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The Role of Calcium Transients in the Collective Migration of Goldfish Epithelial Keratocytes

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The Role of Calcium Transients in the Collective Migration of Goldfish Epithelial Keratocytes

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INTRODUCTION

Collective migration is an integral cell behavior, as it takes place during many biological processes that involve the mass translocation of cells from one region of an organism to another. In tissue morphogenesis during development, as well as in wound healing and cancer metastasis, cells must increase their motility while maintaining the critical cell-cell contacts that define collective migration (Weijer, 2009). For example, the formation of the lateral line during zebrafish development requires a group of epithelial cells to undergo a partial epithelial-to-mesenchymal transition, or EMT, to facilitate migration within the developing embryo (Weijer, 2009). An incomplete EMT promotes collective cell migration by inducing a front-rear polarity, while maintaining strong cell-cell adhesions between adjacent epithelial cells (Theveneau and Mayor, 2013). This partial and reversible EMT is also involved in the migration of epithelial sheets during wound healing. For rounded, non-polarized epithelial cells to acquire a motile phenotype capable of directed migration into the wound space, they must develop a front-rear polarity. Mesenchymal cells owe their migratory phenotype to the localized activity of Cdc42 and Rac1 at the front of the cell to stimulate actin polymerization and subsequent lamellar protrusion (Nelson, 2009). At the same time, localized activity of RhoA at the rear of the cell promotes actomyosin-based contractility to facilitate rear retraction (Nelson, 2009). Epithelial cells polarize in the same manner, while remaining in contact with adjacent cells and maintaining their relative positions with their neighbors. A complete EMT during wound healing would result in the loss of strong cell-cell contacts, disrupting sheet cohesiveness and hindering the proper closure of gaps in the epithelial sheet (Theveneau and Mayor, 2013). Within the context of cancer, however, upregulation of cell motility and simultaneous weakening of cell-cell

adhesions is necessary for the development of a metastatic phenotype in primary tumor cells (Phillips, 2011). As carcinoma cells transition from an epithelial to a mesenchymal phenotype, they are no longer anchored to the primary tumor, but are free to intravasate into blood vessels or migrate to secondary sites as metastatic cells (Phillips, 2011). Although, in certain cancers such as melanoma, breast, and prostate cancer, some metastatic cells do maintain the ability to migrate collectively through the ECM as small cell clusters or streams by maintaining some degree of cell-cell adhesion (Bidard et al., 2008; Aman and Piotrowski, 2008). With such extensive involvement of collective cell migration within the contexts of both cancer and normal cell function, it is critical to understand the mechanisms that drive and regulate the collective migration of cells.

The most defining characteristic of collective cell migration is the presence of cell-cell adhesions that facilitate the synchronized movement of a group of cells. These cell-cell contacts are what distinguish collective cell migration from the simultaneous, yet independent, movement of multiple cells (Rørth, 2009; Rørth, 2012; Theveneau and Mayor, 2013; Ilina and Friedl, 2009). The maintenance of strong but dynamic cell-cell adhesions allows migrating cells of the collective to remain closely associated with one another and influence the motility of neighboring cells (Rørth, 2009; Rørth, 2012; Theveneau and Mayor, 2013). The most prominent type of cell-cell contact among collectively migrating epithelial cells is the adherens junction (Theveneau and Mayor, 2013; Qiagen, 2014). In epithelial cells, adherens junctions are composed primarily of E-cadherin, a transmembrane protein that binds indirectly to F-actin via the cytoplasmic domain, and interacts with E-cadherins of neighboring cells via the extracellular domain (Friedl et al., 2004; Theveneau and Mayor, 2013). The formation and maintenance of adherens junctions are dependent upon the presence of both extracellular and intracellular

calcium (Ko et al., 2001a). Extracellular calcium ions serve to stabilize the rigid hook structure of the extracellular domain of E-cadherin, facilitating the linking of adjacent extracellular domains (Sotomayor et al., 2008; Kim et al., 2011). On the cytoplasmic face of adherens junctions, E-cadherin binds to F-actin via α- and β-catenin, and via adaptor proteins such as vinculin. Influxes in calcium ions promote local actin cytoskeleton rearrangements at sites of adhesion to strengthen the adherens junctions (Ko et al., 2001a; Baum and Georgiou, 2011). While adherens junctions need to be strong in order to maintain cell-cell adhesion, they must also be dynamically regulated so that epithelial cells can move in relation to one another, and sheets can maintain their structural integrity during collective migration (Baum and Georgiou, 2011). E-cadherin is continuously internalized from the plasma membrane via clathrin-dependent endocytosis, and recycled back to the plasma membrane at sites of new adherens junctions (Baum and Georgiou, 2011). On a more rapid timescale, adherens junctions are also regulated by phosphorylation of E-cadherin and associated catenins at specific tyrosine, serine, and threonine residues (Vitorino and Meyer, 2008). For example, it has been found that phosphorylation of β-catenin on tyrosine residue 654 by the tyrosine kinase pp60(c-Src) results in decreased binding to E-cadherin, and subsequent weakening of adherens junctions (Roura et al., 1999). When pp60(c-Src) activity becomes aberrant, as seen in cases of colorectal cancer cells, β-catenin becomes hyperphosphorylated at threonine residues (Coluccia et al., 2006). The resulting dissociation of β-catenin from E-cadherin causes the disassembly of adherens junctions and promotes metastasis (Coluccia et al., 2006). Therefore, it is crucial that cells maintain tight control of such adherens junction remodeling pathways, allowing cells within epithelial sheets to respond to forces while maintaining their strong cell-cell contacts (Baum and Georgiou, 2011).

The composite effect of many adherens junctions linking the cytoskeletons of adjoining cells is a cell body-wide connectivity that is necessary for collective migration (Friedl et al., 2004; Theveneau and Mayor, 2013). Contractile forces produced by actively migrating cells are transmitted to adjoining cells via this interconnected actomyosin network (Friedl et al., 2004; Theveneau and Mayor, 2013). Within epithelial sheets, the most actively motile cells tend to be those at the anterior or periphery of the cell group, as the sheet edge allows them to become polarized by extending lamellipodia into free space, creating the leading edge of the sheet (Friedl et al., 2004; Ilina and Friedl, 2009). Such cells are referred to as leader or pioneer cells because they are thought to signal neighboring cells to follow as they translocate outward from the group (Friedl et al., 2004; Vitorino and Meyer, 2008). The precise nature of such signaling is still under investigation, although it may involve a combination of chemical and mechanical signals (Weijer, 2009; Vitorino and Meyer, 2008). The collective migration of *Dictyostelium discoideum* to form a fruiting body is a well-studied example of how chemical signals can be relayed from cell to cell. Initially, individual cells chemotax towards exogenous cAMP (McCann et al., 2010). The signal is then amplified in the stimulated cells and relayed to neighboring cells in a process called signal relay, allowing the cAMP signal to be transmitted throughout the collective and coordinating directed migration towards the stimulus (McCann et al., 2010). Another possible mechanism coordinating collective migration involves both chemical and mechanical signaling. It has been proposed that leader cells within epithelial sheets are stimulated by growth factors or other chemical signals to undergo a partial EMT and, as they migrate outward from the sheet, follower cells attached via adherens junctions are pulled forward with the retracting rears of the leader cells (Weijer, 2009; Ilina and Friedl, 2009). In endothelial sheets, for example, fibroblast growth factor has been shown to stimulate polarized

lamellipodia formation in leader cells, while internal follower cells respond to the resulting mechanical forces generated by the leader cells, rather than to the growth factor signal directly (Vitorino and Meyer, 2008). It is possible that a similar growth factor-dependent mechanism directs the collective movement of other cell systems as well (Vitorino and Meyer, 2008). Whether cells of a collective communicate with one another via chemical or mechanical signaling processes, calcium ions have been studied extensively as a possible mediator of such signaling.

Calcium transients appear to travel between neighboring cells, and occasionally seem to propagate as waves across multiple adjoining cells within a sheet. However, there has been much debate over what function calcium transients may have in the collective migration of cell sheets, as well as the mechanism of such intercellular calcium signaling. One possible mechanism for calcium signaling within sheets involves intracellular signaling pathways initiated by growth factors. Epidermal growth factor (EGF) binds to the transmembrane growth factor receptor, activating phospholipase C (Chen et al., 1994). This enzyme initiates a reaction that produces the second messenger IP3, which binds to receptors in the endoplasmic reticulum (ER), triggering the release of calcium ions from the ER and producing a calcium transient (Jacques-Fricke, 2008). It has been proposed that either IP3 or calcium ions themselves may also diffuse via gap junctions from cells actively displaying calcium transients to inactive neighboring cells, where new transients are then initiated (Junkin et al., 2014; Paemeleire et al., 2000). This process of intercellular calcium signaling is one possible mechanism for the propagation of multicellular calcium waves (Junkin et al., 2014). In addition to chemical signaling, mechanical forces may also play a role in calcium signaling within sheets. Stretch-activated calcium channels (SACs), for example, rely on cell-generated forces to induce calcium transients via

calcium-induced calcium release, or CICR. When cell membranes come under tension, either from cytoskeletal contractility or from forces exerted by neighboring cells, SACs are stimulated to allow localized influxes of calcium ions into the cytoplasm, which can then trigger a much larger release of calcium ions from the ER, producing a calcium transient. In fibroblasts, the linking of cadherin extracellular domains from adjacent cells has been shown to induce intracellular calcium transients (Ko et al., 2001a). It has been proposed that cell membranes become stretched in response to the cytoskeletal tension produced by the formation of adherens junctions, and consequently activate SACs, leading to intracellular calcium transients (Ko et al., 2001b). The release of intracellular calcium, in turn, strengthens the adherens junctions by promoting actin cytoskeleton rearrangement and recruiting additional E-cadherin and β-catenin to the sites of adhesion (Ko et al., 2001a; Ko et al., 2001b). A similar response to cadherin-cadherin interaction has been seen in epithelial cells in the form of actin polymerization at junction sites (Ko et al., 2001a).

Calcium transients are necessary not only to strengthen calcium-dependent cell-cell adhesions, but also to promote cell motility. Calcium transients facilitate cell motility in single cells by promoting increased cytoskeletal contractility and turnover of cell-substratum adhesions (Doyle et al., 2004). Cytoplasmic calcium ions induce myosin II-dependent contractility of the actin cytoskeleton (Wei et al., 2012). The resulting traction stress forces the release of rear focal adhesions, allowing the cell to translocate forwards (Lee et al., 1999; Doyle et al., 2004). In human U87 astrocytoma cells, the presence of elevated cytosolic calcium also increases the localization of focal adhesion kinase (FAK) to focal adhesions (Giannone et al., 2004). This increases the availability of FAK to phosphorylate its downstream effectors involved in regulating cell retraction and adhesion turnover, including myosin light chain kinase (MLCK)

and calpain (Giannone et al., 2004). Calpain is an enzyme capable of cleaving focal adhesions, thus weakening cell-substratum adhesions (Wei et al., 2012). Therefore, periodic influxes of cytoplasmic calcium, or calcium transients, are necessary to promote continuous cell migration in motile cell types such as leukocytes, Dictyostelium, and keratocytes (Marks and Maxfield, 1990; Lombardi et al., 2008; Doyle and Lee, 2005). Calcium transients in Dictyostelium and keratocytes have been observed as a result of SAC activation during cell motility (Lombardi et al., 2008; Doyle and Lee, 2005).

The role of calcium transients in single motile keratocytes has been studied extensively, while the function of calcium transients within sheets of collectively-migrating keratocytes has yet to be ascertained. The role of calcium in regulating cell-cell adhesions in sheets of keratocytes also remains uncertain. Based on findings from the literature, it is possible that calcium transients have the same function in both single keratocytes and keratocytes within cell sheets, in that the transients facilitate the migration of individual cells by regulating contractility and cell-substratum adhesion turnover (Aman and Piotrowski, 2008). In sheets of keratocytes, however, it is possible that calcium ions are also involved in the formation and maintenance of cell-cell adhesions. Therefore, the goal of this work was to investigate the role of calcium transients in the collective migration of goldfish epithelial keratocytes, with an emphasis on sheet cohesiveness. This was accomplished by observing the effects of depleting external and internal sources of calcium transients, in addition to testing the dependence of calcium transients on signaling from growth factors and SACs.

METHODS

Reagents

Stock solutions of 1 mg/ml type IV collagen (Sigma-Aldrich Co., St Louis, MO) diluted in 0.25% acetic acid were made to 0.05 mg/ml with phosphate-buffered saline (PBS). Stock solutions of Calcium Green^{TM}-1 dextran (3000 MW, Life Technologies, Grand Island, NY) at 8 mg/ml were made to 1 mg/ml working solutions in syringe-filtered PBS supplemented with Ca^{2+} and Mg^{2+} (PBS+/+). Stock solutions of 500 μM Thapsigargin were made up in 100% ethanol. The 500 μM stock was diluted to a 20 μM working concentration in culture medium, and subsequently diluted to a final concentration of 10 μM when an equal volume of culture medium was added to the culture chamber. A stock solution of 1 mM Gadolinium(III) chloride hexahydrate (Sigma-Aldrich Co.) was diluted in Fish Ringer's (FR) solution without serum, and diluted to a final concentration of 10 μM in FR plus 1% serum (FR 1%). Stock solutions of 20 mM EGTA (Sigma-Aldrich Co.) were prepared in PBS and diluted to a final concentration of 2.5 mM in culture medium.

Preparation of collagen-coated coverslips

Squeaky-clean glass coverslips (1.0 thickness, Fisher Scientific, Pittsburg, PA) were prepared as described in (Waterman-Storer, n.d.). Under the tissue culture hood, 100 μl aliquots of a 0.05 mg/ml collagen solution were pipetted onto Parafilm. Dried coverslips were placed on top of the drops and allowed to sit in a covered container for 1-2 hours. Immediately prior to use, coverslips were washed twice with PBS to remove excess collagen, and coverslip edges were blotted on Kimwipes to remove excess fluid.

Cell Culture

Goldfish *Carassius auratus* keratocytes were cultured as described previously (Lee et al., 1993). Briefly, fish scales were removed from the fish and placed in 35 mm diameter culture

dishes containing 2 ml of serum-free RPMI wash solution, consisting of RPMI culture medium supplemented with Penn/Strep and Fungizone at five times the working concentration, pre-warmed to 37°C. Scales were gently swilled and allowed to sit for ~5 minutes. In the tissue culture hood, scales were transferred twice into 2 ml of RPMI supplemented with Penn/Strep, Fungizone, and 10% serum (RPMI+/+) for 3 minutes. After the second wash, scales were transferred to new 35 mm dishes of 60-65 µl RPMI+/+ with 5 to 8 scales per dish, depending on culture size, and covered with a collagen-coated coverslip with the collagen-coated side down. Dishes were incubated in a humid chamber at room temperature for 40-45 hours before use. During this time period, cells that were attached to the extracted scales were able to collectively migrate outwards from the scale to fill the surrounding empty space, much like they would during wound healing. Therefore, the cell culture system we used here can be considered an in vitro model for collective migration during epithelial wound healing.

In the tissue culture hood, 0.5 ml of RPMI+/+ were added to each culture dish and gently swilled. Coverslips were gently lifted from the bottom of the dishes and placed cell-side-up into new 35 mm dishes containing 1.5 ml of RPMI+/+.

Bead-loading of keratocytes

Working solutions of 8 mg/ml Calcium Green™ -1 dextran in syringe-filtered PBS+/+ were prepared and kept in the dark and on ice until use. Coverslips with adhered keratocytes face-up were washed twice in filtered PBS+/+, using an aspirator to gently remove waste medium from the culture dishes. Following aspiration of the second wash, 50 µl of working Calcium Green solutions were pipetted onto each coverslip. Glass beads 425-600 µm in size (Sigma-Aldrich Co.) were sprinkled onto coverslips in a monolayer. Culture dishes containing

the coverslips were gently tapped on the sides to agitate the beads. Filtered PBS+/+ was added to the dishes to sufficiently float beads off of the coverslips, and the coverslips were transferred to new 35 mm dishes containing ~ 2 ml filtered PBS+/+. To rinse off any remaining beads, each coverslip was then transferred to a second dish of ~2 ml filtered PBS+/+. Coverslips were finally transferred to a new 35 mm dish containing 1.5 - 2 ml of RPMI+/+ and incubated in the dark for 30 minutes to allow cell recovery.

Calcium Imaging

Calcium imaging was performed on an inverted microscope (Eclipse TE2000-U; Nikon, Melville, NY, USA) using a Plan Fluor 20x/N.A. 0.5 objective. Calcium Green™-1 dextran was visualized using excitation fluorescence at 470 nm, produced by a Lumencor Spectra X light engine®. Emitted fluorescence was collected using a FITC filter set (Chroma Technology Corp, Bellows Falls, VT). Images were acquired using a sCMOS camera (Orca Flash-4.0; Hammamatsu, Japan). Imaging Software NIS-Elements (Nikon, Inc, Melville, NY) running on a Windows7 platform was used for image acquisition and hardware control. Images of Calcium Green fluorescence were collected at an interval of 300 ms with a 200 ms exposure time for a duration of 1 minute and 30 seconds, and stored on the computer hard drive. Time-lapses were acquired approximately every 20 minutes, unless stated otherwise. Phase contrast images were acquired for the first 1-3 seconds of each time-lapse movie to record the size and shape of the cell sheet.

<u>Calcium Imaging – Serum</u>

For this set of experiments, the duration of each time-lapse acquisition was shortened to 1 minute. This was done in order to minimize cells' exposure to the excitation fluorescence and

reduce phototoxic effects, which had been found to reduce transients in cells before the treatment condition. In each experiment, a group of the same 3 sheets was imaged throughout the pre- and post-treatment conditions. This served to increase the number of cell sheets observed in each experiment, and to further reduce the fluorescence exposure to each sheet over the total observation time. For each experiment, time-lapse videos were acquired approximately every 3 minutes, with a 10-minute interlude between "5 minute post" and "15 minute post" acquisitions to allow the cells to respond to the treatment. According to the initial protocol used, cells were transferred directly from RPMI 10% into RPMI 0% to be imaged. It was occasionally found that, while in RPMI 0%, the initial frequency of calcium transients was elevated compared to subsequent time-lapse sequences in the same condition. The protocol was therefore modified such that cells were incubated in RPMI 0% for 5 minutes before imaging to allow the cells to adjust to being without serum before any images were collected. The post-treatment 50% serum concentration was achieved by adding 400 µl of serum to 400 µl of RPMI 0%.

Calcium Imaging - Gadolinium

For this set of experiments, cells were imaged in 1 ml of Fish Ringers with 1% serum (FR 1%) prior to treatment with 10 µM Gadolinium. Preliminary testing revealed that cells displayed similar transient frequencies in FR 1% as in RPMI 10%. However, FR 1% is a more suitable medium than RPMI 10% for treatment with Gadolinium because FR 1% contains fewer phosphates, which are known to form aggregates with Gadolinium and precipitate it out of solution. The positive trivalent charge of Gadolinium allows it to bind with high affinity to anions, including phosphates, which are prevalent in both RPMI and serum (Caldwell et al., 1998). While these aggregates are not thought to harm the cells, they do interfere with imaging by clouding the field of view. Calcium imaging was conducted as in the serum experiments, but

an additional washout step was added to remove most of the Gadolinium from the medium. Cells were washed twice with 500 µl of FR 1%, leaving a residual concentration of 2.5 µM Gadolinium. Preliminary experiments revealed that the occurrence of calcium transients was not affected at this low concentration of Gadolinium. Cells were then imaged immediately after the washout, and again at 10 and 40 minutes post-washout to allow cells to adjust to the lack of Gadolinium.

Data Analysis

Calcium transient and sheet morphology analyses were performed using Metamorph software (Molecular Devices, LLC) and ImageJ software (W.S. Rasband, NIH, MD). The average untreated cell sheet consists of between 85 and 95 loaded cells, roughly 92% of which actively display calcium transients. The change in average fluorescence intensity for a single cell throughout a time-lapse video was measured using the region of interest tool in Metamorph. As it would be inefficient to produce brightness versus time graphs to quantify the number of transients in each individual cell (Fig. 3), a novel method of analysis was developed to estimate the frequencies of calcium transients in whole sheets (Fig. 1). Stacks of calcium images were built using Metamorph and shortened to include every 5^{th} frame of the original data. This interval represents 2.5 seconds, which optimizes the ease and probability of detecting transients between successive frames. Difference images of shortened stacks were created in ImageJ and thresholded for high brightness values to count the number of bright regions (representative of calcium transients) in each frame. The frequency of calcium transients pre-treatment was calculated by dividing the total number of transients that occurred within one field of view pre-treatment, by the total elapsed time (in minutes) during which the cells were imaged pre-treatment. Note that this time does not include the intervals between the acquisition of each

time-lapse video. The same calculations were used to obtain the post-treatment transient frequencies.

Collective migration involves not only the long-term, large-scale translocation of migrating cell sheets from one location to another, but also the short-term changes in sheet morphology initiated by the finer movements of individual cells within the migrating group that produce these large-scale translocations. In this study, since calcium transients require short-term imaging, we chose to focus on these short-term changes in sheet morphology that could be imaged simultaneously with calcium transients. Focusing on morphological changes also allowed us to quantify changes in sheet cohesiveness. To analyze the changes in cell sheet morphology over time, stacks of phase contrast images corresponding to a 20 minute time interval were built using Metamorph (Fig. 2). Phase contrast images were converted into binary images that could then be thresholded. First, cell sheets were outlined using the free-hand drawing tool in ImageJ, and a custom macro was used to convert the outlines into binary images. These were thresholded to obtain measurements of sheet area and the number of sheets in each frame. To quantify the total area retracted and the number of retracted regions between successive frames, the Align Stack function in Metamorph was used to convert stacks of binary images into stacks of difference images. ImageJ was then used to threshold all of the dark regions corresponding to retracting areas and automatically quantify these two parameters. Changes in area protruded and number of protruded regions were not analyzed here because, as sheet area tended to decrease rather than increase with treatment, retraction was the more appropriate parameter to study the changing sheet morphology.

The dynamics of sheet morphology both pre- and post-treatment was quantified by finding the rate of change in sheet area, the number of sheets, the rate of sheet retraction, and the

number of retracting regions under each condition. The rate of change in sheet area during the pre-treatment phase was calculated by dividing the difference in sheet area between the first and last pre-treatment frame by the total elapsed time pre-treatment. The rate of sheet retraction during the pre-treatment phase was calculated by dividing the total area retracted in all pre-treatment frames by the total elapsed time pre-treatment. The same calculations were used for post-treatment rates of change in sheet area and rates of sheet retraction, respectively. Both the change in sheet area and change in area retracted provided information about changes in sheet size, which is closely related to sheet cohesiveness. The more a sheet begins to break up and retract, the smaller it becomes. Likewise, the number of cell sheets in each frame was used to track sheet cohesiveness and whether fragments of the sheet had broken off. Analysis of the number of retracted regions at the sheet edge pre- and post-treatment provided a measure of the complexity of sheet morphology. As cell sheets begin to break up and lose cohesiveness, the sheet shapes become more irregular due to increasing regions of retraction and protrusion at the edges of the sheets.

RESULTS

When used as a calcium chelating agent, EGTA has been found to prevent calcium transients and disrupt cell-cell adhesions (Lee et al., 1999; Ko et al., 2001a). In chelating extracellular calcium ions, EGTA disrupts the CICR pathway by preventing the localized influx of calcium ions into the cytoplasm via SACs. Preliminary experiments in this lab aimed at studying the effects of calcium-free medium on calcium transients revealed that, in 11 out of 11 observations, switching cells from PBS++ with 3% serum to calcium-free PBS with 3% serum reduced the frequency of calcium transients in cell sheets (data not shown). Conversely, switching cells from calcium-free PBS with 3% serum to PBS++ with 3% serum increased the

frequency of calcium transients during 6 out of 8 observations (data not shown). These results indicate a certain dependence on extracellular calcium ions for producing calcium transients. Therefore, the goal of these experiments was to observe the effects of depleting extracellular calcium, using EGTA, on calcium transients and sheet morphology. The addition of 2.5 mM EGTA to cells in RPMI 10% (calcium concentration at ~0.4 mM) resulted in a noticeable decrease in the number of calcium transients. Individual cells appeared to retract and round up as cells pulled away from one another (Fig. 4A). It was found that cells which were actively displaying transients before treatment exhibited fewer or no transients after the addition of EGTA (Fig. 4B,C). Pre-treatment, the average frequency of calcium transients for the three cells indicated in Fig. 4A was 1.8 transients/min. Immediately following treatment, the average frequency of transients briefly increased to 6.4 transients/min for ~2 minutes, and then decreased to only 0.8 transients/min during the rest of the observation post-treatment. This immediate spike in transient activity was likely due to the cells retracting and becoming stretched upon the addition of EGTA. It is possible that calcium transients could still be initiated immediately following treatment because EGTA may not chelate all of the extracellular calcium ions immediately. However, the subsequent decline in calcium transient frequency to a level below the initial frequency, suggests that the CICR pathway had become disrupted at this time.

In addition to depleting extracellular calcium, another commonly-used approach to eliminating calcium transients is to deplete intracellular calcium stores. In preventing the supply of calcium ions in the ER from replenishing following a calcium transient, Thapsigargin prevents any subsequent effluxes of calcium from the ER into the cytoplasm via the CICR pathway. Thapsigargin has been found to prevent calcium transients (Lee et al., 1999; Klepeis et al., 2001) and to disrupt cell-cell adhesion (Ko et al., 2001a) in cell sheets. The following experiments

were designed to test the effects of depleting calcium transients by using Thapsigargin to prevent the reuptake of calcium ions into the ER. When treated with Thapsigargin, cells displayed a pronounced decrease in the frequency of calcium transients (Fig. 5). Analysis of calcium transients was performed using the methods outlined in Figure 1. In this example, the frequency of calcium transients from each time-lapse video (duration 1.5 min) decreased substantially when the cells were treated with 10 µM Thapsigargin (Fig. 5B-E). The average frequency of calcium transients in this example decreased from 54 transients/min per field of view prior to treatment, to an average of 8 transients/min following the addition of Thapsigargin. Likewise, the average frequency of calcium transients for all of the Thapsigargin experiments declined from 101 transients/min to 14 transients/min following Thapsigargin treatment (Fig. 7D).

Our next goal was to quantify the resulting changes in sheet morphology and cohesiveness associated with this Thapsigargin-induced decrease in the frequency of calcium transients, using the methods described in Figure 2. Thapsigargin treatment resulted in the retraction and dispersion of cell sheets (Fig. 6A). In this example (Fig. 6), sheet area in treated cells began to decrease at an average rate of 1128 μm^2/min, compared to a rate of 965 μm^2/min pre-treatment (Fig. 6B), while the area retracted began to increase at an average rate of 1622 μm^2/min, compared to 1149 μm^2/min pre-treatment (Fig. 6D). It is important to note that, while imaging Calcium Green-loaded cells, calcium transients would occasionally diminish even before Thapsigargin treatment; a decrease suspected to result from repeated exposure to fluorescence in the presence of the Calcium Green indicator, or from cell damage incurred during bead loading. Loss of calcium transients independently of Thapsigargin treatment may obscure any findings related to the Thapsigargin-induced changes in sheet morphology. To control for the potential phototoxic effects of fluorescent light, as well as possible adverse effects

of bead loading, analysis of the change in sheet area was conducted for cells that were either loaded with PBS++ instead of the Calcium Green™-1 dextran indicator ("fake" loaded) or not loaded at all (not loaded), both before and after Thapsigargin treatment. Compared to Calcium Green-loaded cells (Fig. 6B), a similar decrease in sheet area was seen in these fake-loaded and unloaded cells after treatment with Thapsigargin (Fig. 6C). On average, sheet area increased at a rate of 516 μm^2/min before treatment, and decreased at a rate of 798 μm^2/min after Thapsigargin treatment (Fig. 6C). This similarity post-treatment indicates that the morphological effects seen in Calcium Green-loaded cells were the result of Thapsigargin treatment, rather than due to phototoxicity or an adverse reaction to bead loading.

Related to the increase in area retracted in Calcium Green-loaded cells, the number of retracted regions at the sheet edges began to increase for an average of 9 regions post-treatment, compared to 6 regions pre-treatment (Fig. 6D,E). The same trend was seen in the average rate of change in sheet area, the average rate of area retracted, and the average number of retracted regions for all of the sheets analyzed in these experiments (Fig. 7). While there was very little change in sheet area before treatment, sheet area decreased at an average rate of 887 μm^2/min after Thapsigargin treatment (Fig. 7A). In contrast, the average rate of area retracted increased from 826 μm^2/min before treatment to 1789 μm^2/min following addition of Thapsigargin (Fig. 7B). On average, the number of retracted regions increased from 9 regions pre-treatment to 13 regions post-treatment (Fig. 7C). Note that, while the example data set (Fig. 6) exhibited a decrease in sheet area pre-treatment, the pre-treatment changes in sheet area among all of the observed sheets were quite variable, such that some of the sheet areas increased and some decreased. This variability accounts for the very small average change in sheet area pre-treatment (Fig. 7A).

To determine how these four parameters change with respect to one another following thapsigargin treatment, the average percent changes between the pre- and post-treatment were calculated (Fig. 8). The 87% reduction in the frequency of calcium transients was associated with an approximate doubling in the rate of decrease in sheet area, which was related to a nearly 3-fold increase in the rate of area retracted and a 1.5-fold increase in the number of retracted regions.

In preliminary experiments, we had found that the frequency of calcium transients declined markedly when cells were in serum-free medium (RPMI 0%), but not while cells were in medium with 50% serum (RPMI 50%). Therefore, the goal of the following experiments was to determine how the presence or absence of serum-induced calcium transients affects sheet morphology and cohesiveness. When cells in serum-free medium were treated with RPMI 50%, the number of calcium transients quickly increased (Fig. 9B). In this example, the frequency of calcium transients increased to 354 transients/min per field of view at 2 minutes post-treatment (Fig. 9B, orange), compared to only 59 transients/min in RPMI 0% (Fig. 9B, teal). After 20 minutes in RPMI 50%, the frequency of transients remained elevated compared with 2 minutes post-treatment, though slightly reduced, at 233 transients/min (Fig. 9B, blue). On average, the frequency of calcium transients in all of the cell sheets observed increased from 110 transients/min in RPMI 0%, to 193 transients/min after treatment with RPMI 50% (Fig. 10B). After 15 minutes in RPMI 50%, the average frequency of calcium transients had decreased to 143 transients/min, but did not approach the low frequency observed initially in RPMI 0% (Fig. 10B). When observing the resulting short-term (~30 min) changes in sheet morphology, it was found that cell sheets that were treated with RPMI 50% remained cohesive and exhibited minimal dispersion (Fig. 10A).

To compare the response of calcium transients in the presence of abundant serum (RPMI 50%) to that in serum-free medium (RPMI 0%), a subset of experiments was conducted in which cells were maintained in RPMI 0% for the duration of the imaging period. These cells were "treated" with additional RPMI 0% at the point of treatment, rather than with RPMI 50% as in the previous experiments. Compared to cells that were switched to RPMI 50% at the time of treatment, these cells displayed a substantially lower average frequency of transients 5 min after the "treatment" with additional RPMI 0%, which was 43 transients/min (Fig. 11B). The average frequency of transients further declined to only 27 transients/min after 15 min in RPMI 0% (Fig. 11B). These sheets that remained in RPMI 0% (Fig. 11A) began to disperse and lose cohesion more so than the sheets treated with RPMI 50% for an equivalent period of time (Fig. 10A). After ~30 minutes in RPMI 0%, the sheets displayed large regions of retraction and enlarged gaps within the sheets (Fig. 11A). These results confirm that calcium transients and sheet cohesiveness are strongly dependent on serum.

To observe the effects of either the presence or absence of serum on sheet morphology over longer periods of time (1-2 hours), time-lapse imaging was performed on unloaded cells using phase contrast, since this avoids the phototoxic effects of fluorescent light. Maintenance of these cells with or without serum for the duration of the imaging period showed similar results as those seen in the short-term observations on Calcium Green-loaded cells. In either RPMI 10% or RPMI 50%, sheets retracted slightly and remained cohesive, while sheets in RPMI 0% dispersed quickly. The sheet imaged in RPMI 50% retracted slightly at the edges but remained cohesive (Fig. 12A), while in RPMI 0% the cells pulled away from each other and gaps began to develop within the sheet (Fig. 12B). In these experiments, sheet edge complexity was used as a measure of sheet breakup. When cell sheets disperse and lose cohesion, the sheet edges become more

irregular as gaps form within the sheet and fragments of the sheet break off into the surrounding space. To quantify the changes in sheet edge morphology, segments of the sheet edges were traced in each frame, and the length of the resulting traced lines were plotted over time (Fig. 13). The cell sheet that was observed in RPMI 0% for 60 minutes exhibited a rapid, 2.2-fold increase in sheet edge line length, or sheet edge complexity, during the observation period (Fig. 13A,C, orange line). In contrast, the sheet that was observed in RPMI 50% for 100 minutes displayed only a 1.2-fold increase in line length (Fig. 13B,C, red line). On average, all of the sheets observed in the presence of serum displayed a 0.97-fold change in sheet area. Considering the results from both short-term and long-term sheet morphology observations, it appears that sheet cohesion is maintained in the presence of serum, but is compromised when cells are exposed to serum-free medium.

Gadolinium is commonly used to determine the involvement of stretch-activated calcium channels (SACs) in triggering calcium transients. It effectively reduces transients by blocking SACs, thereby preventing the stretch-induced influx of calcium ions into the cytoplasm that initiates calcium transients during CICR (Lee et al., 1999; Caldwell et al., 1998). Therefore, the goal of the following experiments was to observe the effects of blocking SACs on sheet morphology and cohesiveness. When cells were treated with Gadolinium, there was a sharp decrease in the number of transients, with a partial recovery after washing out most of the Gadolinium (Fig. 14). In this representative data set it was found that, 6 minutes following treatment with Gadolinium, the average frequency of calcium transients had been reduced to only 5 transients/min per field of view, compared to 888 transients/min pre-treatment (Fig. 14B,C). Ten minutes after the washout, the average frequency of transients increased to 149 transients/min, and decreased slightly to 100 transients/min at 40 minutes post-washout (Fig.

14B,C). These results are in agreement with the averages obtained from the complete set of Gadolinium experiments. The average frequency of transients before treatment with Gadolinium was 629 transients/min, which decreased to an average of 26 transients/min roughly 4 minutes after treatment with 10 μM Gadolinium (Fig. 15). One minute following the washout with FR 1%, the average frequency of transients remained low, at 18 transients/min. However, this value increased to an average of 75 transients/min after 10 minutes of recovery, and remained elevated at 89 transients/min after 40 minutes of recovery. In summary, treatment of cells with Gadolinium greatly disrupted the production of calcium transients, which could be recovered to some degree by removing most of the Gadolinium from the medium.

While our initial goal for these experiments included studying how this Gadolinium-induced decrease in transients affects sheet morphology, our efforts were hindered by the precipitation of Gadolinium in the presence of serum. This was an experimental limitation, as the cells appear to require a minimum amount of serum for the production of calcium transients, and even trace amounts of serum can produce these aggregates in the presence of Gadolinium. A possible way to circumvent this limitation is discussed later.

DISCUSSION

The goal of this work was to assess the effects of reducing the frequency of calcium transients on the morphology and cohesiveness of goldfish epithelial sheets during collective migration. Our results indicate that calcium transients are necessary for cohesion in migrating epithelial sheets through the formation and disassembly of adherens junctions. We also conclude that stretch-activated calcium channels (SACs) and growth factor-containing serum are both necessary for sheet cohesion and the production of calcium transients in goldfish keratocytes. In

this work we propose a model for the collective migration of goldfish epithelial sheets in which growth factors and SACs function in concert to generate calcium transients, which maintain sheet cohesiveness by facilitating the turnover of adherens junctions.

Our observation that cell sheets began to dissociate in the presence of EGTA implicates extracellular calcium in the maintenance of adherens junctions in goldfish keratocytes. This agrees with previous studies, in which depletion of extracellular calcium with EGTA led to the disruption of adherens junctions (Ko et al., 2001a) due to a weakening of the rigid extracellular domain of E-cadherin responsible for linking neighboring cells together (Sotomayor et al., 2008). Therefore, by reinforcing these domains of E-cadherin, we propose that extracellular calcium helps to maintain cohesiveness in sheets of goldfish keratocytes.

The observed reduction in the frequency of calcium transients and the loss of sheet cohesion when cells were treated with Thapsigargin highlights the importance of calcium transients in maintaining cell sheet integrity. We propose that fluctuations in intracellular calcium allow the formation, maintenance, and disassembly of adherens junctions by regulating intracellular contractile forces. Calcium transients are known to regulate mechanical forces through the calcium-induced activation of MLCK, which then induces myosin II-based contractility of the actin cytoskeleton (Doyle et al., 2004; Wei et al., 2012). Studies have shown that adherens junctions are highly responsive to such cell-generated mechanical forces (Baum and Georgiou, 2011). Under normal circumstances, it is possible that the contractile force produced from a single calcium transient is enough to simultaneously reinforce nascent cell-cell adhesions, while causing the disassembly of pre-established adhesions as they come under tension. Previous studies on sheets of human gingival fibroblasts have implicated calcium transients in the strengthening of adherens junctions between neighboring cells (Ko et al., 2001a;

Ko et al., 2001b). The formation of nascent adherens junctions between cells results in the stretching of cell membranes, stimulating calcium transients in the adjoined cells (Ko et al., 2001a; Ko et al., 2001b). The subsequent influxes in calcium ions promote actin cytoskeletal rearrangement at the adhesion sites and recruit additional cadherin and β-catenin to strengthen the nascent adhesions (Ko et al., 2001a; Ko et al., 2001b). Calcium-induced increases in contractility may also facilitate the disassembly of adherens junctions, either directly by rupturing the adhesions, or indirectly through a force-mediated increase in the dissociation rate of adherens junctions (Wolfenson et al., 2011). However, circumstances which interfere with the calcium-induced regulation of contractility may lead to the misregulation of adherens junction assembly and disassembly. If too much or not enough force is applied to adherens junctions, they can become disrupted and disassemble (Theveneau and Mayor, 2013). Thapsigargin has been found to not only inhibit fluctuations in intracellular calcium, but to cause elevated cytosolic calcium levels as calcium ions are prevented from re-entering the ER (Lytton et al., 1991). We observed a unique two-part response in cells treated with Thapsigargin. During the initial response, keratocytes initially took on an elongated and stretched appearance as they began to retract and dissociate from one another; a response indicative of sustained, increased cytoskeletal contractility and poor cell-substratum adhesion turnover. It is possible that, if cell-substratum and cell-cell adhesions are both regulated in the same manner by calcium-induced fluctuations in contractility, then cell-cell adhesion turnover may also have been impacted by Thapsigargin. We suggest that the sustained, elevated level of cytosolic calcium in treated keratocytes was responsible for rupturing their adherens junctions by inducing strong contractile forces in these cells. To our surprise, cells exhibited a secondary response to Thapsigargin roughly 30 to 50 minutes after treatment, in which cells became less elongated and began to

protrude into free space. This supports our suggestion that the contractility-induced rupture of adherens junctions led to the initial response to Thapsigargin, which subsequently alleviated some degree of cytoskeletal tension, allowing cells to protrude more freely. However, cells still appeared to have difficulty retracting at the rear, indicative of poor turnover of cell-substratum adhesions. A similar response to Thapsigargin was observed in single migrating keratocytes (Lee et al., 1999). The effects of Thapsigargin on keratocyte sheets illustrate that the proper regulation of adherens junction turnover is critical for the maintenance of cohesive cell sheets. With regular intracellular fluctuations in calcium, individual adherens junctions are constantly assembled and disassembled. This is critical for relieving tension within cell sheets, as it allows the sheet morphology to change without rupturing cell-cell adhesions and compromising sheet integrity (Baum and Georgiou, 2011). However, when calcium transients are inhibited following Thapsigargin treatment and adherens junctions cannot disassemble in a controlled manner, they may simply rupture such that the entire cell-cell adhesion is lost.

We have found that blocking SACs or depleting serum from the medium results in a reduced frequency of calcium transients in goldfish keratocytes. These findings are in agreement with previous studies on single migrating fish keratocytes, in which Gadolinium treatment resulted in a reduced frequency of calcium transients (Lee et al., 1999). We also observed a decrease in sheet cohesiveness in keratocytes maintained in serum-free medium. Based on our results and those from previous studies, we propose that SACs and serum work in concert to regulate cytoskeletal contractility, and thus cell-cell adhesion turnover, through the generation of calcium transients. We further suggest that SACs and serum help regulate contractility through a rapid MLCK (calcium-dependent) and slower Rho kinase (calcium-independent) mechanisms, respectively (Morin et al., 2014). The rapid calcium-dependent fluctuations in contractility

produced by SACs may support sheet cohesiveness. Cells that experience a higher frequency of calcium transients may be better able to turn over their adherens junctions and respond to external forces while maintaining cell-cell contacts. Our observation that calcium transients are dependent on the presence of serum has also been observed in human vascular smooth muscle cells, and may be attributed to the presence of growth factors (Scherberich et al., 2000). In a previous study on calcium signaling during wound healing in corneal epithelial cells, it was found that cells exhibited a greater number of calcium transients upon wounding in the presence of epidermal growth factor (EGF) than upon wounding without growth factors present (Klepeis et al., 2001). It is possible that the serum-dependent calcium transients we observe here are a result of elevated cytoskeletal contractility, induced by growth factors. Previous studies on fish keratocyte motility have implicated Rho kinase in maintaining long-term cytoskeletal contractility via indirect activation of myosin II (Morin et al., 2014). Therefore, we propose that Rho kinase signaling may raise the baseline level of cytoskeletal tension such that only small increases in contractility are needed to activate SACs. This would support more frequent production of stretch-induced calcium transients. In this manner, long-term maintenance of a baseline level of contractility may help to maintain sheet cohesion by promoting the regular turnover of adherens junctions.

We have devised a model for the collective migration of fish epithelial keratocytes where sheets maintain their cohesiveness through the concerted actions of SACs and growth factors in serum, which induce either short-term or long-term fluctuations in contractility, respectively. Cell-cell adhesions require rapid, controlled fluctuations in contractility for regular assembly and disassembly.

In light of our findings and proposed model for sheet cohesion, several experiments could be done in the future to expand our current understanding of collective migration in goldfish keratocytes. We would first test the effects on calcium transients and sheet morphology of chelating intracellular calcium using the cell-permeable chelating agent, BAPTA. As BAPTA chelates intracellular calcium specifically, this experiment would confirm the effects of dampening fluctuations in intracellular calcium levels while extracellular calcium is still present. As observed in previous studies (Klepeis et al., 2001), we would expect to see a reduction in calcium transients following BAPTA treatment. Based on our findings here, we would also expect a subsequent decrease in sheet cohesiveness.

We would also like to further examine the effects of blocking SACs on cell-cell adhesions. As our current work with Gadolinium was hindered by the formation of aggregates, we would need to select an alternative SAC blocker to allow us to study changes in sheet morphology. To identify the most prevalent SAC proteins expressed in goldfish keratocytes, we could perform a real-time RT-PCR reaction using known primers specific to several possible transient receptor potential (TRP) family proteins, which have previously been detected in zebrafish keratocytes (Graham et al., n.d.). This procedure would allow us to then select a non-lanthanide inhibitor that is specific to the identified SAC proteins and would not produce aggregates with components of serum. We expect that blocking SACs and preventing the generation of stretch-induced calcium transients would disrupt the rapid cycling of contractility and thus adhesion turnover, causing cell sheets to disperse.

To further investigate whether the observed effects of serum are due to growth factor signaling or to some other component of serum, we would like to treat cells with individual, purified growth factors, such as EGF or TGF, which are known to indirectly activate RhoA.

Assuming our model is correct, we would expect to see an increase in calcium transient frequency and maintenance of sheet cohesion comparable to treating cells with 50% serum. An alternative approach to testing whether or not growth factors are responsible for the observed loss of sheet cohesion would be to focus further downstream in the signaling pathway at Rho kinase specifically. Using a Rho kinase inhibitor, such as Y-27326, to inactivate Rho kinase, we would expect the baseline cytoskeletal contractility to decrease, thus disrupting the production of calcium transients and cell-cell adhesions. If purified growth factors and a Rho kinase inhibitor can both reproduce the results found from adding and depleting serum, respectively, this would support our model for the requirement of serum for sheet cohesion.

In the future we would also like to investigate the possibility of calcium transients traveling as intercellular waves, and whether such wave propagation plays a role in maintaining sheet cohesiveness. Other studies have reported this phenomenon in various cell types, and propose that calcium ions or IP3 may travel between cells through gap junctions to induce transients in neighboring cells (Junkin et al., 2014; Paemeleire et al., 2000). Given what we know about the necessity of calcium transients for cell-cell adhesion, it is possible that such a mechanism of calcium transient propagation throughout the cell sheet helps coordinate calcium transient activity, and thus contractile forces and adhesion turnover, in connected cells. We would like to test this theory by inhibiting gap junctions with 18-α-glycerretnic acid and observing the effects on calcium transients and sheet morphology.

The findings we present here contribute to our understanding of how calcium transients help cells within sheets to remain connected during collective migration. The regulation of calcium transients, as well as their role in cell-cell adhesion, are of great importance in identifying the underlying processes that drive cancer metastasis, thus providing clues to its

prevention. The clearer our understanding of the mechanisms that keep migrating bodies of cells connected, the better we can control such mechanisms when they go awry.

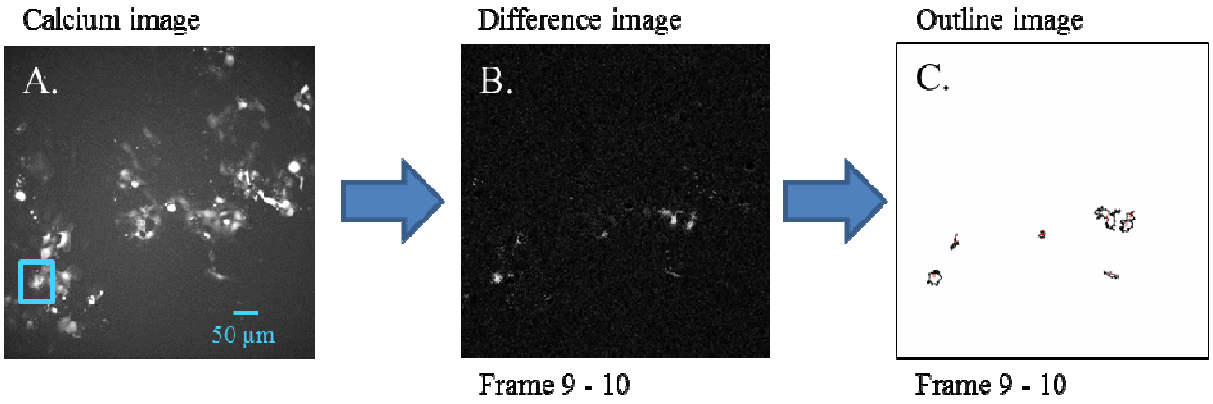
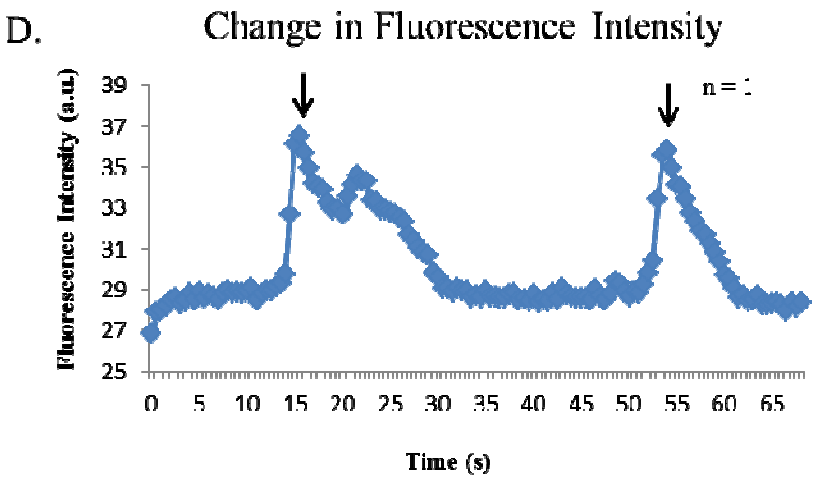
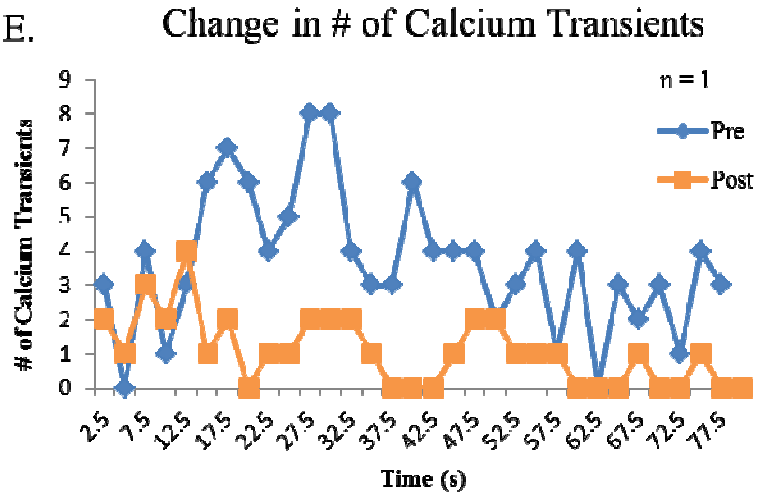


Fig. 1 – Diagrammatic representation of the methods for analyzing changes in calcium transients. (A) Calcium fluorescence image of a cell sheet loaded with Calcium Green indicator. Bar, 50 µm. (B) Difference image of calcium transients occurring between frames 9 and 10 of the time-lapse sequence. (C) Outline image of calcium transients in (B). (D) Plot of the average fluorescence intensity over time in the cell in (A) (box, n=1). Two transients occur during a 65 s observation period (arrows). (E) Plot of the number of calcium transients over time in cells from (A), pre-treatment (blue line) and post-treatment (orange line) (n=1).





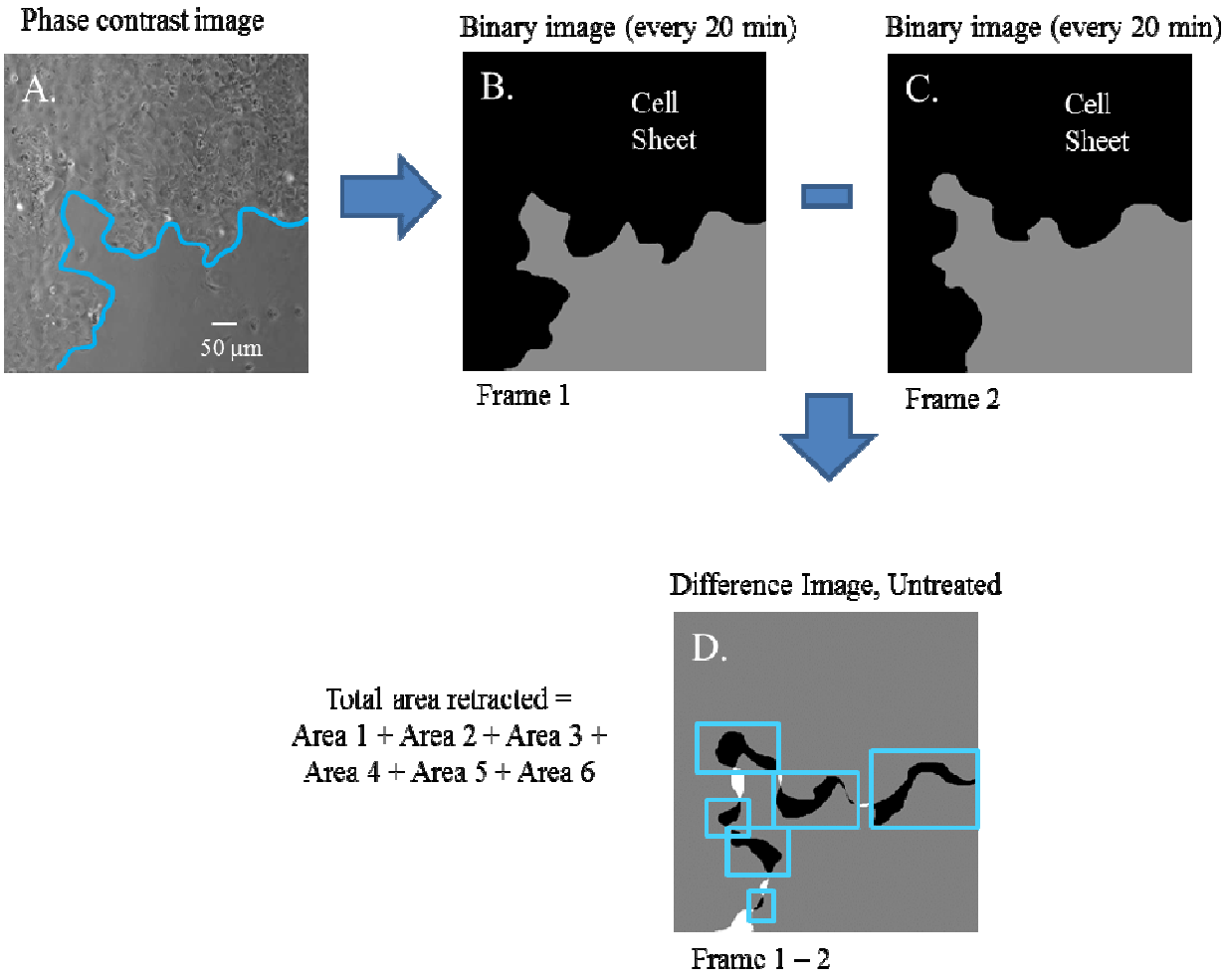


Fig. 2 – Diagrammatic representation of the methods for analyzing changes in sheet morphology. (A) Phase contrast image of a cell sheet pre-treatment. Blue line indicates sheet edge. Bar 50 µm. (B) Binary image of frame in (A). (C) Binary image of next frame in sequence after (B). (D) Difference image showing regions of retraction (dark regions, blue boxes) between frames in (B-C).

A. Untreated (in RPMI 10%)

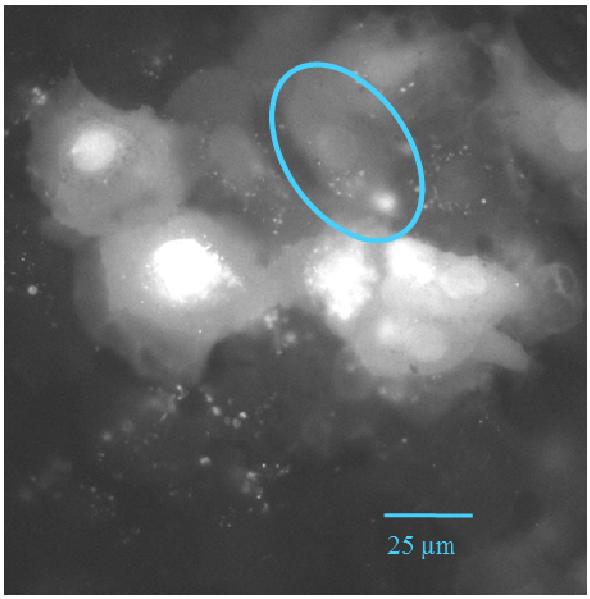
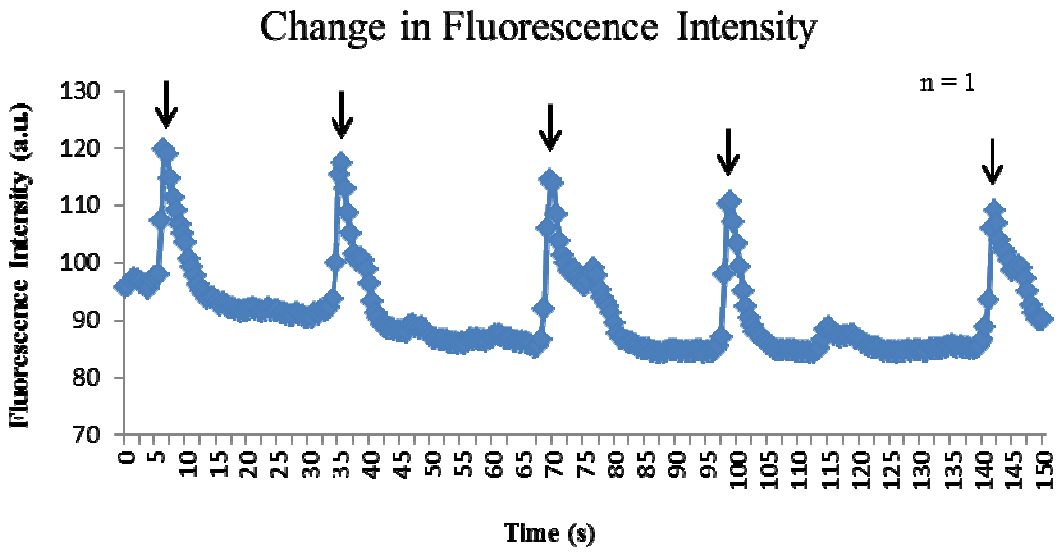


Fig. 3 – Calcium transients in untreated cells. (A) A calcium fluorescence image of an untreated cell sheet loaded with Calcium Green indicator. Bar, 25 µm. (B) Plot of fluorescence intensity versus time from the cell (blue oval) in (A). Five calcium transients (arrows) occur during the 2.5 min of observation (n=1).

B.



A.

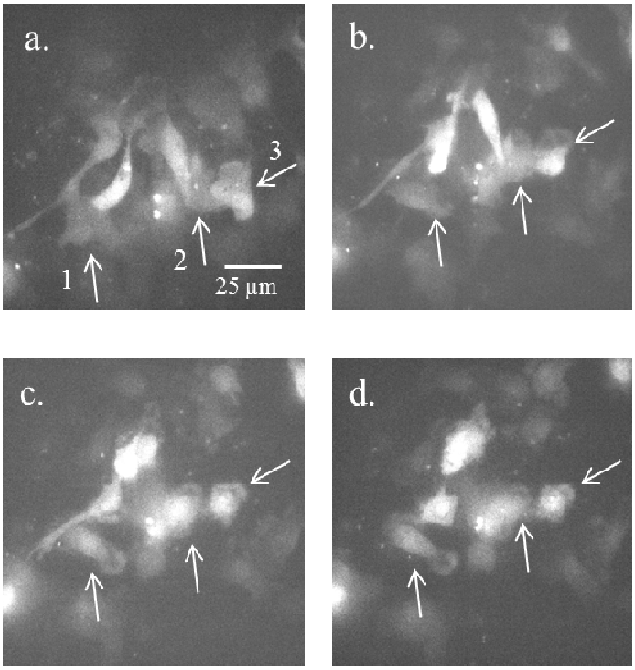
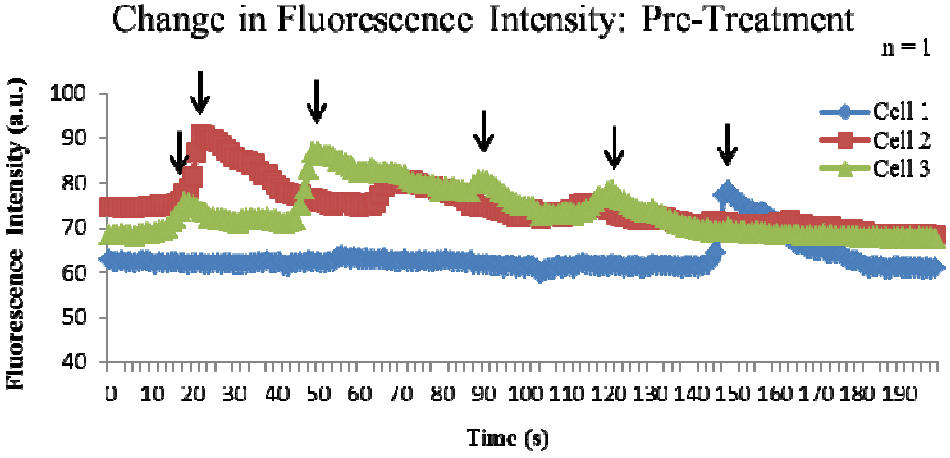
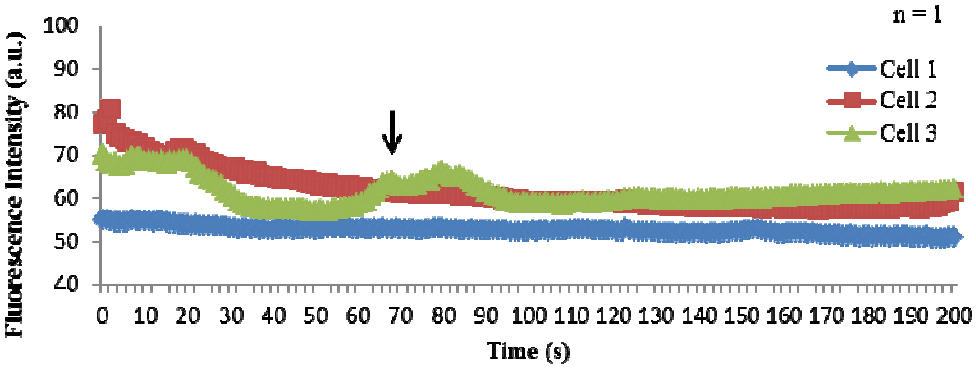


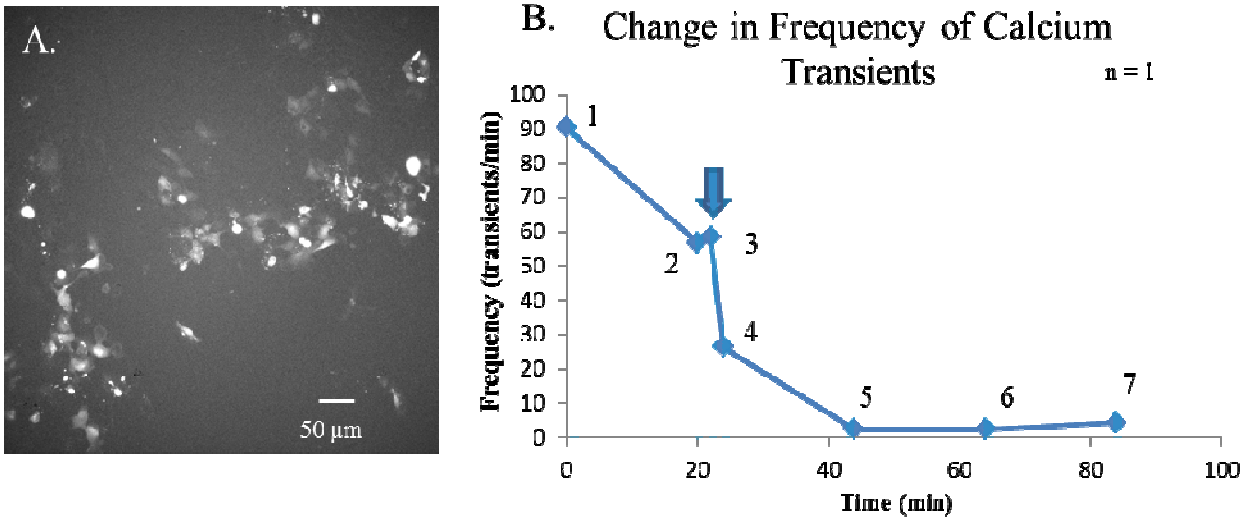
Fig. 4 – Effects of EGTA on the number of calcium transients and sheet morphology. (A, a-d) Calcium fluorescence images of cells loaded with Calcium Green. Bar, 25 µm. (A, a) Cell sheet 3 min before EGTA addition. (A, b-d) Cell sheet 60 s, 110 s, and 175 s after EGTA addition, respectively. (B-C) Plot of fluorescence intensity over time in the cells indicated in (A - cell 1, 2, and 3, white arrows) (n=1). (B) A 200 s sample of observation pre-treatment. One transient (black arrows) occurs in two of the cells (red, blue lines), and 4 transients occur in the third cell (green line). (C) A 200 s sample of observation post-treatment. One transients occurs in the third cell (green line). Average baseline intensity of each cell declined following the addition of EGTA, which may be attributed to fading of the Calcium Green indicator fluorescence.

B.



C.





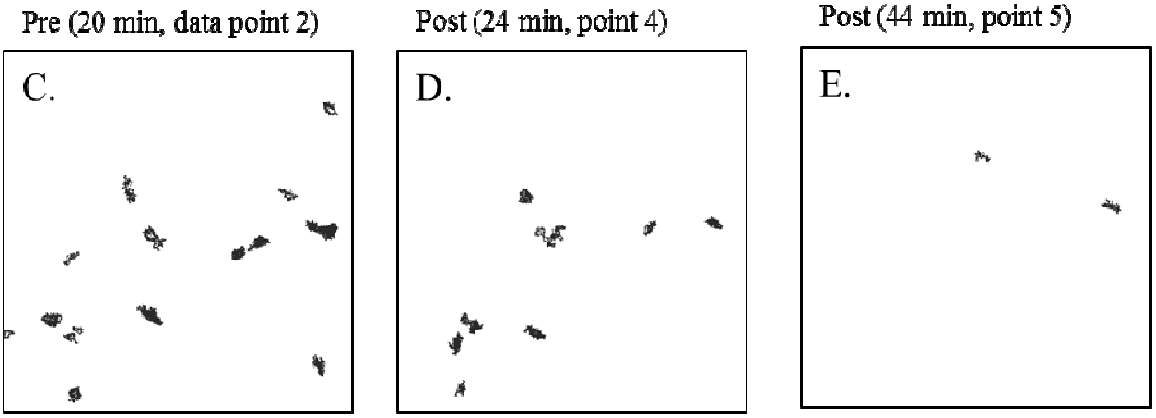
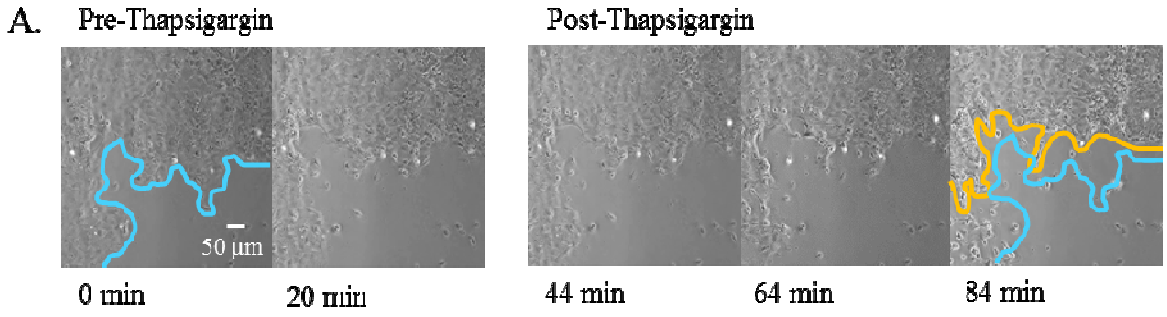


Fig. 5 – Representative example of the effects of Thapsigargin on the frequency of calcium transients. (A) Calcium fluorescence image of a cell sheet loaded with Calcium Green indicator, representing one frame from a time-lapse video. Bar, 50 µm. (B) Plot of the average frequency of calcium transients from each video (1.5 min duration, blue bars), for the entire sheet (A) before and after the addition (blue arrow) of Thapsigargin (n=1). (C-E) Outline images of the sum of transients from each video (1.5 min duration), represented by data points 2, 4, and 5 respectively (B).



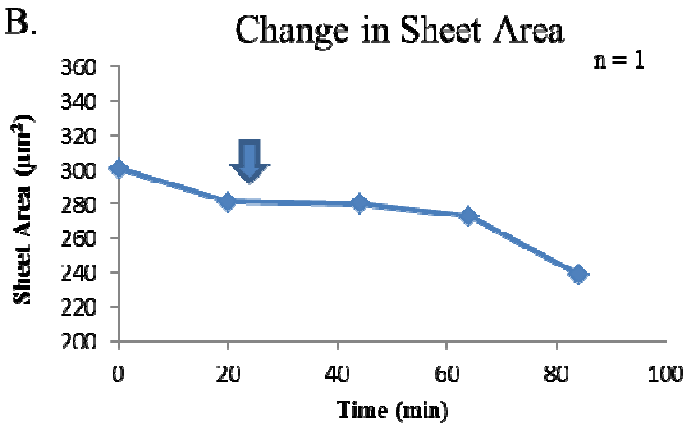
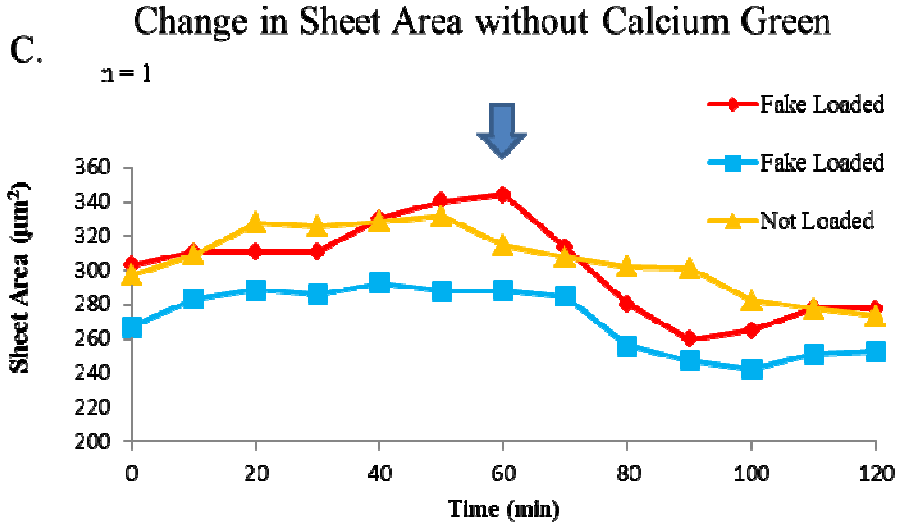


Fig. 6 (A-C) – Representative example of the effects of Thapsigargin on sheet morphology. (A) Phase contrast images of a cell sheet (Fig. 5A) with emphasis on the change in position of the sheet edge before (blue line, 0 min and 84 min) and after (orange line, 84 min) treatment with Thapsigargin. Bar, 50 µm. (B-C) Change in sheet area in cells loaded (B) and not loaded (C) with Calcium Green, before and after the addition (blue arrow) of Thapsigargin (n=1). Y-axis values (B-C) have been divided by 1000 for illustrative purposes. (D-F) Plot of the area retracted (D) and number of retracted regions (E) over time, before and after the addition (blue arrow) of Thapsigargin (n=1).



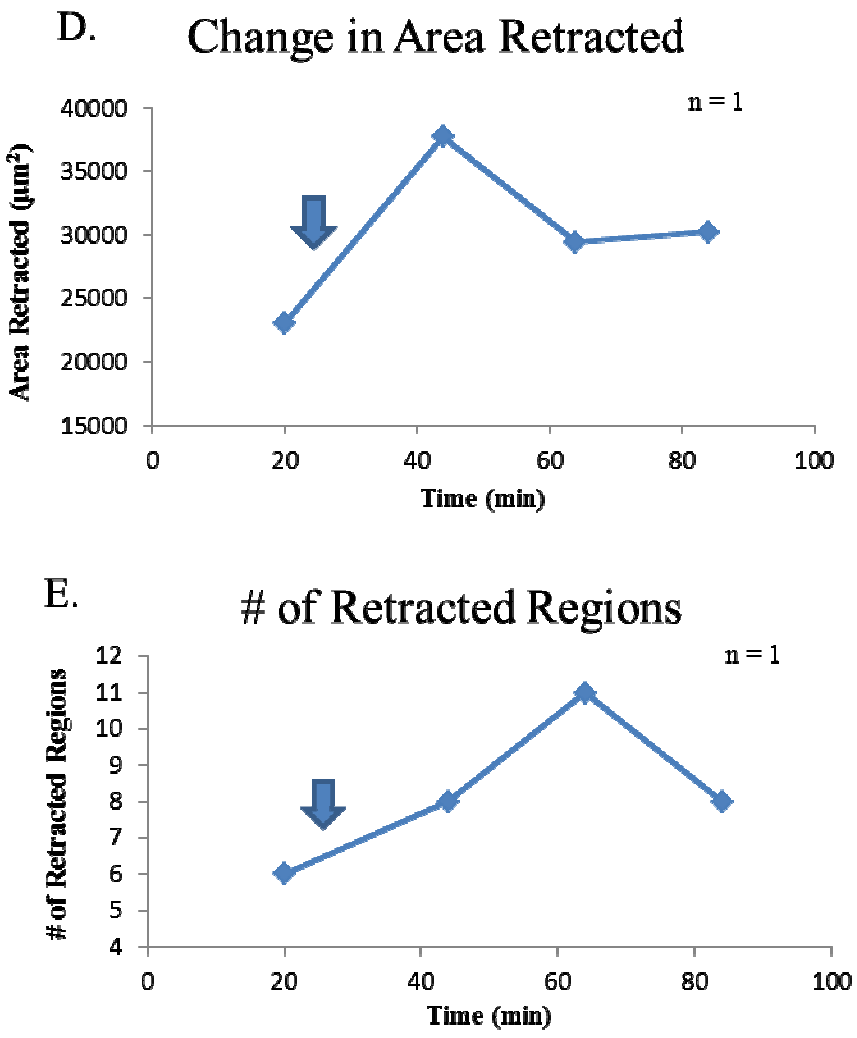


Fig. 6 (D-E) – Representative example of the effects of Thapsigargin on sheet morphology.

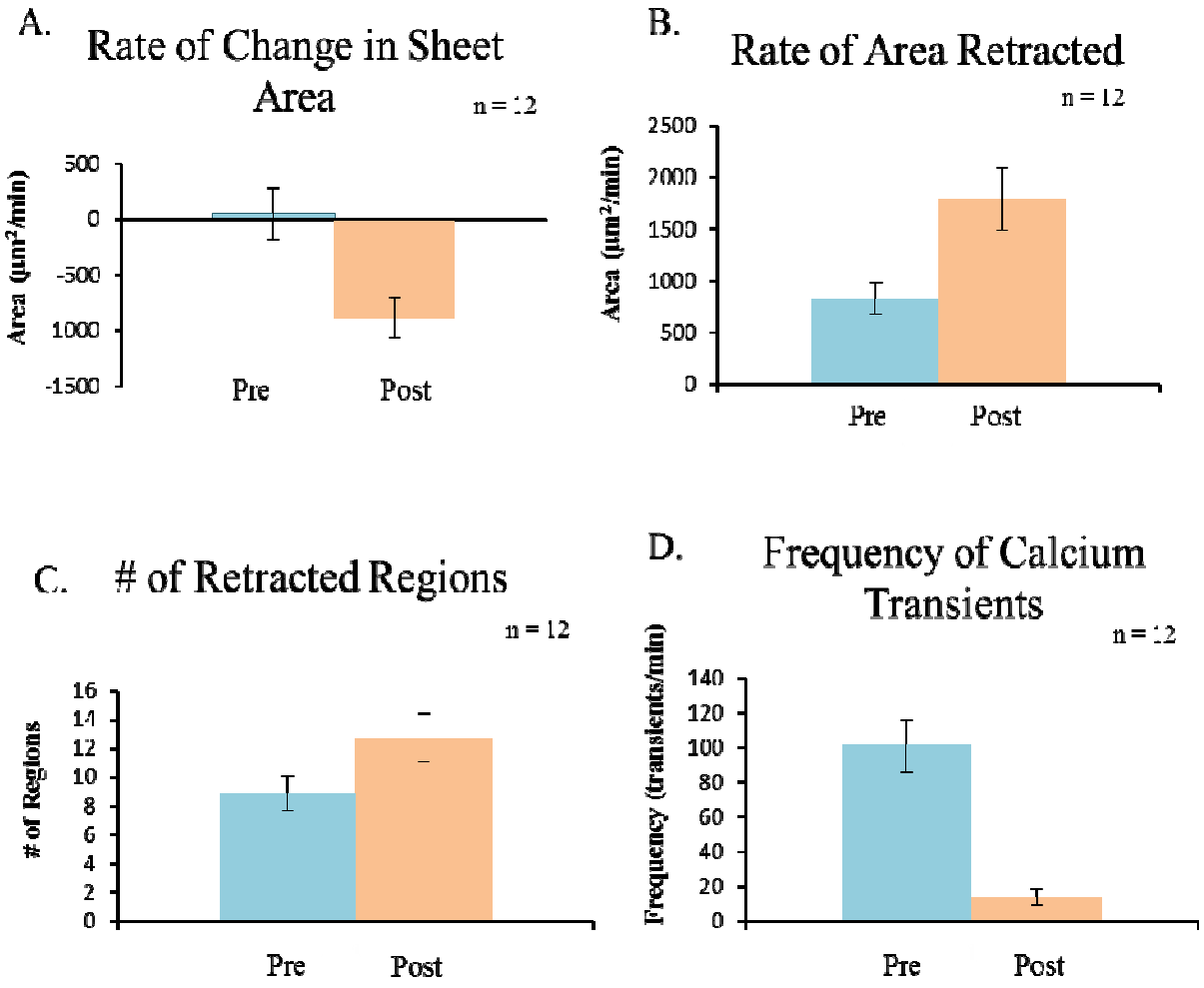


Fig. 7 – The average effects of Thapsigargin on the frequency of calcium transients and sheet morphology. (A-D) Bar graphs of the average rates of change in sheet area (A), rate of area retracted (B), number of retracted regions (C), and frequency of calcium transients (D), pre (teal) and post (orange) Thapsigargin treatment (n=12). Note that the pre-treatment (teal) y-axis value in (A) has been multiplied by 3 for illustrative purposes.

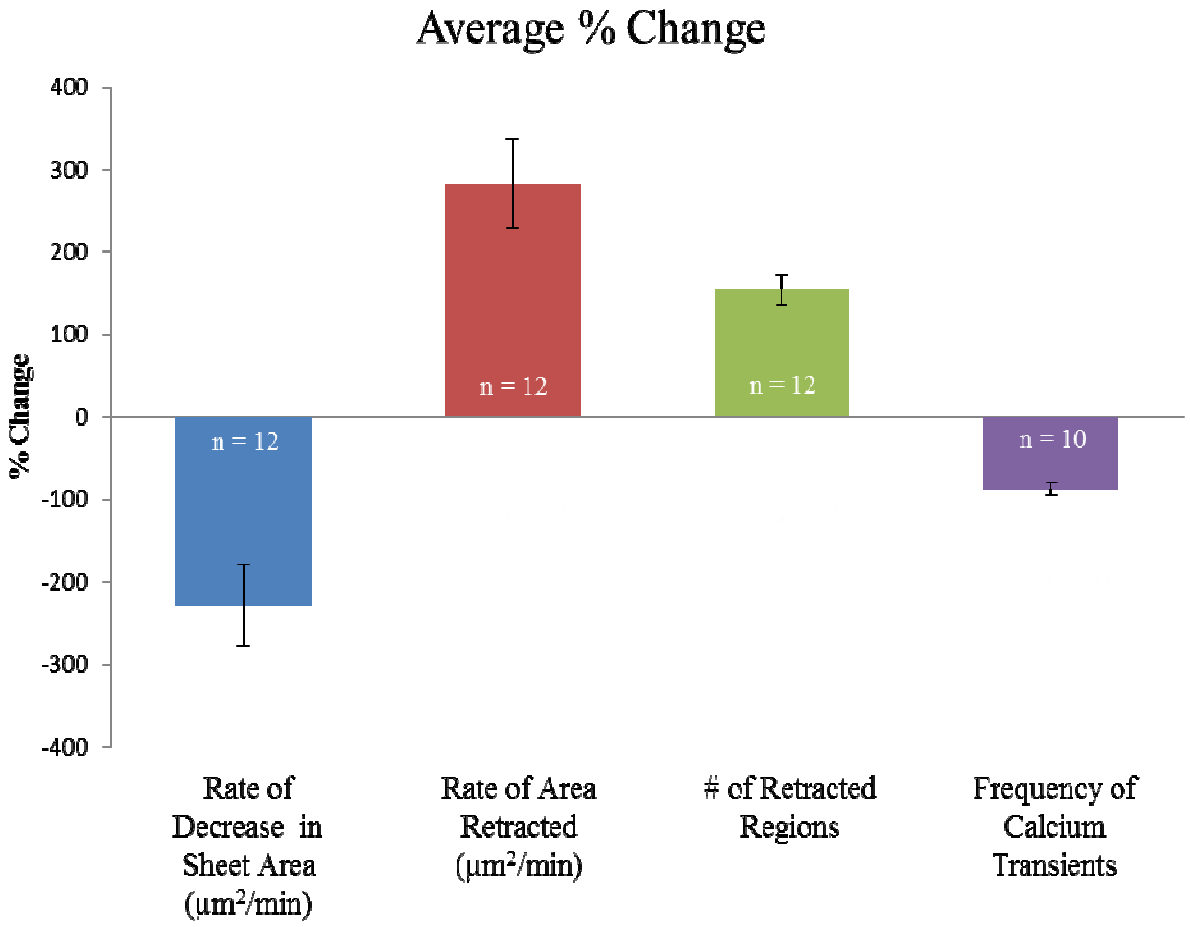
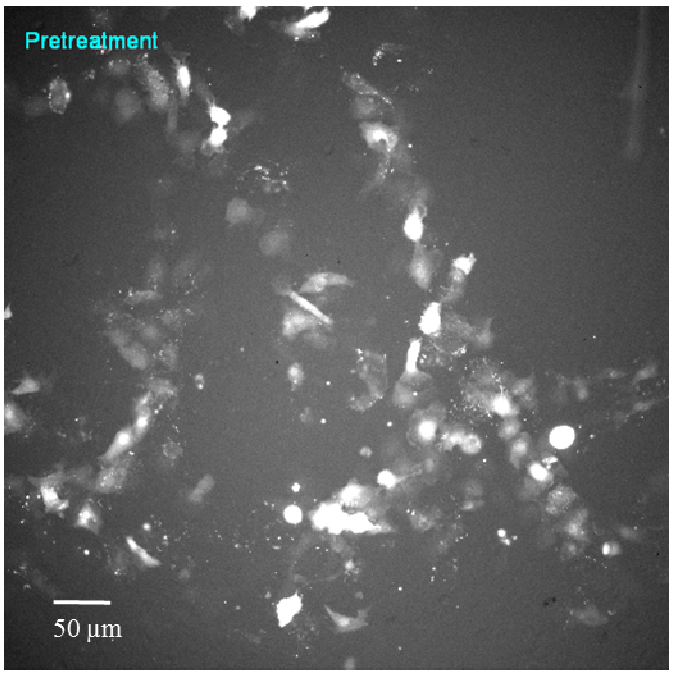


Fig. 8 – Summary of the average percent changes between pre- and post-Thapsigargin treatment for each parameter tested. Bar graph of the average percent change in the rate of decrease in sheet area (blue bar, n=12), the rate of area retracted (red bar, n=12), the number of retracted regions (green bar, n=12), and the frequency of calcium transients (purple bar, n=10). Note that the standard error of the mean for average percent change in frequency of calcium transients has been multiplied by 3 for illustrative purposes.

A.



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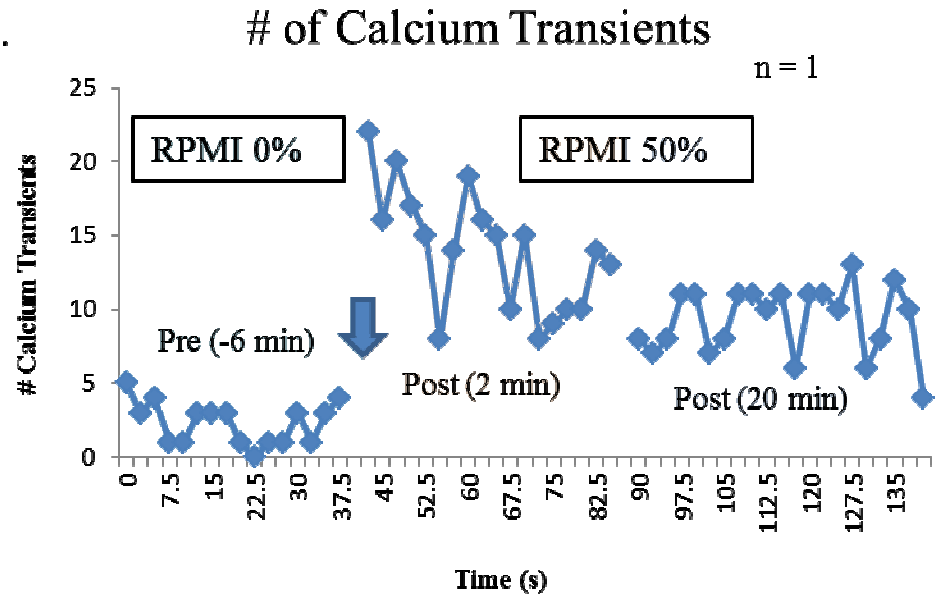
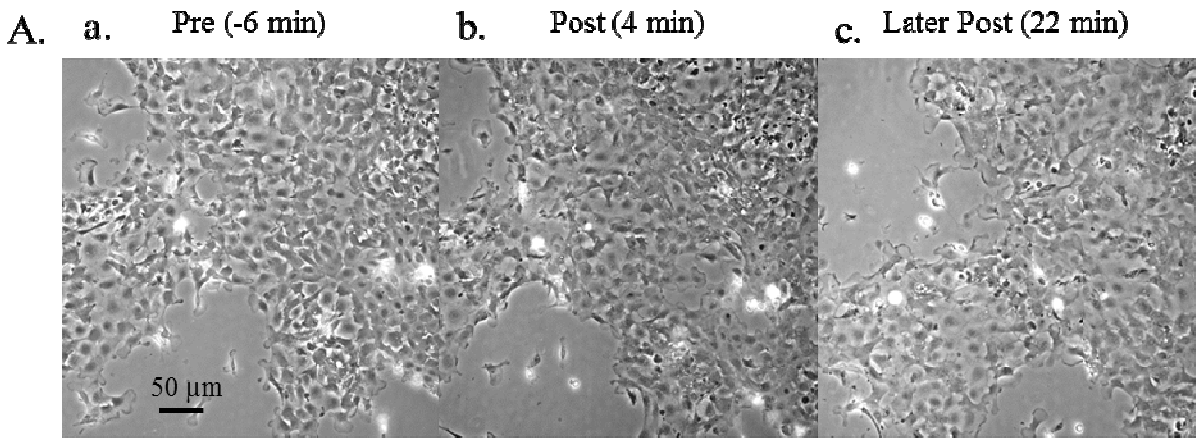


Fig. 9 – A representative example of the short-term effects of serum addition on the number of calcium transients. (A) Calcium fluorescence image of cells loaded with Calcium Green indicator, in RPMI 0%. Bar, 50 µm. (B) Plot of the number of calcium transients in the cell sheet (A) over time from time-lapse videos acquired 6 minutes before (teal), 2 minutes after (orange), and 20 minutes after (blue) the switch (blue arrow) to RPMI 50% serum. (n=1).



Pre-treatment in RPMI 0% serum, Post-treatment in RPMI 50% serum

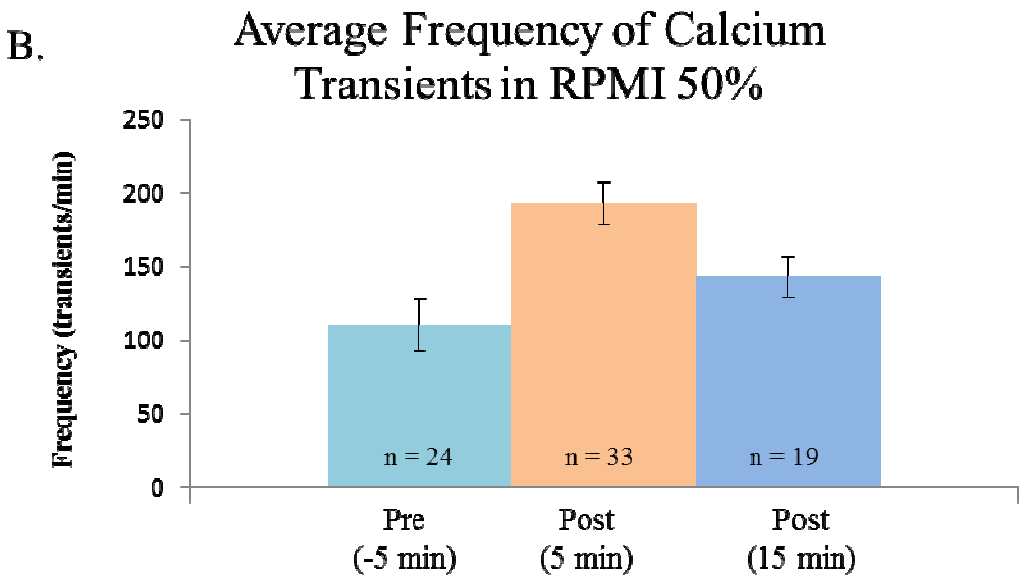
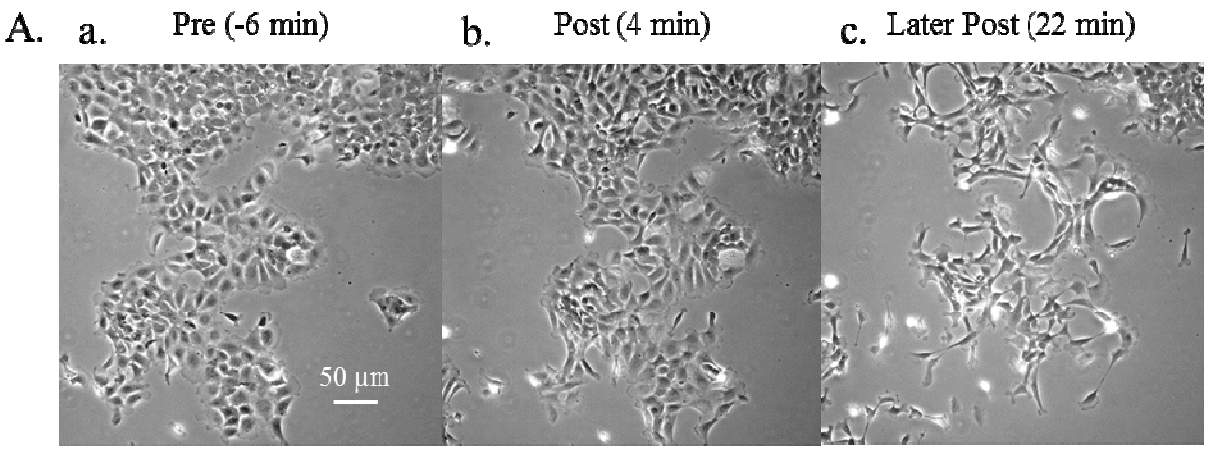


Fig. 10 – The short-term effects of treatment with RPMI 50% on the frequency of calcium transients and sheet morphology. (A) Phase contrast images of a representative cell sheet 6 min before (a), 4 min after (b), and 22 min after (c) treatment with RPMI 50%. (B) Bar graph of the average frequency of calcium transients in cell sheets, on average 5 min before (teal bar, n=24), 5 min post (orange bar, n=33), and 15 min post (blue bar, n=19) treatment with RPMI 50%.



Serum-Free: Pre-treatment in RPMI 0% serum, Post-treatment in RPMI 0% serum

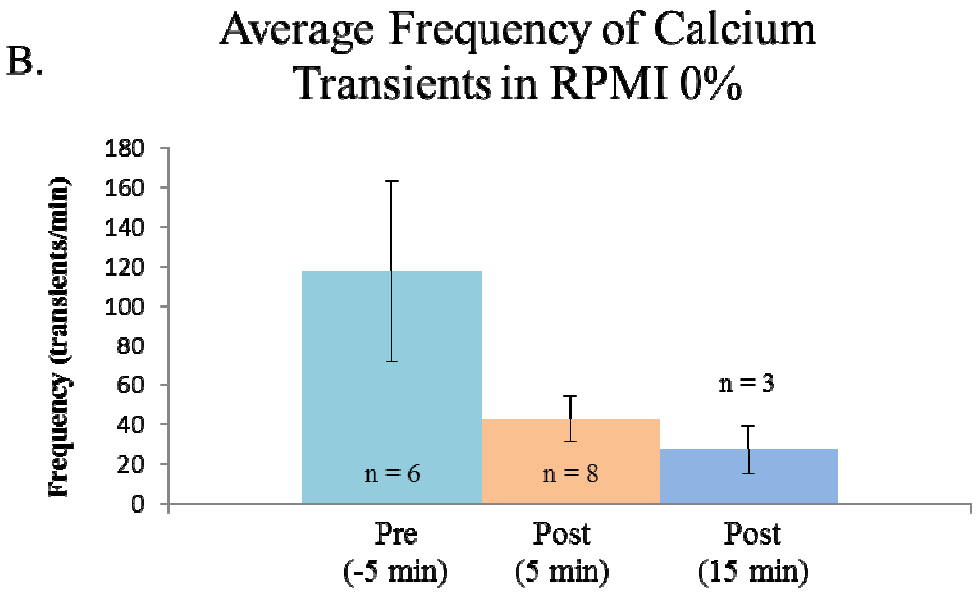


Fig. 11 – The short-term effects of treatment with RPMI 0% on the frequency of calcium transients and sheet morphology. (A) Phase contrast images of a representative cell sheet 6 min before (a), 4 min after (b), and 22 min after (c) treatment with additional RPMI 0%. (B) Bar graph of the average frequency of calcium transients in cell sheets, on average 5 min before (teal bar, n=6), 5 min post (orange bar, n=8), and 15 min post (blue bar, n=3) treatment with additional RPMI 0%.

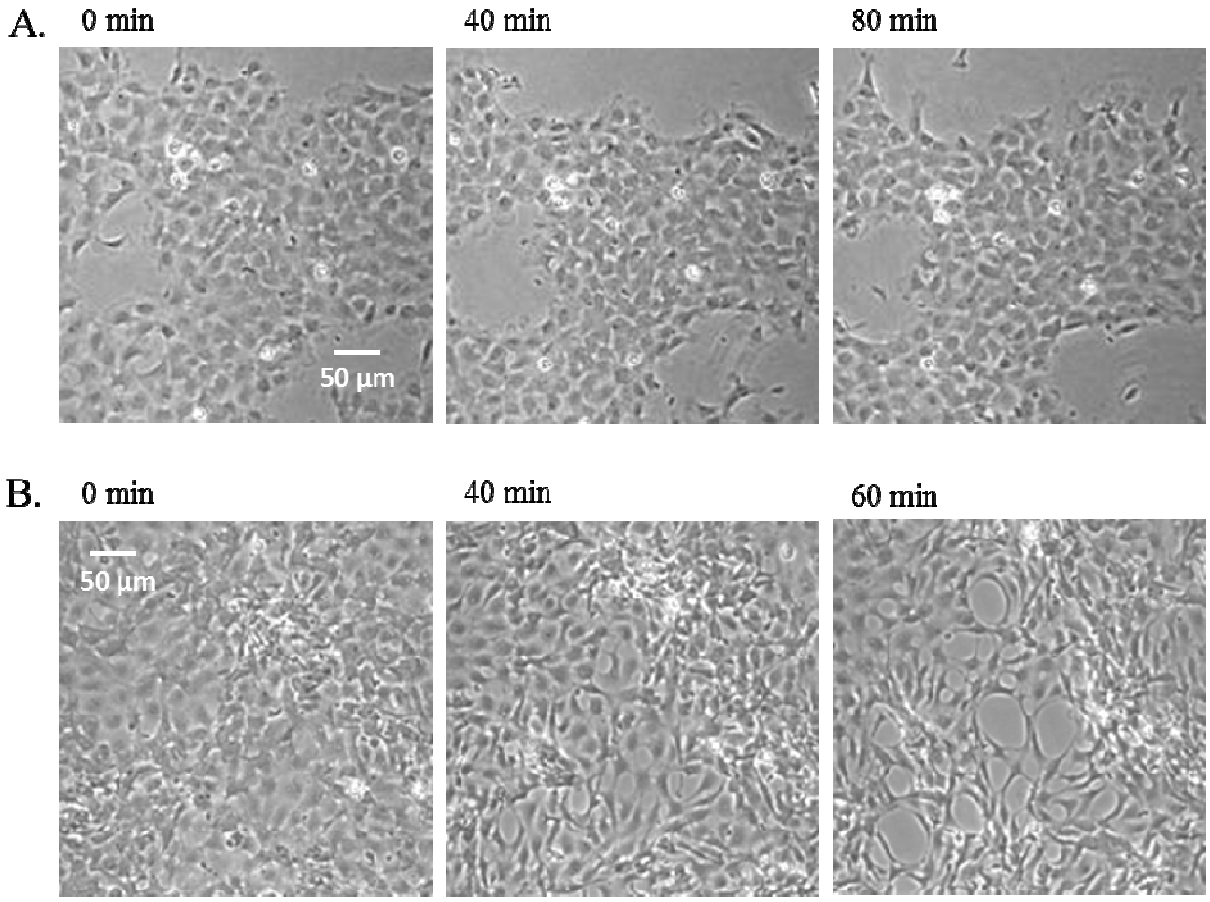


Fig. 12 – The long-term effects on sheet morphology of treating cells with serum (RPMI 10% or RPMI 50%) or without serum (RPMI 0%). (A and B) Phase contrast images cropped to illustrate changes in morphology in RPMI 50% (A) at 0, 40, and 80 min of observation, or in RPMI 0% (B) at 0, 40, and 60 min of observation. Bar, 50 µm.

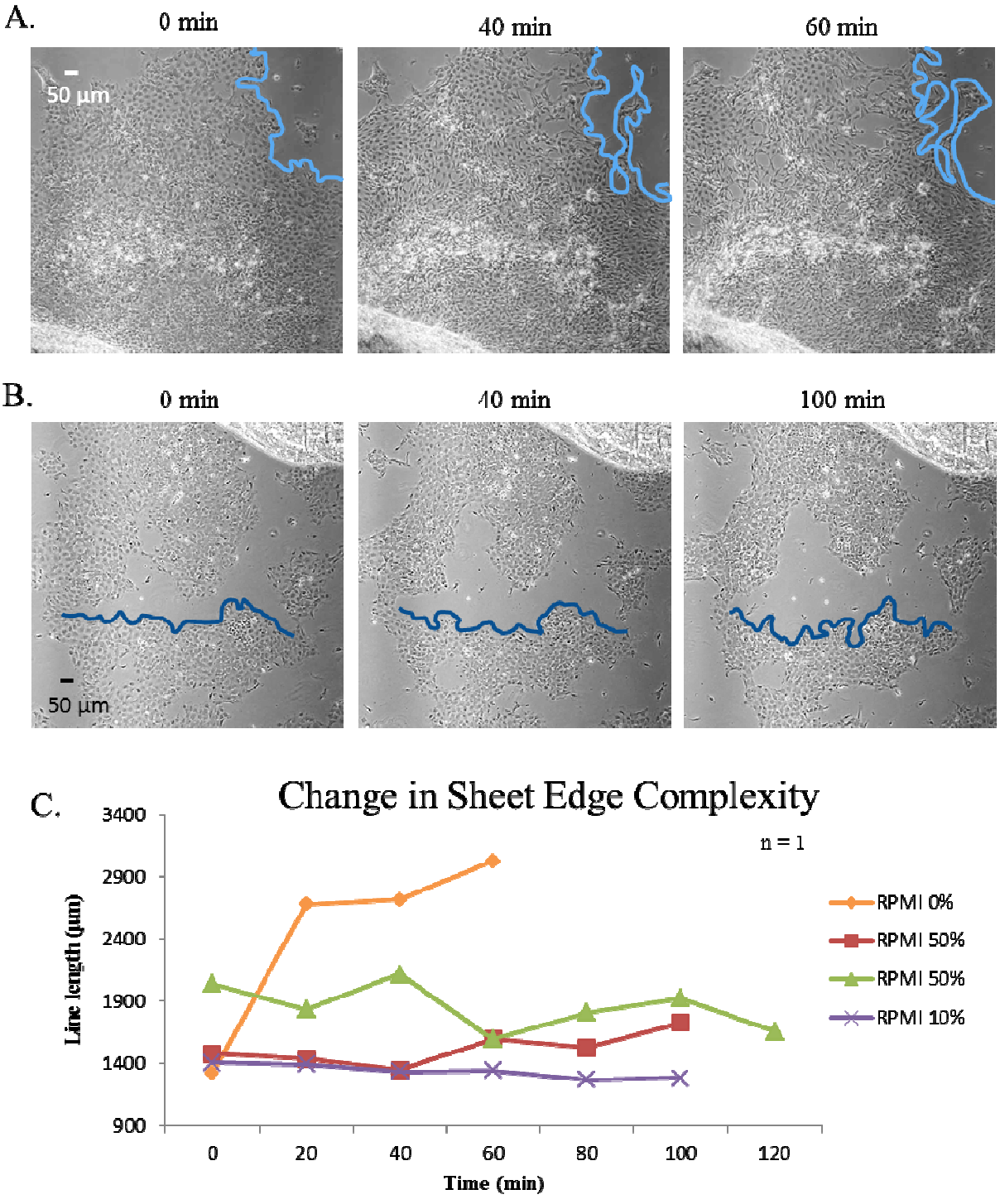


Fig. 13 – The long-term effects on sheet morphology of treating cells with serum (RPMI 10% or RPMI 50%) or without serum (RPMI 0%). (A and B) Phase contrast images illustrating changes in sheet edge complexity (blue outlines) in RPMI 0% (A) or RPMI 50% (B). Bar, 50 µm. (C) Plot of change in sheet edge line length (µm) over time (min) in RPMI 0% (A, orange line), RPMI 50% (B, red line; green line), and RPMI 10% (purple line). n=1.

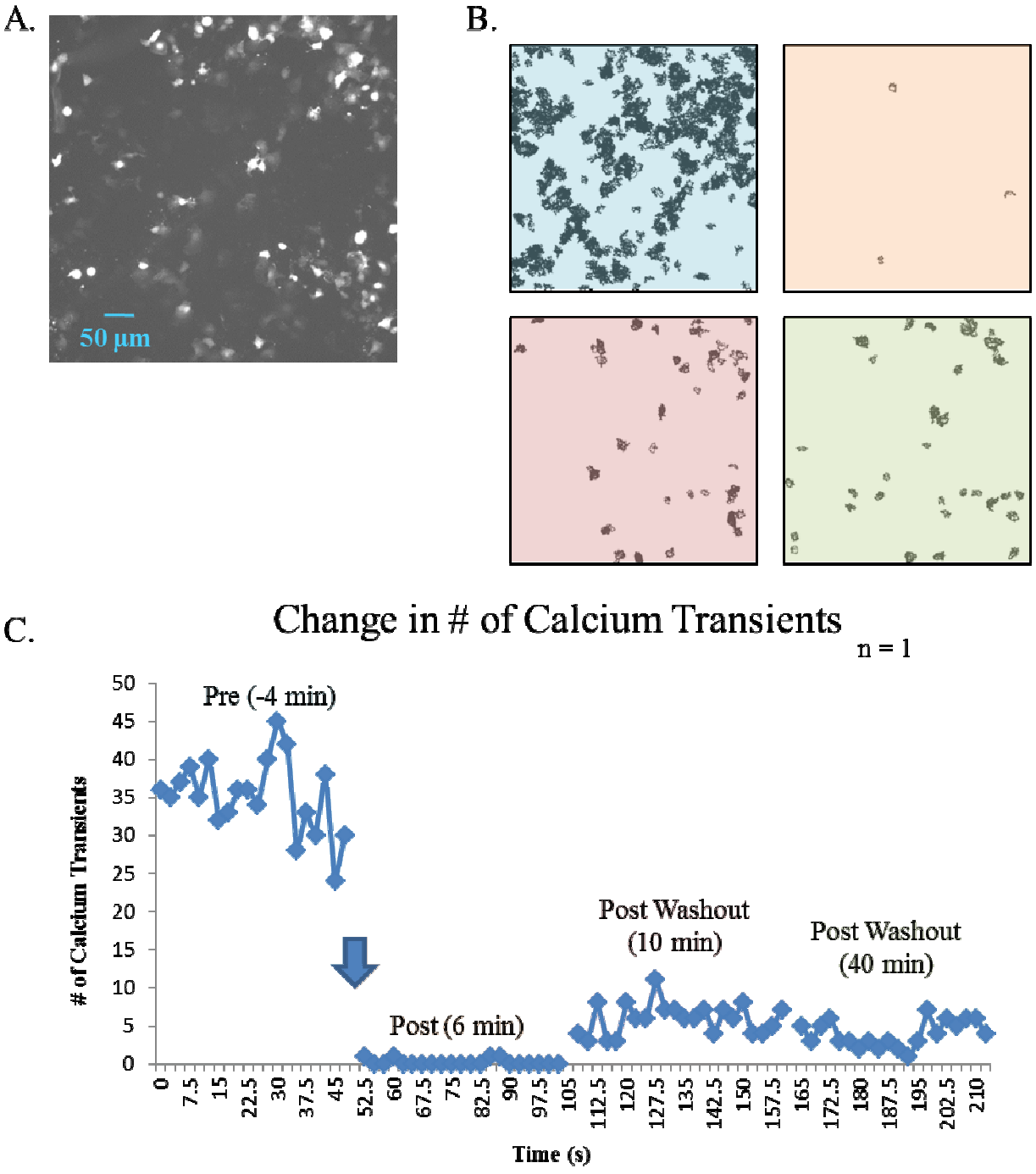


Fig. 14 – Representative example of the effects of Gadolinium treatment and washout on the number of calcium transients. (A) Calcium fluorescence image of cells loaded with Calcium Green indicator, in FR 1% serum. Bar, 50 µm. (B) Outline images of the sum of calcium transients in the 4 conditions plotted (C). (C) Plot of the number of calcium transients in the cell sheet (A) during each time-lapse video (duration ~1 min), 4 min before (teal) and 6 min after (orange) the addition of Gadolinium (blue arrow), as well as 10 min (red) and 40 min (green) after washing out Gadolinium. (n=1).

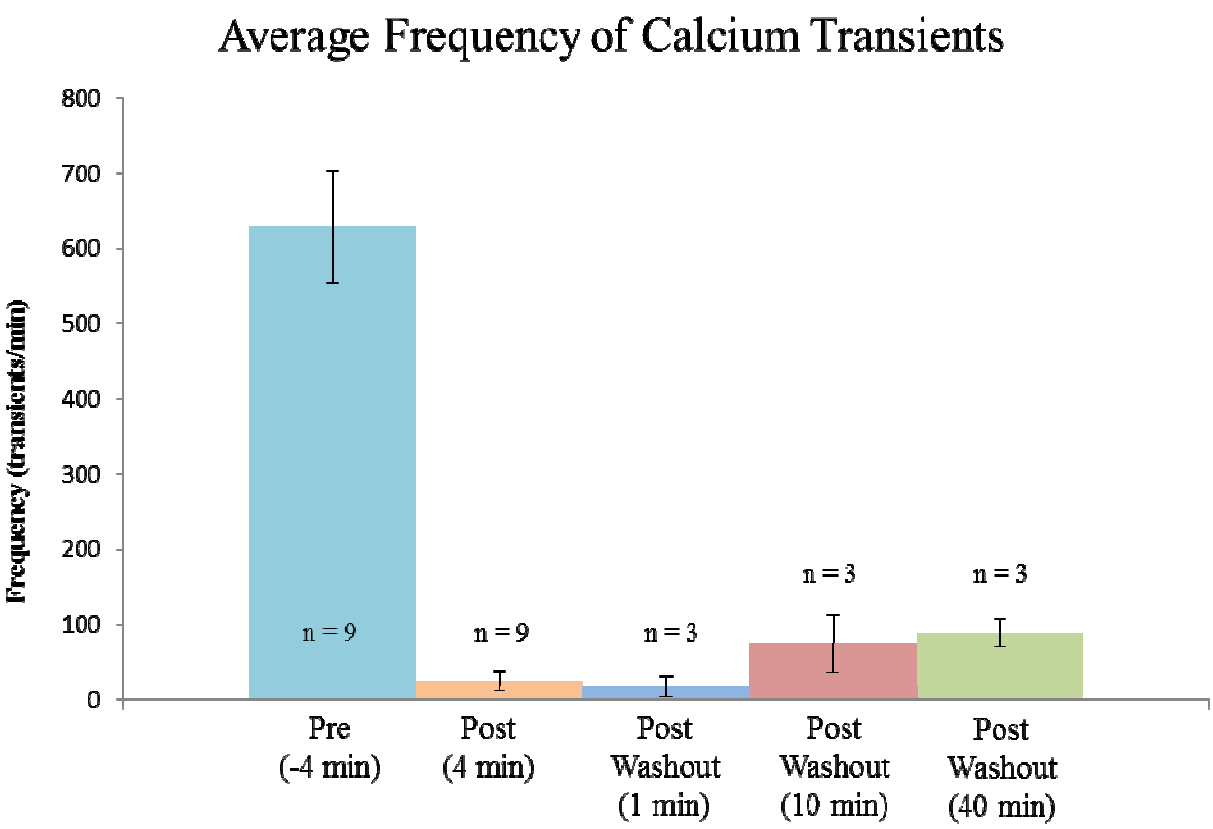


Fig. 15 – The average effects of Gadolinium treatment and washout on the frequency of calcium transients. Bar graph of the average frequency of calcium transients in cell sheets 4 min before (teal bar, n=9) and 4 min after (orange bar, n=9) the addition of Gadolinium, as well as 1 min (blue bar, n=3), 10 min (red bar, n=3) and 40 min (green bar, n=3) after washout of Gadolinium.

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