Deep nuclear invaginations linked to cytoskeletal filaments: Integrated Bioimaging of epithelial cells in 3D culture

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Summary

Fluorescence light and electron microscopy of 3D-grown HMECs reveal deep nuclear invaginations and tunnels. These invaginations are wrapped around intermediate filaments and actin cables that touch nuclear lamina connecting the microenvironment to the nucleus.
Abstract

The importance of context in regulation of gene expression is now an accepted principle; yet the mechanism by which the microenvironment communicates with the nucleus and chromatin in healthy tissues is poorly understood. A functional role for nuclear and cytoskeletal architecture is suggested by the phenotypic differences observed between epithelial and mesenchymal cells. Capitalizing on recent advances in cryogenic techniques, volume electron microscopy, and super-resolution light microscopy, we studied human mammary epithelial cells in 3D cultures forming growth-arrested acini. Intriguingly, we found deep nuclear invaginations and tunnels traversing the nucleus, encasing cytoskeletal actin and/or intermediate filaments, which connect to the outer nuclear envelope. The cytoskeleton is connected also to both other cells via desmosome adhesion complexes and to the cell’s ECM via hemidesmosomes. This finding supports a physical/mechanical link from the desmosomes and hemidesmosomes to the nucleus, which previously was hypothesized but now is visualized for the first time. These unique structures including the nuclear invaginations and the cytoskeletal connectivity to the cell nucleus is consistent with a dynamic reciprocity between the nucleus and the outside of epithelial cells and tissues.
Introduction

The three-dimensional (3D) architecture adopted by cells in response to microenvironmental stimuli plays a crucial role for their function within tissues. When cells are removed from their native organs and placed in two-dimensional (2D) cultures, they essentially lose all tissue-specific functions. When malignant cells are induced to form phenotypic normal-like structures in 3D culture, these structures are phenotypically reverted and behave like non-malignant cells. They fail to form tumors (Bissell and Hines, 2011; Bussard and Smith, 2012; Weaver et al., 1997) and normalize their pattern of gene expression (Becker-Weimann et al., 2013). The findings from our and other laboratories show clearly that the 3D architecture of the tissues, and the cells within them, regulate and are regulated by their biological state. We submit that cell and tissue polarity, through the cytoskeleton, allows for a signaling connection from the extracellular matrix (ECM) via the organization of the plasma membrane, to the nuclear envelope and the nucleoplasm.

Recent work has shown clearly that many previously-unexplored aspects of 3D architecture, such as the organization of the nuclear envelope is not stochastic or cell intrinsic: the structure and composition of nuclear envelope responds to microenvironmental stimuli with important consequences for gene regulation (Bissell and Labarge, 2005; Boudreau et al., 1995b; Hagios et al., 1998; Kim and Wirtz, 2015; Spencer et al., 2011; Versaevel et al., 2014; Xu et al., 2009; Xu et al., 2010). Even before the advent of 3D cultures, Bissell and colleagues proposed that ECM must signal through receptors, via cytoskeleton through nuclear matrix to chromatin to control tissue specific function and vice versa (Bissell et al., 1982), with the concept being further extended by Boudreau et al. in 1995 (Boudreau et al., 1995a). This possibility has found much support through the literature both from Bissell laboratory as well as others that there is physical linkage between the extracellular matrix through ECM receptors to the cytoskeleton, which in turn connects to the nuclear envelope receptors to physically couple to chromatin (Tapley and Starr, 2013a). Even in traditional cell culture on flat substrata (2D cell culture) intriguing studies performed nearly 20 years ago made clear the nucleus is mechanically linked to the plasma membrane via the cytoskeleton, and further, that under mechanical strain the nuclear morphology can be highly altered (Bhat and Bissell, 2014; Hu et al., 2005; Maniotis et al., 1997; Osorio and Gomes, 2014; Zhu et al., 2014). These and a number of other studies raise the question, what are the essential 3D architectural elements in the interconnected network of the nucleus,
cytoskeleton, and ECM in cells forming quiescent, organotypic 3D structures akin to those in vivo?

Mammary gland specific functions of epithelia, such as quiescence, basal/ apical polarity or, in murine cells, production of milk proteins can be recapitulated in biomimetic cultures in 3D. In laminin-rich gels single non- malignant mammary epithelial cells undergo ‘cohesive angular motion’, immediately adopt apical-basolateral polarity, divide with unequal stem-like divisions while adhering to each other all the while rotating continually until between 7-10 days they become quiescent (Tanner et al., 2012). Whereas non-malignant epithelial cells in biomimetic cultures develop this polarized 3D architecture over time and resemble mammary acini, cells cultured on traditional flat polystyrene show only limited polarity in monolayers when reaching confluence. While it is clear that the cellular architecture is regulated by the microenvironment, how the ECM connects to the nucleus remains incompletely understood. This is because there are serious resolution limits to traditional optical microscopy and the challenges of using sub-100 nm resolution techniques in crowded, 3D embedded cultures.

To address the issues of architecture at the level of the whole cell, we analyzed the 3D architecture of non-malignant human breast epithelial cells in acinar-like structures using a number of complementary imaging approaches, including high-resolution 2D transmission electron microscopy (TEM), electron tomographic volume microscopy, focused ion beam scanning electron microscopy (FIB-SEM), as well as confocal and super-resolution immunofluorescence imaging, including stochastic optical reconstruction microscopy (STORM). We found that non-malignant cells in 3D showed deep invaginations into- and tunnels through- the nucleus that house cytoskeletal filaments that connect to the nuclear envelope. These findings establish for the first time the structure of the physical connection between the nucleus and cell adhesion sites, providing a promising mechanical link between the cell’s microenvironment and the nucleus.
Results

S1 cells in biomimetic 3D tissue culture show growth arrest and unexpected organization

In order to study the high-resolution nuclear and cytoskeletal architecture of mammary epithelial cells we chose to study a well-characterized non-malignant human mammary epithelial cell (HMEC) line, HMT-3522-S1 (S1) (Briand et al., 1987; Petersen et al., 1992). We cultured the S1 cells in a laminin-rich extracellular matrix (lrECM) 3D tissue culture over the course of 10 days with EGF withdrawal at day 7, when the acini were formed. We found nearly all of cells in acini at day 10 were growth-arrested (Fig 1A). Only two in 5,317 cells examined incorporated EdU during a 24 hour labeling period, and thus were determined to be proliferating (0.0003%). In contrast, in tumorigenic HMT-3522-T4 cells, stained as a positive control, 97% of cells incorporated the EdU label during the 24 hour period (Fig. 1A, insert). S1 acini varied in size and the number of nuclei per acinus ranged from 5 to 20, with most of the acini showing 9-12 nuclei per acinus (Fig. 1B). Consistent with our previous work, we observed immunofluorescent staining for the Golgi (GM130) that was positioned towards the acinar center as well as the tight junction protein zona occludens-1 (ZO-1) in 57.7% of the acini (Fig. 1C - E). β-catenin was positioned basolaterally (Fig. 1F) and the hemidesmosome associated protein α6 integrin as well as actual hemidesmosomes (Fig. 1G, H) localized to the acinar periphery (i.e. basal surface of the cells of the acinus) suggesting appropriate apical-basal polarity consistent with previous work (Plachot et al., 2009; Weaver et al., 2002; Weaver et al., 1997). Furthermore, we found the ultrastructural phenotype described to be very robust, even when acini were grown on-top of the 3D culture instead of being fully immersed in the 3D extracellular matrix (Lee et al., 2007).

We examined embedded acini as whole-mounts by light microscopy, and noted a range of different sizes typically ranging from ~20-50 µm (Fig. 1I). Semi-thin sections of 500 nm were imaged with optical light microscopy upon toluidine blue staining (Fig. 1J). We found that the size of each cell (in cross section) in acini was fairly similar, and that size differences of acini were explained by differences in the number of cells per acinus (Fig. 1J-L), which is consistent with our results on nuclei number per acinus shown in Figure 1B. With transmission electron microscopy we imaged acini of different sizes (Fig. 1K-M) and
discovered the cells were not as closely associated as expected from the immunofluorescence data. In order to evaluate a larger volume of a typical acinus than is possible by traditional TEM, we turned to a serial sectioning approach, followed by serial TEM imaging of 22 thin resin sections, each with a nominal thickness of 100 nm. Serial section TEM (Fig. 1M-M") across a total depth of ~2 µm revealed the cells had intermittent junction points along their lateral surfaces and whereas there is apical polarity as measured by ZO-1 in the apical space, no true lumen with tight junctions is observed (Movie 1). Interestingly, whereas optical light microscopy of toluidine blue stained semi-thin sections and electron micrographs clearly show the intercellular spaces, confocal immunofluorescence microscopy of β-catenin shows continuous staining revealing that the resolution limit of light in fluorescence microscopy can lead to a false interpretation of lateral membrane organization (Fig. 1F, J, K, L).

**Basolateral organization with desmosomes flanked by membrane protrusions in acini with optimal preservation**

To examine the cell-cell interactions in more detail, we performed high-resolution transmission electron microscopy (TEM) imaging (Fig. 2). The cellular ultrastructure showed exquisite preservation owing to the ultra-rapid freezing and the freeze-substitution process (McDonald and Auer, 2006; Triffo et al., 2008), otherwise known as high-pressure freezing with freeze substitution (HPF-FS). The compact spacing of elements within the cytoplasm (Fig. 2A) displays exquisite preservation, as indicated by dense cytoplasm free of signs of extraction or aggregation, which contains cytoskeleton radiating from cell-cell adhesion sites (Fig. 2B, C). The sites of cell-cell adhesion occur in a spot-like pattern flanked on either side by microvilli-like structures (Fig. 2A-C). The adhesion complexes typically bridge a ~30-35 nm wide gap, with prominent and well defined ~10-15 nm thick dark bands on the cytoplasmic side of the plasma membrane (Fig. 2D, E).

To gain insight into the 3D macromolecular architecture of such adhesion sites, we employed electron tomography (ET) (Fig. 2F) with ~1 nm thin slices through the 3D tomogram revealing straight filaments crossing the gaps perpendicular to the flanking plasma membranes. Given the filament’s dimensions, bridging a 30-35 nm wide gap, as well as the uniform cytoplasmic thickness and the cytoskeletal connectivity, we identified these junctions to be predominantly macula adherens (desmosomes) (Movie 2) (Al-Amoudi et al., 2007). The junctions identified in Figure 2 are unlikely to be another type of common cell-cell junctional complex present in epithelial cells, zonula adherens junctions. We do observe
occasional small adherens junctions which span ~15-25 nm between adjacent cell membranes, but by ultrastructural analysis do not correspond to the expected numbers of zonula adherens junctions suggested by the β-catenin staining (Fig. 1F). It is worth noting that despite the apical ZO-1 (Fig. 1C) we rarely observed zonula occludens (tight junctions).

In the areas between the desmosomal adhesion sites, the cells were typically much further apart (several hundred nanometers; Fig. 2A-C). In these spaces we found plasma membrane bound protrusions that, when cut longitudinally or in cross-section, resemble microvilli. The fact that desmosomes, which represent a basolateral marker, and microvilli, which represent an apical marker, are in close proximity without separation by a tight junction (Fig. 2B, C), suggest that acinar cells no longer display the strict apical-basolateral polarity that is a hallmark of fully differentiated epithelial tissues in vivo. Instead cells show a reduced polarity with cellular surfaces facing the extracellular matrix still forming hemidesmosomes (Fig. 1H) yet they are lacking continuous and apically located tight junctions, and thus do not have a sealed lumen. It should be pointed out however, that based on studies of the mammary glands of virgin and early pregnant mice, highly ordered tight junctions and completely closed lumen appear only on day 15 of pregnancy (Barcellos-Hoff et al., 1989; Pitelka et al., 1973), when components of other milk proteins such as whey acidic protein appear (Bissell and Ram, 1989; Chen and Bissell, 1989; Lin and Bissell, 1993).

Membrane protrusions in the intercellular space form an extensive 3D network

When imaging large areas we found the internally located intercellular spaces to be continuous with the 3D extracellular matrix space without tight junctional complexes, needed to form a tight luminal seal (Fig. 3A, B). Many of the intercellular protrusions resembling microvilli contain short randomly oriented thin filaments (Fig. 3C, arrows) consistent with actin filaments typically found in microvilli lining the luminal space in epithelial tissues. However, unlike typical microvilli, which given a certain cell type adopt a consistent shape and length within the same cell (Sauvanet et al., 2015), we find membrane protrusions of various length, some of which are branched, further indicating that their architecture may be more complex.

To address this complexity and obtain high resolution insight into their 3D architecture, we performed FIB-SEM imaging (Fig. 3D-F), which allows the 3D reconstruction of large cellular volumes at ~10nm resolution. FIB-SEM generates 3D data by cycling between back-scattered electron imaging of a sample block surface and focused ion
beam ablation of the surface, iterating on this process thousands of times to sequentially cut through a volume of sample. Each slice or ablation of the specimen surface is tightly controlled to be a specific thickness thus generating a defined z-dimension for the voxel size (i.e. the z-slice thickness for the FIB-SEM datasets in this manuscript are each 4 nm and thus provides a voxel dimension of 4 nm$^3$). Such images (a single 4nm image from a stack is shown in Fig. 3D) can then be combined into a 3D volume, revealing the complexity of these membrane protrusions that can be traced in 3D through manual segmentation of each individual slice (Fig. 3E). The resulting 3D map shows that these membrane protrusions adopt a complex 3D architectural network of interacting membrane protrusions (Fig. 3F) similar to those found in mouse mammary epithelial cells in organotypic 3D culture undergoing branching morphogenesis (Ewald et al., 2012). The protrusions observed in 3D also branch often, fold back into the plasma membrane like ruffles, have bulges and uneven thicknesses, and are of differing lengths.

Nuclear invaginations in thin sections are 3-dimensional crevices and tunnels, as observed by FIB-SEM, STORM and confocal immunofluorescence microscopy

2D TEM and 3D FIB-SEM imaging of multiple S1 acini grown in 3D lrECM revealed deep invaginations of the nuclear membrane into the nucleus (TEM: Fig. 4A, B; FIB-SEM: Fig. 4C - F). These nuclear invaginations are known as type II nucleoplasmic reticulum (NR) (Malhas et al., 2011; Malhas and Vaux, 2014). Some NR invaginations in S1 acini appeared rather wide (~300 nm) (Fig. 4A) and contained ~25-30 nm sized particles which we interpret to be ribosomes (Fig. 4B). Inside the nucleus, sites of invaginations often displayed electron dense heterochromatin in close proximity to the inner nuclear membrane flanking complexes that span the inner and outer nuclear membrane, and thus are indicative of nuclear pore complexes (Fig. 4B). Note that due to the specific sample preparation method chosen for the 2D TEM imaging, internal membranes showed low contrast (Giddings, 2003; McDonald and Morphew, 1993; Triffo et al., 2008). Also, due to the difference in heavy metal staining needed for imaging samples by FIB-SEM, the texture of the chromatin within the nucleus appears less heterogeneous (Fig. 4C-F).

Other NR invaginations appeared as narrow (~25-50 nm) clefts and often contained a thin filament ~30-40 nm diameter in cross-section near the base of the invagination deep inside the nucleus (left cell of Fig. 4C, left inset), which runs parallel to the cleft membranes without ever entering the nucleus (Fig. 4C-E, Movie 3). Likewise, the perpendicular,
longitudinal view resolves this filamentous structure as a fiber. Note the inserts providing a close-up view of two filaments in two different cells recorded in cross-section and in longitudinal orientation, respectively (Fig. 4C). Another view of cross-section filaments in cells is shown in Figure 4D. Here three thin filaments can be observed within the nuclear invagination, with one being in very close proximity to the outer nuclear membrane (Fig. 4E). Figures 4C - E are 4 nm 2D slices of 3D volumes imaged by FIB-SEM. The narrow crevice-like nature of these invaginations becomes clearer upon 3D rendering of the segmented nuclear envelope depicted in Figure 4F. Primary cilia and centrosomes were observed in four of the cells at the apical surface and away from the nucleus; and further, golgi were previously observed in distinctly apical locations by confocal microscopy and by volume EM were not observed to be exclusively located near invaginations, thus indicating that these invaginations are likely not traditional nuclear clefts (Figs. 1 and S1, and Movie 3)(Bourgeois et al., 1979; Elkouby et al., 2016; Hulspas et al., 1994).

In order to confirm that these nuclear crevices were indeed NR invaginations in the nuclear envelope we labeled S1 cells grown in 2D culture with antibodies against the nuclear envelope protein, lamin B1 then imaged these cells using conventional confocal and 3D STORM microscopy, which allows ~20 nm resolution protein localization in three dimensions (Huang et al., 2008; Rust et al., 2006). While conventional confocal immunofluorescence microscopy showed a punctate signal for lamin B1 apparently residing inside the nuclear volume (Fig. 4G), STORM 3D imaging resolved these structures as hollow tubes that can be followed in 3D for over 600 nm length (Fig. 4H, I). The hollow tubes revealed by 3D STORM resemble the nuclear crevice invaginations shown by electron microscopy. Note that the color coding reflects position in the axial z-direction.

**Invaginations of the nuclear membrane in human breast epithelial cells**

Electron microscopy of normal human breast epithelium has consistently revealed that the nuclei of normal HMECs display type II NR nuclear invaginations (Eyden B., 2013; Lingle and Salisbury, 1999; Malhas et al., 2011; Ozzello, 1974; Stirling and Chandler, 1976; Tsuchiya and Li, 2005). We found that immunostaining of normal human breast tissue with lamin B1 antibody illuminates the uneven topology of the NR of the nuclei of HMECs in vivo (Fig. 5A). Type II NRs are apparent in both mammary ductal and acinar cells (Fig. 5A, B, Movies 4, 5) and in both myoepithelial cells and luminal epithelial cells, consistent with published electron microscopy data and our electron microscopy of mouse mammary
epithelial tissue (Fig. S2). Confocal stacks of lamin B1 staining illuminates NR invaginations in the z-plane (Fig. 5C). A NR invagination in an acinar cell clearly reveals the uneven topology of the nucleus (Fig. 5D, arrow). The appearance of the nuclear membrane in cells from the same sample is strikingly different, lacking type II NR, as shown with lamin B1 staining of vascular smooth muscle cells (Fig. 5E). This suggests that the unique shape of the nuclei as influenced by the NR invaginations observed in mammary epithelium is unlikely to be a preparation artifact.

**Actin- and keratin-based cytoskeletal filaments inside nuclear tunnels form bridges to the nucleus and mechanically connect the outer nuclear envelope membrane with cell adhesion complexes**

A dense cage of cytoskeletal elements surrounding the nucleus and extending out to cell-cell junctional complexes were observed in multiple 3D volume EM datasets. Cytoskeletal elements furthermore traversed nuclear tunnels formed by the nuclear envelope (Fig. 6). As shown in Figure 6A, we found tunnels through the nucleus in the 3D FIB-SEM data sets (Movies 3 and 6). Such tunnel-like partitions of the nucleus can be also observed in 2D TEM images (e.g. Fig. 1K-M, Movie 1), but these features can only be fully appreciated upon 3D FIB-SEM imaging (Fig. 6A-D, Movie 3). Close inspection of a filament in the nuclear tunnel (Fig. 6B) suggests it may be an intermediate filament bundle based on its 3D morphology, however no similarly sized conical bundles are observed in the cytoplasm as demonstrated in the 3D rendering of the segmented filament network (Fig. 6C, Movie 6).

Interestingly, we noticed that the main thick cytoskeletal filament traversing a nuclear tunnel displayed a number of small branches, sometimes almost perpendicular to the main filament axis direction (Fig. 6C, D). We found that these small filaments terminate on the nuclear envelope membrane (Fig. 6D-E), suggesting a mechanical link between cell-cell adhesion sites and regions deep inside the nucleus through the intermediate filament network. Figure 6F is a 3D rendering of both the cytoskeletal network as well as the nuclear membrane. Note that all 3D models are shown on top of a bottom 2D 4 nm slice of the 3D FIB-SEM volume (shown in greyscale) in order to provide adequate ultrastructural context. A scroll-through movie of the 3D volume and segmentation is provided (Movies 3 and 6).

We studied entire S1 acini at somewhat lower resolution using serial block face SEM imaging (SBF-SEM), which uses a diamond knife instead of an ion beam to trim and thus expose a new block surface for SEM imaging (Movie 7). Over the course of imaging multiple
acini we observed nuclear envelope folding and tunnels in a number of cells, confirming that the NR topology was present in a significant proportion of cells (Movies 7, S1, S2). Given the novelty of these cytoskeletal transnuclear tunnels, we wanted to confirm these structures using more traditional approaches, such as anti-lamin B1 immunofluorescence confocal microscopy. We imaged 15 acini totaling 203 cells (from 2 experiments) and counted cells with nuclear envelope folding and tunnels (Fig. 7A, Movie 8). We found that all studied cells displayed nuclear membrane wrinkling and 51% of cells studied displayed one or more nuclear tunnels, defined as a lamin B1 nuclear membrane signal traversing the nucleus thus indicating tunneling of the nuclear membrane through the nucleus (Fig. 7B arrows, C, Movies 9 - 11). In addition, we quantified tunnels with volume EM and found that in 32 cells from 5 acini examined 10 cells (31.25%) displayed one or more nuclear tunnel (Fig. 7C, Movie 7). By both volume EM and confocal microscopy we found ~9% of cells had 2 tunnels, and 2-3% displayed 3 or more tunnels traversing the nucleus (Fig. 7C, Movie S3). Furthermore, live cell imaging of HMT-3522-S1 cells using lipophilic membrane dyes shows the nuclear membrane clearly traversing the nucleus demonstrating, that the tunnels observed are not an artifact of fixation or processing in the sample work up for electron microscopy or immunofluorescence (Fig. S3, Movie 12).

Based on the dimensions of the cytoskeletal fibers observed in nuclear tunnels, we suspected the fibers observed in nuclear tunnels contained intermediate filaments, though they could also contain actin. In order to address whether these cytoskeletal elements were actin- and/or cytokeratin-based, we performed 3D STORM imaging on S1 cells labeled with either phalloidin-Alexa Fluor 647 or an anti-pan-cytokeratin primary antibody detected by an Alexa Fluor 647-conjugated secondary antibody. Surprisingly, we found evidence for the presence of both actin (Fig. 7D) and cytokeratins (Fig. 7E) in the cytoskeletal filaments traversing the nucleus, which is also detectable in conventional fluorescence imaging (see Fig. 7D, E inserts, respectively), but becomes much more prominent upon 3D STORM imaging.

SUN-domain proteins have been recognized widely as mechanical linkers between the cytoskeleton and the nuclear envelope (Lombardi et al., 2011; Starr and Fridolfsson, 2010; Tapley and Starr, 2013b; Tzur et al., 2006). Mediated by KASH proteins and nesprins, SUN-domain proteins act as anchors within the nuclear membrane that mechanically couple the nucleus with cytoskeletal actin and intermediate filaments (Tzur et al., 2006). Recent work
has shown that SUN proteins play a critical role in transmitting mechanical stimuli from the extracellular environment into intracellular chemical signals, including those affecting cell polarization (Lombardi et al., 2011).

We hypothesized that SUN-proteins can act as a linker between deep nuclear invaginations and the cytoskeleton, which in turn links to desmosomes and hemidesmosomes, thus transducing forces between neighboring cells in acini deep into the nucleus. By performing two-color STORM imaging of SUN-1 and cytokeratin, we found that SUN-1 is concentrated at the nuclear tunnels and invaginations, and encircles the cytokeratin filaments that tunnel into and through the nucleus (Fig. 7F-H). This result indicates that the cytoskeletal filaments we detect by EM and 3D STORM do not merely tunnel into and traverse the nucleus, but rather directly link the nuclear membrane deep within invaginations to the external cellular environment. We speculate that the unique spatial distribution of SUN and cytoskeletal proteins deep within the nucleus allows for extracellular mechanical signals to strongly influence the collective polarization of cellular units within acini.

Discussion

In this work, we investigated at ultra-high resolution, the structure of cells organized into tissue. We chose to image the HMT-3522 S1 cell line, which is part of a well-studied in vitro model of breast cancer progression (Briand et al., 1996; Briand et al., 1987; Petersen et al., 1992; Weaver et al., 1997). The S1 non-malignant HMEC cell line, recapitulates several important aspects of the mammary gland in vivo such as growth arrest and aspects of polarity while affording reproducibility and control not possible with native tissue. Furthermore, the relative small size and yet multicellular complexity of the acinus makes it an ideal model system for high-resolution 2D and 3D analysis.

We identified a number of features of growth arrested polarized cultures which had not previously been associated with normal function and for the first time imaged the physical structure of the cytoskeletal linkage from the desmosomes and hemidesmosomes to the nuclear membrane. Using high resolution electron microscopy (EM) imaging technologies, including 2D TEM, 3D ET and FIB-SEM, along with optical microscopy including epifluorescence, confocal and 3D STORM, we were able to image the architectural features of growth arrested acinar cultures at multiple scales and unprecedented resolution.
By immunofluorescence, growth-arrested S1 structures displayed a number of important features of luminal epithelial acini in vivo, including localization of both basal and apical polarity markers both in this- and in our previous- work. By integrating multiple modes of microscopy, we constructed a much higher resolution view of the 3D architecture of growth arrested acinus-like structure in S1 breast cell line. We found aspects of basal polarity and the presence of an extensive membrane protrusion network, which may allow neighboring cells to maximize cell-cell contact areas without generating junctional commitments. Although the structures could growth arrest, we did not observe tight junctions or an organized lumen.

Using large 3D volume EM techniques, we observed deep invaginations into the nucleus, some of which completely traverse the nucleus. We observed that such tunnels frequently enclosed cytoskeletal elements, which we identified as both actin and cytokeratin intermediate filaments. FIB-SEM 3D analysis revealed the cytoskeletal filaments to end and possibly connect to the nuclear envelope without entering directly into the nucleus space. Our observations are consistent with previous work that has suggested that cytoskeletal elements in nuclear invaginations may be rich sites for LINC complexes, i.e. bridges between the cytoskeleton and chromatin (Versaevel et al., 2014).

An important aspect of this work is our demonstration that the cytoskeletal connection from desmosomes and hemidesmosomes to the nucleus can take on unexpected and surprising shapes. A few papers had described a mechanical linkage between the plasma membrane and the nuclear membrane (Kim and Wirtz, 2015; Lombardi et al., 2011; Maniotis et al., 1997). Our finding of an intermediate filament cage surrounding the nucleus, and occasionally passing into the nuclear space via invaginations or tunnels in the nucleus suggests a unique mechanical coupling between receptors on the plasma membrane and nuclear not found in previous work in mesenchymal type cells cultured on rigid substrata highlighting the power of the form of imaging we’ve undertaken. Furthermore, 2-color STORM imaging demonstrates that nuclear membrane linkers like SUN-1 can be observed in both the nuclear periphery and nuclear tunnels. Reports of nuclear invaginations have been in the literature for a long time (for review please see (Malhas et al., 2011)). Whereas, the reason behind why nuclei in epithelial cells have folds and invaginations was not well understood, we believe that in this work we have provided a possible explanation.
We submit that our findings of nuclear invaginations that often contain cytoskeletal filaments, linked to the nuclear envelope, suggests a direct link between regions deep inside the nuclei and cell-cell and/or cell-ECM adhesion sites, which would allow mechanical ECM signaling to the nucleus and vice versa.

We further propose that integrated bioimaging is uniquely suitable to the study of other complex systems at different scales and resolution with multi-modal imaging and subsequent data integration at the model level. Having studied the HMT-3522-S1 in such detail, we are now ready to perform a comparative study between 2D-, 3D- cultures and tissues in vivo for the presence of the intriguing structures in order to understand their functional significance.
Materials and Methods

HMT-3522 cell culture

2D: HMT-3522-S1 and HMT-3522-T4-2 mammary epithelial cells were grown as previously described (Briand et al., 1996; Briand et al., 1987; Petersen et al., 1992; Weaver et al., 1997). Cells were routinely tested for contamination with the Lonza MycoAlert Assay.

3D: S1 cells on 2D were trypsinized and plated into 3D at 0.8 million cells per milliliter of lrECM (Matrigel®) inside lrECM. Each assay was composed of 240K S1 cells resuspended into 300uL of lrECM and pipetted into a 4-well Nunc plate. Matrigel was allowed to polymerize, 500uL H14 medium was added and assays were cultured at 37˚C in a humidified atmosphere with 5% CO₂. Assays were subsequently fed every 2-3 days, EGF was withdrawn from the medium at day 7 and acini were harvested at day 10.

Human breast tissue

Human breast tissue from reduction mammoplasty was collected in compliance with relevant ethical regulations and approved by the Lawrence Berkeley National Laboratory Human Subjects Committee. Informed consent was obtained from all subjects.

Mouse mammary tissue

Three week old female *Mus musculus*, C57BL/6 4th inguinal mammary glands were collected and processed for EM as described below. All tissues were collected in compliance with relevant ethical regulations and approved by the Lawrence Berkeley National Laboratory Animal Wellness Research Committee.

Immunofluorescence

Thirty micron sections of O.C.T. embedded human breast tissue from reduction mammoplasty or Day 10 HMT-3522-S1 acini were immunostained as follows: S1 acini were harvested from 3D lrECM cultures either by pipetting the lrECM + acini directly onto a glass slide or by partially digesting the lrECM with a mixture of ice cold PBS + 5mM EDTA for 15-30 minutes. S1 acini or human tissue sections were fixed in 4% paraformaldehyde for 20 minutes at room temperature or fixed in 1:1 methanol: acetone for 20 minutes at -20˚C. Slides were blocked in 10% goat serum in IF buffer (see (Lee et al., 2007)) + anti-mouse IgG Fab fragments (1:250 dilution; BD Biosciences 553998). Primary antibody was applied in blocking solution over night at 4˚C. Slides were washed 3 x 2 hours in IF buffer. Secondary
antibody was applied overnight at 4˚C. Slides were washed 3 x 2 hours in IF buffer and DAPI was applied. Acini were imaged on a Zeiss LSM 710 laser scanning confocal microscope equipped with 100x/1.4NA objective and 405 and 488 laser lines. The pinhole was set to 1 AU for all experiments. Data was acquired and analyzed with Zen software or a Zeiss Axioskop Imaging platform with SPOT Basic software. Samples were stained with Alexa Fluor 546 phalloidin (Invitrogen A12379, 1:200) to detect F-actin or with the following antibodies: rat anti-α6 integrin clone GoH3 (BD Pharmingen 555734, 1:300), rabbit anti-ZO-1 (Life Technologies 61-7300, 1:100), rabbit anti-lamin-B1 (Abcam ab16048, 1:500), Rabbit anti-GM130 (Cell Signaling Technology 12480; 1:3,000), β-catenin (BD 610154, 1:200). AF488-conjugated secondary antibodies (at 5 µg mL⁻¹) were used to label the primary antibodies.

**EdU staining**

S1 cells were grown in 3D lrECM culture for 10 days, on day 7 EGF was withdrawn from the medium and 24 hours prior to harvest EdU was added (5µM). Tumorigenic HMT-3522-T4-2 cells were grown on 2D and labeled with EdU (5µM) for 24 hours. EdU incorporation was detected with the Click-iT EdU Alexa Fluor 488 Imaging Kit (Invitrogen, C10337). Cells were counterstained with DAPI and proliferation was determined by counting EdU positive cells and total cells.

**High pressure freezing with freeze substitution**

HMEC acini, unfixed in matrigel, were placed 1 mm wide by 200 µm deep aluminum freezing hats, cryoprotected in 20% bovine albumin for freezing. The acini were then cryo-immobilized using a BAL-TEC HPM-010 high-pressure freezer (BAL-TEC, Inc., Carlsbad, CA). The samples that had been unfixed prior to HPF were placed in freeze-substitution medium made up of 1% osmium tetroxide, 0.1% uranyl acetate, and 5% ddH₂O in acetone. All samples were freeze-substituted either by using a Leica AFS2 (Leica Microsystems, Wetzlar, Germany) following a previously described protocol (McDonald et al., 2007) or by super quick freeze substitution (McDonald and Webb, 2011). Following freeze-substitution, the acini samples were rinsed in pure acetone and then progressively infiltrated with an epon-araldite resin (McDonald and Muller-Reichert, 2002). Samples were flat or thin embedded before being polymerized overnight at 60°C (Muller-Reichert et al., 2003).
Volume electron microscopy sample preparation

HMEC acini embedded within matrigel were chemically fixed with EM-grade 2% paraformaldehyde and 0.5% glutaraldehyde. The acini were stained with an osmium-thiocarbohydrazide-osmium (OTO) method (Friedman and Ellisman, 1981; Willingham and Rutherford, 1984) in combination with microwave assisted processing, followed by HPF-FS, as previous described (Ewald et al., 2012). Briefly, samples were subjected to HPF as described above and freeze-substituted with 4% osmium tetroxide, 0.1% uranyl acetate, and 5% ddH$_2$O in acetone; then flat embedded and polymerized in hard epon resin.

Transmission electron microscopy

70-100 nm sections were collected on formvar-coated grids using a Reichert UltracutE ultramicrotome (Leica Microsystems, Germany). For serial section TEM, 100 nm sections were collected in ribbons of ~5 sections upon each grid to preserve the order and orientation of the sectioned material. Sections were post-stained using 2% uranyl acetate in 70% methanol followed by Reynold’s lead citrate. The sections were imaged in an FEI Tecnai 12 TEM (FEI, Hillsboro, OR) operated at 120 kV. Images were recorded using a Gatan CCD with Digital Micrograph software (Gatan Inc., Pleasanton, CA). ImageJ software (Abràmoff et al., 2004) and Adobe Photoshop CS4 (Adobe Systems Inc., San Jose, CA) were used for further image processing and for the registration of the serial section images for 3D analysis.

Volume electron microscopy imaging

For FIB-SEM, resin-embedded samples were polished with a dry diamond knife tool to expose the area of interest on both the top and one side of the block and then mounted to a 45° pre-tilt SEM stub using colloidal silver paint. Milling and imaging of the block was performed on a FEI Helios Nanolab 650 Dual Beam FIB with Slice and View software (FEI, Hillsboro, OR). 6k by 4k images were collected with Elstar in-lens BSE detector at 2 kV with horizontal field width of 23 µm at a working distance of 2.46 mm; FIB milling was performed at 77 pA to generate a z-dimension step size of 4 nm; 1200 total slices for a complete depth of 4.5 µm. Due to errors with the Slice and View software, the FIB-SEM run was stopped and had to be restarted multiple times, thus there are some gaps in the entire dataset. For SBF-SEM, acini embedded in resin were mounted onto an aluminum pin with a cyanoacrylate adhesive and loaded into a sample holder for the Gatan 3View (Gatan Inc.,
Serial block face scanning electron microscopy was carried out as previously described (Denk and Horstmann, 2004). SBF-SEM data was collected using a 3View system mounted on a FEI Quanta 600 FEG SEM; serial images were 4k x 4k and acquired at 5 kV; z-dimension slices of 50 nm. Volume representations and manual segmentations of the volume EM datasets were performed using FEI Amira software (FEI, Hillsboro, OR).

**Electron tomography**

For Electron tomography, thin sections (100 nm) were imaged in a FEI Tecnai F30 microscope operating at 300 kV (Boulder Laboratory for 3D Electron Microscopy of Cells, University of Colorado). Binned 2k by 2k tilt series were collected on 4k by 4k charge-coupled-device Gatan camera (Gatan, Inc., Pleasanton, CA) every 1° from 70° to 70°, using the UCSF tomography (http://www.msg.ucsf.edu/Tomography/tomography_main.html) or SerialEM software package (http://bio3d.colorado.edu/SerialEM/). The nominal setting of defocus was 1 micrometer, and the pixel size of the data corresponded to 1 nm. Series were aligned with the help of either 10-nm or 15-nm gold fiducial markers (BBI Research, Inc., WI), and 3D reconstructed using the IMOD software package (http://bio3d.colorado.edu/imod/).

**Immunofluorescence labeling for STORM imaging**

S1 cells were cultured on #1.5 glass coverslips (12 mm dia.) as described above for 2D cell culture. For experiments aimed at visualizing the actin cytoskeleton, cells were initially fixed and extracted for 1 min with a solution of 0.3% (v/v) glutaraldehyde and 0.25% (v/v) Triton X-100 in cytoskeleton buffer (CB, see (Xu et al., 2012)), and then post-fixed for 20 min in 2% (v/v) glutaraldehyde in CB (Small et al., 1999; Svitkina, 2007; Xu et al., 2012). For experiments visualizing pan-keratin, cells were fixed for 15 min in ice-cold methanol. For immunofluorescence labeling, cells were blocked with a solution of 3% bovine serum albumin and 0.1% Triton X-100 in PBS, and then stained with primary and secondary antibodies. The primary antibodies used were mouse anti-cytokeratin, pan (4,5,6,8,10,13,18) (Cell Signaling Technologies 4545P; 1:20), rabbit anti-Lamin B1 (Abcam ab16048; 1:400), and rabbit anti-SUN1 (GeneTex GTX63537; 1:400). Alexa Fluor 647 (AF647)-conjugated secondary antibodies (at 5 µg mL⁻¹) were used to label primary antibodies for single color experiments. For 2-color experiments, AF647-conjugated secondary antibodies (at 5 µg mL⁻¹) were used to label pan-cytokeratin primary antibodies and CF568-conjugated secondary
antibodies (at 5 µg mL\(^{-1}\)) were used to label SUN1 primary antibodies. For fluorescent labeling of actin filaments, samples were incubated (Xu et al., 2012) with AF647-conjugated phalloidin (Invitrogen A22287) at ~0.4 µM.

**STORM imaging**

3D STORM imaging (Huang et al., 2008; Rust et al., 2006) was performed on a homebuilt setup based on a Nikon Eclipse Ti-U inverted optical microscope using an oil immersion objective (Nikon CFI Plan Apochromat λ 100x, NA 1.45). Lasers at 647 nm (MPB Communications), 560 nm (MPB Communications), and 405 nm (Coherent) were coupled into an optical fiber after an acousto-optic tunable filter and then introduced into the sample through the back focal plane of the microscope. Using a translation stage, the laser beams were shifted toward the edge of the objective so that emerging light reached the sample at incidence angles slightly smaller than the critical angle of the glass-water interface. Continuous illumination of 647-nm laser (~2 kW cm\(^{-2}\); for AF647), or 560-nm laser (~2 kW cm\(^{-2}\); for CF568) was used to excite fluorescence from labeled dye molecules and switch them into the dark state. Concurrent illumination of the 405-nm laser was used to reactivate the fluorophores to the emitting state. The power of the 405-nm laser (typical range 0-1 W cm\(^{-2}\)) was adjusted during image acquisition so that at any given instant, only a small, optically resolvable fraction of the fluorophores in the sample were in the emitting state. For 3D STORM imaging, a cylindrical lens was inserted into the imaging path so that images of single molecules were elongated in \(x\) and \(y\) for molecules on the proximal and distal sides of the focal plane (relative to the objective), respectively (Huang et al., 2008). For imaging buffer see (Xu et al., 2012).
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Competing interests
The authors declare no competing or financial interests.

Author Contributions
Concept and design of research and writing were by M.A. and M.J.B.; K.X. conceived and designed STORM experiments; D.M.J. and J.L.I. performed most experiments and data analyses, and co-wrote the manuscript. M.W. performed STORM imaging, with help from H.H. and A.B.C.; H.P. performed some electron microscopy and W.T. visualized volume EM datasets; C.S.L. collected focused ion beam SEM data, with help from J.R.; C.R. performed confocal microscopy, live cell imaging and co-wrote the manuscript.

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Figures
Figure 1. Growth-arrested S1 acini show little evidence of lumen and unexpected cell-cell interactions by 2D EM and serial section TEM. (A) At 10 days of growth in lrECM, S1 acini demonstrate growth arrest with 98.8% of cells not incorporating EdU (n=5,317 cells in 3 independent experiments), (inset) control T4-2 cell incorporation of EdU at 24 hours (n=2,006 cells in 2 replicates). (B) Using DAPI staining the average number of nuclei per acini was determined to be 11.16 (n=55 acini counted in 2 independent experiments). Acini displayed apical polarity based on (C) ZO-1 staining oriented at apical surface (D) when quantified 57.7% of the acini displayed apical polarity (n=592 acini counted in 2 independent experiments). Representative immunofluorescence staining for apico-basal polarity in acini with (E) Golgi (GM130), (F) beta-catenin, and (G) alpha 6 integrin (inset, higher magnification). (H) Transmission electron microscopy (TEM) of basal surface of acini reveals hemidesmosomes. Light microscopy views of S1 acini prepared for TEM: (I) polymerized in Epon-Araldite resin and (J) and 500 nm semi-thin cross-section through acini. TEM of acini (K - M) reveal electron lucid intercellular spaces, which (M-M'”) serial sectioning demonstrates are continuously present along the cell surfaces, as viewed every 0.5 μm. Scale bars for (A) 100 μm, (C, E, F, G) 20 μm, inset of (F) 10 μm, (H) 200 nm, (I) 25 μm, (J) 25 μm, (K – L) 5 μm, (M – M’”) 5 μm.
Figure 2. Basolateral desmosomes are flanked by membrane protrusions in acini with optimal preservation. (A) Thin section TEM reveals the intercellular space contains copious membrane protrusions. (B) Boxed region of (A), displays complex mix of protrusion side by side with spot-like junctions. (C) The punctate cell-cell adhesion complexes framing the membrane protrusions are likely desmosomes. (D) High magnification view from thin section TEM of the electron dense plaques. (E) In the boxed region of (D) the electron dense plaques of the desmosome are apparent and well separated filaments span the gap between them. Using electron tomography (F), a single 1 nm slice through the tomogram affirmed the gap between plaques spanned 30-35 nm, the filaments between plaques to be on average 5-10 nm thick; the midline of cadherin dimer overlap in the intercellular space can be visualized by the tomogram (F) and thin section TEM (E). Scale bars for (A) 5 µm, (B) 1 µm, (C) 100 nm, (D) 100 nm, (E) 30 nm.
Figure 3. Extensive membrane protrusions fill the intercellular space. (A - B) Thin section TEM reveals the 2-dimensional disorganization of membrane protrusions that fill the intercellular space. (C) Higher magnification of the protrusions in (B) reveal membrane bound structures, which appear to be randomly oriented. The protrusions are filled with cytoplasmic density and occasionally contain organized filaments (arrows). To further understand the full organization of these protrusions FIB-SEM imaging and feature segmentation was performed (D – F, black arrows follow a single region for orientation). A representative single 4 nm slice of (D) the raw dataset and (E) the pattern of masking cell from the cytoplasm through the protrusions are shown. (F) The whole segmented volume of cytoplasm and protrusions for the two masked cells in (E) reveals the complex and unexpected inter-connectivity between the protrusions of opposing cells. The 3D architecture displayed by membrane protrusions includes branched, looping, and interwoven structures. Scale bars for (A) 5 µm, (B) 2 µm, (C) 250 nm, (D) 1 µm.
Figure 4. Nuclear invaginations observed in thin sections are 3-dimensional tunnels and crevices as shown by FIB-SEM and STORM. (A) In thin section TEM large nuclear invaginations were observed, and (B, boxed region of (A)) when imaged at higher magnification cytoplasmic material (ribosomes, arrow heads) and nuclear pore (arrow) are observed in the crevice, while heterochromatin (asterisk) is shown on either side of the nuclear pore. To more clearly understand the ultrastructure of these invaginations, (C – F) FIB-SEM imaging was performed. (C, D) Single 4 nm slices are shown from the volume dataset; (C) the insets demonstrate a filamentous structure appears to track along the curvature of invagination of the nucleus, left inset filament in cross-section and right inset filament obliquely sliced. (E) Boxed region of (D), multiple filaments (arrows) are observed, with one making close contact to the nuclear envelope likely a point of termination at the nuclear envelope (black arrow); a mitochondrion (M) is next to the nuclear crevice. (F) 3D rendering of the nuclear envelope demonstrates that the nuclear invaginations are crevices propagated across the surface of the nucleus. To get at whether these structures can be detected by light microscopy, (G) epifluorescence and (H, I) STORM imaging were performed on S1 cells immunolabeled for Lamin B1. Again, nuclear invaginations are observed and are propagated into the nucleus over at least 600 nm, as shown by in (I). Z-
position is represented by color from -400 nm (violet) to +400 nm (red). Scale bars for (A) 5 µm, (B) 500 nm, (C) 5 µm, (D) 5 µm, (E) 500 nm, (H) 5 µm, (I) 500 nm.
Figure 5. Nuclear invaginations of the NR in normal human breast tissue. (A-E) Images of lamin B1 immunostaining in normal human mammary tissue. (A) Maximum intensity projection of mammary epithelial ductal and (B) acinar cells, both areas have cells with uneven nuclear membranes indicative of invaginations of the NR. (C) Three confocal slices of the ductal epithelium (white box) showing invaginations and uneven surface topology (white arrow). DAPI stained nuclei in blue. (D) Confocal slice of the acinar epithelium (white box) showing nuclear invagination (white arrow). DAPI stained nuclei in blue. (E) Vascular smooth muscle from the same tissue section displaying convex nuclei absent of NR invaginations. Scale bars for (A) 5 µm, (B) 5 µm, (C) 3 µm, (D) 3 µm, (E) 10 µm.
Figure 6. Cytoskeletal organization in 3D bridges the nuclear tunnels. (A - F) FIB-SEM dataset displaying nuclear tunnel, with a traversing thick cytoskeletal filament. (A) A single 4 nm slice of a FIB-SEM in which the electron density of the tunneling filament is observed and (B, boxed region in A) a close up view of the filament, displaying the density and wispy texture. (C) The filament, rendered in 3D from the volume EM FIB-SEM dataset, linking into the cytoarchitecture of the cell, most of which is made up of intermediate filaments. (D) View of the nucleus spanning region of the filament in which branches are observed terminating at the nuclear envelope (blue and black arrows). (E – E”) Cartesian coordinate cross-sections through the filament, displaying 2 separate branches (D, blue arrows) off the filament with (E) original EM density or (E’, yellow) as segmented volumes with the branches connected directly to the nuclear envelope (E”, nuclear envelope in blue). (F) Cytoskeleton (yellow) segmented and overlaid with the nuclear envelope (blue), rendered transparent, demonstrating the nuclear envelope completely encloses the cytoskeletal filament. Scale bars for (A) 1 µm, (B) 0.25µm.
Figure 7. Cytoskeletal organization in 3D bridges the nuclear tunnels and contain both actin and keratin. (A) Confocal imaging of a growth arrested acinus, labeled for Lamin B1 and DAPI. All nuclei display a wrinkled envelope. (B) A step-wise progression through the Lamin B1 staining highlights nuclear tunnels in multiple nuclei (B, arrows). (C) Quantification of nuclear tunnels per cell in confocal stacks (solid bars) and volume EM (open dotted bars). (D) 2D STORM and epifluorescence (inset) image of actin filaments labeled by phalloidin, with focal plane at the center of the nucleus. Shows actin filaments going through the center of the nucleus in a side-view perspective, as well as small patches of actin filaments that likely entered the focal plane via invaginations of the nuclear envelop. (E) 3D STORM and epifluorescence (inset) images of cytokeratin in another sample, showing keratin filaments going through the center of the nucleus in a side-view perspective. Color was used to present the height (z) information according to the color scale bar, with violet being closest to the coverslip and red being farthest away,
respectively. (F) 2-color STORM image of cytokeratin (green) and SUN-1 (magenta) with the focal plane at the center of the nucleus. (G, H) The separated cytokeratin and SUN-1 channels, respectively. SUN-1 is concentrated at nuclear tunnels and invaginations, and encircles keratin filaments that traverse the nuclear space. Scale bars (A) 5µm (B) 10µm (D-H) 2 µm.
References


Figure S1. Apically oriented centrosomes and primary cilia in growth arrested acinus. (A – D) FIB-SEM dataset of growth arrested acinus in which upon the apical surface of four cells either primary cilia or centrioles are observed. (A) A single 4 nm slice of a FIB-SEM dataset, with (A’) primary cilium in apical location of central cell (blue arrows, basal body components). (A”) The primary cilium 3D structure displayed as a segmented volume (blue, cilium; collar, yellow; basal body, orange). (B – B’) Centrosome (blue arrows) of the left-most cell is visible. (C – C’) One basal body (blue arrow) in the right-most cell is observed. (D – D’) The primary cilium (blue arrows, defined upper length of the cilium) of the uppermost cell is visible.
Figure S2. Wild type murine mammary gland TEM displays nucleoplasmic reticulum type II. (A) Cross-sectional view of the luminal space of a mammary duct in which two cells (B, C) have prominent type II nucleoplasmic reticula (NR). The cytoplasm is dense and filled with ribosomes in both cells. (D) Longitudinal view of a mammary duct with few cells (D’) displaying NR type II. Both (A) and (D) are TEM of 90 nm sections and indicate that NR can be difficult to properly visualize by 2D imaging alone. Scale bars for (A) 5 µm, (B) 1 µm, (C) 1 µm, (D) 10 µm, (D’) 5 µm.
Figure S3. Live cell imaging of nuclear membrane in S1 cells. S1 cells were labeled with lipophilic membrane dye Vybrant Dil 24 hours prior to plating in 3D lrECM. Once plated in 3D lrECM cells were allowed to grow for 24 hours and then imaged. We find after 48 hours of labeling the Vybrant Dil membrane dye is internalized and labels internal membranes. Imaging of the dye shows multiple nuclear membrane tunnels traversing S1 nuclei. In some cases Golgi (brightly labeled organelles) appear to reside inside the tunnels. Scale bar 5 μm.
Supplemental Movies

The full collection of Supplemental Movies can be viewed on FigShare:
https://figshare.com/s/8c3880ac8a03dbbd13ee

**Movie S1: S1 acinus imaged by SBF-SEM.** SBF-SEM data set collected of a S1 acinus, displaying eight whole and partially imaged cells. Five of the cells contain nuclear invaginations, three cells (37%) have nuclear tunnels with a total of four tunnels observed (two in one cell and one in each other cell). One cell has neither feature. Pixel size is ~100 nm\(^3\). Movie S1 (doi: 10.6084/m9.figshare.3466046) can be viewed at FigShare:
https://figshare.com/s/048709bb791cd795146c

**Movie S2: S1 acinus imaged by SBF-SEM.** SBF-SEM data set collected of a S1 acinus, displaying six whole and partially imaged cells. Four of the cells contain nuclear invaginations, two cells (33%) have nuclear tunnels with a total of four tunnels observed (three in one cell and one in the other). Two cells do not display either feature. Pixel size is ~100 nm\(^3\). Movie S2 (doi: 10.6084/m9.figshare.3466049) can be viewed at FigShare:
https://figshare.com/s/7ddb4a0fa2ed0d47d3af

**Movie S3: Lamin B1 stain illuminates four nuclear tunnels in a single S1 cell.** Close up view of a single cell in an acinus stained for lamin B1 (green) and DNA (DAPI, blue) and imaged by confocal microscopy. Four tunnels traversing a single nucleus are observed. Movie S3 (doi: 10.6084/m9.figshare.3466052) can be viewed at FigShare:
https://figshare.com/s/a09eeb9cc8f609e743d
The full movie collection can be viewed on FigShare:
https://figshare.com/s/c5aae652c5e129840319

**Movie 1: Serial section TEM of S1 acinus.** Total depth of 2.2 micron of S1 acinus surveyed by serial section TEM. Twenty-two 100 nm thin sections sequentially cut and imaged by TEM. Images were overlaid with ImageJ (https://imagej.nih.gov/ij/). Serial sectioning provided 3-dimensional view across middle of an acinus, demonstrating lack of apical polarity and highlighting the spot-like adhesion points on lateral cell surfaces. Movie 1 (doi: 10.6084/m9.figshare.3141928) can be viewed at FigShare: https://figshare.com/s/7db3e62d6c36992f048e

**Movie 2: High resolution electron tomography of desmosome cell junction.** Electron tomogram of a 100 nm thin section imaged every 1° from 70° to 70°. The nominal setting of defocus was 1 micrometer, and the pixel size of the data corresponded to 1 nm. The ultrastructural composition of the desmosome junction is visualized. Filaments between electron dense plaques on adjacent cell membranes are observed to span a gap of ~ 30 nm. Cytoskeletal filaments are observed linking into the electron dense plaques. Movie 2 (doi: 10.6084/m9.figshare.3167950) can be viewed at FigShare: https://figshare.com/s/b5f9dabb665811b91954

**Movie 3: FIB-SEM of part of a S1 acinus with nuclear invaginations and tunnels.** FIB-SEM was collected on the S1 acinus for a final voxel dimension of 4 nm³. The left and right most cells each have deep nuclear invaginations (NR type II). A primary cilium is observed at the beginning of the dataset on the apical surface of the central cell. The central displays both nuclear invaginations and cytoskeletal filaments transversing the nucleus within a tunnel. Movie 3 (doi: 10.6084/m9.figshare.3145006) can be viewed at FigShare: https://figshare.com/s/6f14eb6853c9d13c600c

**Movie 4: Lamin B1 stain of human mammary ductal cells.** Confocal stack of a duct in a cryosection of human mammary tissue stained for DNA (DAPI, blue), lamin B1 (green), & F-actin (phalloidin, red). Movie 4 (doi: 10.6084/m9.figshare.3145030) can be viewed at FigShare: https://figshare.com/s/b230939a5a4834a2c33

**Movie 5: Lamin B1 stain of human mammary acinar cells.** Confocal stack of an acinus in a cryosection of human mammary tissue stained for DNA (DAPI, blue), lamin B1 (green), & F-actin (phalloidin, red). Movie 5 (doi: 10.6084/m9.figshare.3145027) can be viewed at FigShare: https://figshare.com/s/47acbd0d1e10aa0e2eb5

**Movie 6: Segmented nuclear tunnel and cytoskeletal filament from FIB-SEM.** The central cell nuclei from Movie 3 is segmented to reveal the 3D architecture of the cytoskeletal filament transversing the nucleus within a tunnel, rendered in yellow. Visualization of the 3D segmentation was performed with Amira software (FEI, Inc.). Movie 6 (doi:
Movie 7: SBF-SEM of a S1 acinus with nuclear invaginations and tunnels. An acinus was imaged via SBF-SEM for a final voxel dimension of 50 nm³. Cells are observed at various z-depths without and with deep nuclear invaginations (NR type II) and two cells display nuclear tunnels with cytoskeletal filaments. Movie 7 (doi: 10.6084/m9.figshare.3145012) can be viewed at FigShare: https://figshare.com/s/601e64196ed20212fde2

Movie 8: Lamin B1 stain of S1 acinus illuminates multiple nuclear tunnels and the NR. Confocal stack of staining for lamin B1 (green) and DNA (DAPI, blue) in growth arrested S1 acini. NR type II is observed in multiple cells as well as nuclear tunnels transversing the nucleus. Movie 8 (doi: 10.6084/m9.figshare.3145003) can be viewed at FigShare: https://figshare.com/s/375a00065eaa3c54602f

Movie 9: Lamin B1 stain of single cell illuminates a nuclear tunnel in a S1 acinus. Close up view of a single cell from the acinus in Movie 8 stained for lamin B1 (green) and DNA (DAPI, blue). A tunnel transversing the nucleus is observed. Movie 9 (doi: 10.6084/m9.figshare.3145021) can be viewed at FigShare: https://figshare.com/s/1cf3f3a543fd60f081cc

Movie 10: Lamin B1 stain of single cell illuminates a nuclear tunnel in a S1 acinus. Close up view of a second cell from the acinus in Movie 8 stained for lamin B1 (green) and DNA (DAPI, blue). A tunnel transversing the nucleus is observed. Movie 10 (doi: 10.6084/m9.figshare.3145018) can be viewed at FigShare: https://figshare.com/s/20218c99196600461760

Movie 11: Lamin B1 stain of single cell illuminates a nuclear tunnel in a S1 acinus. Close up view of a third cell from the acinus in Movie 8 stained for lamin B1 (green) and DNA (DAPI, blue). A tunnel transversing the nucleus is observed. Movie 11 (doi: 10.6084/m9.figshare.3145015) can be viewed at FigShare: https://figshare.com/s/fc9da060edc6af6f54c4
Movie 12: Live S1 cells stained with a membrane dye. Confocal stack of live S1 cells stained with the lipophilic membrane dye Vybrant Dil after 24 hours in 3D lrECM culture. The membrane dye stains the nuclear membrane illuminating multiple tunnels transversing the nucleus. Movie 12 (doi: 10.6084/m9.figshare.3145024) can be viewed at FigShare: https://figshare.com/s/d6864e5c476f70c4ba77