High-resolution helium ion microscopy of epididymal epithelial cells and their interaction with spermatozoa

Teodor G. Păunescu, Winnie W.C. Shum, Chuong Huynh, Lorenz Lechner, Bernhard Goetze, Dennis Brown, and Sylvie Breton

**ABSTRACT:** We examined the rat and mouse epididymis using helium ion microscopy (HIM), a novel imaging technology that uses a scanning beam of He$^+$ ions to produce nanometer resolution images of uncoated biological samples. Various tissue fixation, sectioning and dehydration methods were evaluated for their ability to preserve tissue architecture. The cauda epididymidis was luminally perfused in vivo to remove most spermatozoa and the apical surface of the epithelial lining was exposed. Fixed epididymis samples were then subjected to critical point drying (CPD) and HIM. Apical stereocilia in principal cells and smaller apical membrane extensions in clear cells were clearly distinguishable in both rat and mouse epididymis using this technology. After perfusion with an activating solution containing CPT-cAMP, a permeant analog of cAMP, clear cells exhibited an increase in the number and size of membrane ruffles or microplicae. In contrast, principal cells did not exhibit detectable structural modifications.

**Key words:** epididymis / spermatozoa / helium ion microscopy / principal and clear cells / epididymosomes

**Introduction**

The epididymis is a long and highly convoluted tubule connecting the efferent ducts to the vas deferens (Jones, 1998). It is divided into four segments with distinct anatomical properties and gene expression patterns: the initial segment (IS), the caput (head), the corpus and the cauda epididymis (for review see Cooper, 1998; Robaire et al., 2006; Cornwall, 2009; Shum et al., 2009; Belleannee et al., 2012). Four different cell types are present in the epididymal epithelium: principal, clear (in all regions except the IS), narrow (restricted to the IS) and basal cells (Shum et al., 2009).

Functionally, clear cells are responsible for the acidification of the epididymal lumen via vacuolar proton-pumping ATPase (V-ATPase)-mediated H$^+$ secretion (Brown et al., 1992; Breton et al., 1996), whereas principal cells are mainly implicated in protein secretion, and ion and water transepithelial transport (Da Silva et al., 2006; Belleannee et al., 2012). Apocrine secretion of proteins in this tissue involves membranous microvesicles named epididymosomes (Sullivan and Saez, 2013), which were first reported almost three decades ago in the Chinese hamster (Yanagimachi et al., 1985). Generally, microvesicles include exosomes that are secreted following fusion of multivesicular bodies with the plasma membrane, and larger vesicles that are shed directly from the cell plasma membrane (Cocucci et al., 2009). They are found in various biological fluids, including blood (Caby et al., 2005), urine (Psitkun et al., 2004; Keller et al., 2007; Miranda et al., 2010), saliva (Palanisamy et al., 2010), amniotic fluid (Keller et al., 2007) and cerebrospinal fluid (Harrington et al., 2009). Epididymosomes are vesicles measuring 20–500 nm in diameter (Krapf et al., 2012; Belleannee et al., 2013; Sullivan and Saez, 2013) that can be isolated from the epididymal fluid (Frenette et al., 2005; Rejraji et al., 2006). They were shown to be the mediators of the protein transfer between the epididymal epithelium and the sperm cells, which underlies the post-testicular maturation of spermatozoa (Saez et al., 2003; Sullivan et al., 2007; Thimon et al., 2008; Oh et al., 2009; Frenette et al., 2010; Sullivan and Saez, 2013).
Imaging the epididymis in various mammalian species has a long history, starting with light and electron microscopy (EM) studies (Horstmann, 1962; Holstein, 1964; Leeson and Leeson, 1964; Nicander, 1964; Schmidt, 1964; Fahrmann and Schuchardt, 1966; Horstmann et al., 1966; Murakami et al., 1976; Hamilton et al., 1977). Pioneering studies using EM to investigate the ultrastructure of sperm cells are even older (Reed and Reed, 1947, 1948; Sanders, 1948; Anberg, 1957). To this day, bright field immunocytochemistry and immunofluorescence microscopy (Da Silva et al., 2006; Robaire et al., 2006; Cyr et al., 2007; Cornwall, 2009; Joseph et al., 2011; Shum et al., 2011b; Belleannee et al., 2012), as well as scanning and transmission EM (Zhou et al., 1987; Forne and De Rosas, 1991; Rajalakshmi et al., 1993; Stoffel and Friess, 1994; Villalpando et al., 2000; Hermo and Jacks, 2002; Koga and Ushiki, 2006; Takahashi et al., 2006; Lorenzana et al., 2007; Parent et al., 2011; Lin et al., 2013) remain the methods of choice for investigation of the epididymal epithelium and spermatozoa.

In the present study we used for the first time helium ion microscopy (HIM) to investigate the structure and morphology of epididymal epithelial cells and sperm. HIM is a novel technology that can yield high-resolution (nanometer or potentially even sub-nanometer) images by scanning the samples with a beam of He\(^+\) ions. The technical characteristics of the He\(^+\) beam allow it to focus to \(<\)0.75 nm and possibly down to \(~\)0.25 nm (Ward et al., 2007; Bell, 2009). For a review of the advantages of the HIM imaging technology compared with established scanning or transmission EM see (Bell, 2009). Traditionally used in materials science, HIM has been applied successfully in recent years to the study of various types of biological samples, including cancer cells (Bazou et al., 2011a), platelets (Bazou et al., 2011b) and kidney glomeruli and tubule cells (Rice et al., 2013).

We demonstrate here that when applied to the study of rodent epididymis and spermatozoa, HIM allows for the high-resolution imaging of stereocilia (the long microvilli of principal cells in all regions of the epididymis) in principal cells and reveals the presence of membrane ruffles or microplicae in clear cells that increase in number and size under activated conditions, as well as the ultrastructure of sperm cells, including their physical interaction with the epithelium. HIM also reveals that the surface of the sperm cytoplasmic droplet is covered with vesicle-like structures whose size and shape is consistent with that of epididymosomes.

**Methods**

**Ethics approval**

Animal experiments were approved by the Massachusetts General Hospital Subcommittee on Research Animal Care, in accordance with the National Institutes of Health, Department of Agriculture, and AAALAC requirements.

---

**Figure 1** Vibratome sections of the cauda epididymidis of B1-EGFP mouse, expressing EGFP specifically in clear cells, visualized by light (A) and epifluorescence (B) microscopy. The cauda tubule was cut longitudinally to expose the apical surface of the epithelium. Several EGFP-positive clear cells are seen. (C–E) Higher magnification of the area shown in the box (A and B). Scale bars: A, B: 100 \(\mu\)m; C, D, E: 50 \(\mu\)m.
Tissue harvesting
Adult (>10 week old) male Sprague–Dawley rats (Charles River Laboratories International, Wilmington, MA, USA), adult (>12 week old) male C57BL6 mice (Jackson Laboratory, Bar Harbor, ME, USA) and B1-EGFP transgenic mice expressing enhanced green fluorescent protein (EGFP) in clear and narrow cells (Miller et al., 2005) were housed under standard conditions, had free access to water and were maintained on a standard rodent diet. The animals were anesthetized with pentobarbital sodium (60 mg/kg body wt i.p., Nembutal, Abbott Laboratories, Abbott Park, IL, USA) and the cauda epididymis was luminally perfused under a dissecting microscope with a small catheter connected to a syringe infusion pump (model 100, KD Scientific, Holliston, MA, USA) at a rate of 0.25 ml/h as previously described (Shum et al., 2008, 2011a; Belleannee et al., 2010). The lumen of the mouse epididymis was flushed free of spermatozoa for 5 min using a physiological solution based on previous reports (Levine and Marsh, 1971; Jenkins et al., 1980; Clulow et al., 1994), containing (in mM) 50 NaCl, 50 K gluconate, 1.2 MgSO4, 0.6 CaCl2, 4 Na acetate, 1 Na2 citrate, 6.4 NaH2PO4, 3.6 Na2HPO4 (pH 6.6). The osmolality was adjusted to 360 mmol/kg with raffinose (Shum et al., 2011a). All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Experimental mice were subsequently perfused for 20 min with an alkaline solution (pH 7.8) containing 1 mM 8-(4-chlorophenylthio)adenosine 3′,5′-cyclic monophosphate (CPT-cAMP, Sigma-Aldrich), 1.0 mM NaH2PO4 and 9.6 mM Na2HPO4. The rat epididymal lumen was perfused for 15 min with a similar buffered perfusion solution, except for Na2HPO4 (8.8 mM) and Na2HPO4 (1.29 mM) (pH 6.0, 330 mmol/kg). Control rats were subsequently perfused with the same solution for another 30 min, whereas experimental rats were perfused for the same duration with an activating solution containing 1 mM CPT-cAMP and 15 mM NaHCO3. The pH was adjusted to 7.2 by modifying the concentration of NaH2PO4 (2.67 mM) and Na2HPO4 (7.33 mM). Osmolality was adjusted to 330 mmol/kg with raffinose to preserve the morphology of the epithelium.

For HIM imaging, all control and CPT-cAMP-treated tissues were fixed by luminal perfusion with 4% glutaraldehyde (GA) in 0.1 M sodium cacodylate buffer, and subsequently post-fixed in the same fixative overnight at 4°C. The following day, the tissues were washed extensively in phosphate-buffered saline (PBS, 0.9% NaCl in 10 mM phosphate buffer, pH 7.4), cut with a razor blade to expose the apical surface of the epididymal epithelium and stored at 4°C in PBS containing 0.02% NaN3 until further processing.

For conventional fluorescence microscopy, some tissues were fixed in paraformaldehyde-lysine-periodate (PLP) fixative containing 4% paraformaldehyde, 10 mM sodium periodate, 75 mM lysine and 5% sucrose in 0.1 M sodium phosphate buffer, as described previously (Shum et al., 2008). After three to five washes in PBS, the cauda epididymis was cut using a TPI PELCO 101 series 1000 vibratome (Technical Products International, Inc., St. Louis, MO, USA). Some tissues were immunofluorescently labeled for the V-ATPase, using an affinity purified chicken antibody against the E subunit of the V-ATPase as we described previously (Breton et al., 2006). We previously reported that dehydration in a series of graded methanol solutions allows for good quality post-CDP tissue morphology (Rice et al., 2013). Epididymal tissues were subjected to a similar dehydration schedule: 25% (v/v) MeOH in PBS for 60 min, 40% (v/v) MeOH in PBS for 45 min and 60% (v/v) MeOH in double-distilled water (ddH2O) for 45 min, all at room temperature. The MeOH solution was refreshed halfway through each incubation step. The tissues were then incubated in 80% (v/v) MeOH in ddH2O for 45 min at room temperature, followed by another 80% (v/v) MeOH overnight at 4°C. The following day, the tissues were placed in 100% MeOH for at least 1 h, and the pure MeOH was replaced once again before performing CPD.

Epididymal tissues were placed in metal baskets and subjected to CPD in a Samdrin-795 CPD apparatus (Tousimis Research Corporation, Rockville, MD, USA) as previously described (Rice et al., 2013). During this process, the tissues were maintained at supercritical parameter values (1200 psi, 42°C) for at least 4 min, and subsequently the pressure was reduced slowly (<100 psi/min).

The protocols described here were selected after evaluating various fixatives, including GA and modified PLP (Paunesku et al., 2004; Rice et al., 2013), for optimal labeling using 2 or 4% v/v GA, fixation methods, i.e. luminal perfusion of the epididymis versus whole animal transcardial perfusion (Paunesku et al., 2004), post-fixation and dehydration protocols, for their ability to preserve tissue architecture (Rice et al., 2013).

Helium ion microscopy
HIM was performed as previously reported (Rice et al., 2013) using an Orion helium ion microscope (Carl Zeiss Microscopy, Peabody, MA, USA) at a 35 keV beam energy, with a probe current of 0.1–1.5 pA and with no conductive coating of the samples. HIM images of uncoated samples, acquired by collecting the secondary electrons produced by the He+ beam/sample interaction, feature unmasked and unobscured surface morphological details (reviewed in Bell, 2009). A low-energy electron flood gun was used after each individual line pass of the imaging beam in order to achieve charge neutralization.

Digital images were not post-processed, except for brightness and contrast adjustment (Vanden Berg-Foels et al., 2011; Rice et al., 2013). We previously reported that dehydration in a series of graded methanol solutions allows for good quality post-CDP tissue morphology (Rice et al., 2013). Epididymal tissues were subjected to a similar dehydration schedule: 25% (v/v) MeOH in PBS for 60 min, 40% (v/v) MeOH in PBS for 45 min and 60% (v/v) MeOH in double-distilled water (ddH2O) for 45 min, all at room temperature. The MeOH solution was refreshed halfway through each incubation step. The tissues were then incubated in 80% (v/v) MeOH in ddH2O for 45 min at room temperature, followed by another 80% (v/v) MeOH overnight at 4°C. The following day, the tissues were placed in 100% MeOH for at least 1 h, and the pure MeOH was replaced once again before performing CPD.

Epididymal tissues were placed in metal baskets and subjected to CPD in a Samdrin-795 CPD apparatus (Tousimis Research Corporation, Rockville, MD, USA) as previously described (Rice et al., 2013). During this process, the tissues were maintained at supercritical parameter values (1200 psi, 42°C) for at least 4 min, and subsequently the pressure was reduced slowly (<100 psi/min).

The protocols described here were selected after evaluating various fixatives, including GA and modified PLP (Paunesku et al., 2004; Rice et al., 2013), for optimal labeling using 2 or 4% v/v GA, fixation methods, i.e. luminal perfusion of the epididymis versus whole animal transcardial perfusion (Paunesku et al., 2004), post-fixation and dehydration protocols, for their ability to preserve tissue architecture (Rice et al., 2013).

Tissue processing for HIM
To preserve tissue morphology, critical point drying (CPD) requires a pre-treatment with a suitable solvent (such as methanol, acetone or ethanol) to replace tissue water (Bazou et al., 2011a). We previously reported that dehydration in a series of graded methanol solutions allows for good quality post-CPD tissue morphology (Rice et al., 2013). Epididymal tissues were subjected to a similar dehydration schedule: 25% (v/v) MeOH in PBS for 60 min, 40% (v/v) MeOH in PBS for 45 min and 60% (v/v) MeOH in double-distilled water (ddH2O) for 45 min, all at room temperature. The MeOH solution was refreshed halfway through each incubation step. The tissues were then incubated in 80% (v/v) MeOH in ddH2O for 45 min at room temperature, followed by another 80% (v/v) MeOH overnight at 4°C. The following day, the tissues were placed in 100% MeOH for at least 1 h, and the pure MeOH was replaced once again before performing CPD.

Epididymal tissues were placed in metal baskets and subjected to CPD in a Samdrin-795 CPD apparatus (Tousimis Research Corporation, Rockville, MD, USA) as previously described (Rice et al., 2013). During this process, the tissues were maintained at supercritical parameter values (1200 psi, 42°C) for at least 4 min, and subsequently the pressure was reduced slowly (<100 psi/min).

The protocols described here were selected after evaluating various fixatives, including GA and modified PLP (Paunesku et al., 2004; Rice et al., 2013), for optimal labeling using 2 or 4% v/v GA, fixation methods, i.e. luminal perfusion of the epididymis versus whole animal transcardial perfusion (Paunesku et al., 2004), post-fixation and dehydration protocols, for their ability to preserve tissue architecture (Rice et al., 2013).

Helium ion microscopy
HIM was performed as previously reported (Rice et al., 2013) using an Orion helium ion microscope (Carl Zeiss Microscopy, Peabody, MA, USA) at a 35 keV beam energy, with a probe current of 0.1–1.5 pA and with no conductive coating of the samples. HIM images of uncoated samples, acquired by collecting the secondary electrons produced by the He+ beam/sample interaction, feature unmasked and unobscured surface morphological details (reviewed in Bell, 2009). A low-energy electron flood gun was used after each individual line pass of the imaging beam in order to achieve charge neutralization.

Digital images were not post-processed, except for brightness and contrast adjustment (Vanden Berg-Foels et al., 2011; Rice et al., 2013) performed
Results

We first examined the apical surface of the epididymal epithelium using conventional microscopy. Many EGFP-expressing clear cells that are dispersed among non-fluorescent principal cells can be seen in vibratome sections of the cauda epididymidis of B1-EGFP mice (Fig. 1).

Figure 2 shows an HIM image of a control rat cauda epididymidis. The high resolution, sharpness and depth of field of HIM images are remarkable even when taken at low magnification. In most orientations, the field of view reveals epididymal clear cells as darker spots of irregular shape and variable size ranging from 5 to 30 μm (arrows), interspersed among principal cells that appear brighter, due to their numerous stereocilia. The inset in Fig. 2 shows an enface view of the epithelium lining the rat cauda epididymidis, immunostained for the vacuolar proton-pumping ATPase, V-ATPase (red), which is expressed in the apical membrane of clear cells.

Higher magnification images (Fig. 3) further emphasize the distinction between clear cells and the surrounding principal cells. Under control conditions (Fig. 3A and C), the clear cell apical membrane features microvilli and a few, relatively short microplacae, in contrast to the longer stereocilia of the principal cells. The majority of the clear cell apical membrane is, however, clearly visible and allows for the visualization of indentations. Several factors, including cAMP, alkaline pH and bicarbonate are known to induce an increase in the number and length of apical membrane protrusions. High-magnification HIM imaging of activated clear cells from rat epididymis perfused with a solution containing 1 mM CPT-cAMP and 15 mM bicarbonate shows that these protrusions appear to be microspacae or small membrane ruffles (Fig. 3B and D). The density and size of these microspacae is increased in activated cells to such an extent that the flatter areas of the plasma membrane between the microspacae are virtually no longer visible (Fig. 3D). Similar apical microspacae were detected following cAMP treatment in...
clear cells of the mouse cauda epididymidis (Fig. 4). Unlike in the rat epididymis, where most clear cells are surrounded exclusively by principal cells, mouse clear cells can be seen clustered together. Interestingly, several membrane protrusions with morphological characteristics of membrane budding during apocrine secretion were detected in clear cells (arrows).

Despite extensive luminal perfusion of the cauda epididymidis before the fixation step, we made the surprising observation that not all sperm cells had been flushed from the lumen. Most of these remaining spermatozoa had their heads anchored within the layer of principal cell stereocilia (Fig. 5; arrows), indicating a close physical interaction between spermatozoa and the epithelial cells. The morphology of certain sperm cells suggests a distinct kind of interaction with the epididymal epithelium. Figure 6 shows a mouse spermatozoon whose head is embedded within the layer of principal cell stereocilia (panel A), as also illustrated in Fig. 5. Increasing the magnification reveals concentric rings on the sperm mid-piece (Fig. 6B; arrows), as well as small, vesicle-like structures on the surface of the cytosolic droplet located at the mid-principal piece junction (Fig. 6B). The latter can be seen more clearly at an even higher magnification (Fig. 6C and D), where numerous surface depressions also become visible. The vesicle-like structures are round and measure \( \sim 90 \) nm in diameter. A number of these structures feature a rough surface morphology (arrowheads in C and D), although smooth membranous vesicles can also be detected (arrows and double arrow in C and D). These structures might represent epididymosomes in the process of fusing with the spermatozoon membrane and/or vesicles in the process of budding off the cytoplasmic droplet.

**Discussion**

We describe in this report the first application of HIM technology to the study of the structure and morphology of the rodent epididymis. HIM allows high-resolution imaging of epididymal epithelial cells and spermatozoa. The distinction between epididymal clear and principal cells is obvious in HIM images, based on the brightness of the signal. The dense apical stereocilia of principal cells result in a brighter appearance, as we reported previously for the brush border of the renal proximal tubule, in which the apical microvilli are even more densely packed (Rice et al., 2013). The architecture of the epididymal epithelium, as determined by HIM, confirms previous results obtained using scanning EM (Murakami et al., 1976; Hamilton et al., 1977; Zhou et al., 1987; Fornes and De Rosas, 1991; Susheela and Kumar, 1991; Stoffel and Friess, 1994; Aire and Soley, 2000; Lorenzana et al., 2007), but the images in the current study demonstrate the significant improvement in resolution and overall clarity offered by this technology over previous imaging methods.

We have previously shown that basal cells send a narrow body projection that can extend all the way to the luminal side of the epithelium of rat and mouse epididymis (Shum et al., 2008, 2013, 2014). These luminal-reaching projections are present only in the IS of the mouse epididymis, and in the distal corpus and very proximal cauda epididymidis in the rat. We visualized here a more distal region (mostly the distal cauda), where these projections are very rare, and we therefore attributed the different appearance of the epithelial apical surface to two cell types: principal cells and clear cells. HIM imaging confirmed that clear cells possess a surface...
morphology that is quite distinct from principal cells. Under resting conditions, the majority of the clear cell membrane is visible and shows the presence of several indentations, reminiscent of renal collecting duct cell membrane features, which are thought to represent various configurations of endo- and/or exocytotic events (Rice et al., 2013). We have previously shown that several factors, including a permeant analog of cAMP (CPT-cAMP) and luminal bicarbonate, stimulate proton secretion in clear cells, via elongation of membrane protrusions that contain a high density of the proton pumping V-ATPase (Pastor-Soler et al., 2003; Beau lieu et al., 2005; Shum et al., 2009). Based on their appearance on 5 μm cryostat sections, we initially labeled these protrusions as ‘microvilli’ (Brown et al., 1992; Breton et al., 1996). HIM imaging now shows that these protrusions are in fact microvilli or small membrane ruffles, in agreement with an earlier study by the Cooper group, who used scanning EM to show the presence of ‘leaf-like’ microvilli on the surface of clear cells (Hamilton et al., 1977). A similar response to cAMP treatment has been detected when using HIM to image intercalated cells of the renal collecting duct (our unpublished observations), which extended the characterization of the previously reported effect of cAMP on the length of apical microvilli in this cell type (Paunescu et al., 2010). In contrast to the dramatic effect of CPT-cAMP and bicarbonate on clear cells, we did not detect morphological changes in epididymal principal cells after perfusion under these conditions in either rat or mouse epididymis.

In the mouse epididymis, in contrast to the rat epididymis, clusters of adjacent clear cells were detected. In previous studies, these clusters appeared as rows of clear cells in 5 μm cryostat sections (Miller et al., 2005). In mouse clear cells, in addition to microvilli we also detected the presence of large membrane protrusions. These might reflect budding of vesicles from the apical plasma membrane. While the process of vesicle shedding via plasma membrane budding was originally thought to represent an artifact caused by poor fixation, these vesicles are now considered to play significant roles in the communication between neighboring cells (Cocucci et al., 2009). Epididymosomes are important contributors to sperm maturation in the epididymis, allowing the transfer of new proteins from epithelial cells to the sperm cells (Belleeanee et al., 2013; Sullivan and Saez, 2013). They are composed of different types of extracellular vesicles, originating either from the fusion of multivesicular bodies with the apical membrane followed by

Figure 6 (A) A low-magnification HIM image showing a mouse cauda spermatozoon, whose head is embedded within the epididymal epithelium. This spermatozoon has a prominent cytoplasmic droplet located at the mid-principal piece junction (arrow). (B) An intermediate magnification image of the same sperm cell reveals concentric rings on its midpiece (arrows) and vesicle-like structures on the surface of the cytoplasmic droplet. (C and D) High-magnification HIM imaging of the cytoplasmic droplet shows these vesicle-like membranous structures more clearly, alongside a number of surface depressions. Some vesicles have a rough appearance (arrowheads), while other have a smooth surface (arrows). A more complex structure (double arrow) compatible with a membrane protrusion that is budding from the cytoplasmic droplet is also visible. Scale bars: A: 5 μm; B: 1 μm; C: 200 nm; D: 100 nm.
the release of exosomes, or from the budding of larger vesicles from the plasma membrane. While principal cells are currently thought to be the main contributors of epididymosomes to the luminal fluid, our data suggest that clear cells might also participate in the formation of at least one type of microvesicle—via budding of their plasma membrane. Interestingly, we have recently shown that cSrc, which is abundantly expressed in clear cells, is incorporated into spermatozoa during their transit through the mouse cauda epididymidis (Kraft et al., 2012). cSrc might, therefore, be delivered to spermatozoa via vesicles that budded off the plasma membrane of clear cells. The presence of a very dense array of stereocilia in principal cells precluded the detection of any exocytoctic or fusion event that would take place at the level of the plasma membrane.

The ultrastructure of sperm cells and their interaction with the epithelium were also investigated using HIM technology. High-resolution images confirmed structural features of spermatozoa, such as the presence of concentric rings on the sperm midpiece, which have been previously described using scanning EM (Villalpando et al., 2000). In addition, in agreement with previous studies (Cooper, 2011; Xu et al., 2013), some spermatozoa had a cytoplasmic droplet located at the midprincipal piece junction (Fig. 6), while others did not have a discernable cytoplasmic droplet (Fig. 5). We describe here two aspects of the interaction between the spermatozoa and the epididymal epithelium. The concept of a direct physical interaction is supported by the presence of isolated sperm cells that remained attached to the epididymal tubule even after a lengthy period of luminal perfusion. These spermatozoa had their heads firmly anchored within the epithelium, indicating a very close interaction with epithelial cells. In a scanning EM study, a close interaction between spermatozoa and epididymal epithelium has also been previously reported in the rat epididymis (Fornes and De Rosas, 1991). Whether this interaction is indicative of the growing (albeit controversial) notion that active selection, removal and degradation of spermatozoa occur during epididymal transit (Sutovsky, 2003; Axner, 2006; Jrad-Lamine et al., 2011) will require further investigation. Interestingly, a different, indirect type of interaction is suggested by the fact that the surface of the sperm cytoplasmic droplet is covered with vesicle-like structures. The roughly spherical shape and the size (∼90 nm in diameter) of some of these structures suggest that they are epididymosomes imaged while fusing with the spermatozoa membrane, to deliver proteins originating from the epithelium during epididymal maturation (Saez et al., 2003; Sullivan et al., 2007; Thimon et al., 2008; Oh et al., 2009; Frenette et al., 2010; Sullivan and Saez, 2013). In addition, we also detected some structures that appeared to be in the process of budding from the cytoplasmic droplet.

In conclusion, our results confirm that the HIM technology advances the imaging of biological samples significantly and can be applied successfully to study the male reproductive tract and spermatozoa, thus enhancing our understanding of their structure, function and intercellular communication.

Acknowledgements

We are grateful to Dr Paul Kelly of Salem State University, Salem, MA, USA and to Ms. Ann Tisdale of the Schepens Eye Research Institute, Boston, MA, USA for providing access to their critical point drying apparatus.

Authors’ roles

T.G.P., D.B. and S.B. conceived and designed the study. T.G.P., W.W.C.S., C.H., L.L., B.G. and S.B. performed the experiments and collected the data. T.G.P., D.B. and S.B. performed the data analysis. T.G.P. and S.B. wrote the draft manuscript. All authors contributed to the final version of the manuscript.

Funding

This work was supported by NIH grants DK073266 to T.G.P., DK042956 to D.B., HD040793, DK097124 and DK085715 to S.B. Additional funding was provided by a sponsored research agreement from D.B. from the Zeiss Corporation.

Conflict of interest

T.G.P. and D.B. received partial funding from a sponsored research agreement with the Zeiss Corporation. The co-authors Chiung-Hyunh, Lorenz Lechner and Bernhard Goetze are employees of the Zeiss Corporation.

References


Reed CI, Reed BP. Comparative study of human and bovine sperm by electron microscopy. Anat Rec 1948; 100:1–7.


