. However, after 10 min LPS treatment, von Willebrand factor (vWF)-labeled WPB became externalized and was surrounded by the halo areas of lost HS-decorated ESG (Figure 5A, bottom row). Only the super-resolution STORM could make it possible to observe such a nano-scaled picture for the ESG and exocytotic vWF/ WPBs. Figure 5B shows the relative intensity of anti-HS and vWF/ WPBs after LPS treatment compared with the control.

**CONCLUSION**

Although the ESG has been studied for over a half-century since the 1960s for its composition, structure and function, its detailed components and ultrastructure in its native cellular environment have just started to be investigated owing to the recent development of super-resolution optical microscopy such as STORM. Due to its heterogeneous structures, complex composition of various proteoglycans, GAGs, and absorbed plasma proteins, attachment to the transmembrane glycoproteins and receptors, as well as EC cytoskeleton and plasma membrane, its functions as a mechanosensor, a barrier to the interaction between circulating cells to ECs lining the vascular wall and a molecular sieve to water and solutes are expected to be governed by multiple mechanisms. By employing STORM, we can first elucidate its various components and their organization under physiological conditions and explore the molecular and cellular mechanisms by which these structures and organizations are modulated under pathological conditions.

Since the spatial resolution of the STORM is determined not only by the optical parts of the microscope, but largely by the suitable photo-switchable probes, *i.e.*, the half-life of the fluorophore as well as the size of the specific biomarker such as antibodies used in the current studies. In the future studies for the ESG, we will further improve the resolution by finding longer half-life fluorophores and smaller biomarkers such as small peptides and fragments of antibodies. The STORM resolution is also limited by how fast a computer can acquire and analyze the images. In addition to updating high performance computers, we will create more efficient computational algorithms. The resolution can also be improved by post-image processing techniques.

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