Supplementary Information for:
Tutorial Review: From the Cellular Perspective:
Exploring differences in the cellular baseline in macroscale and microfluidic cultures

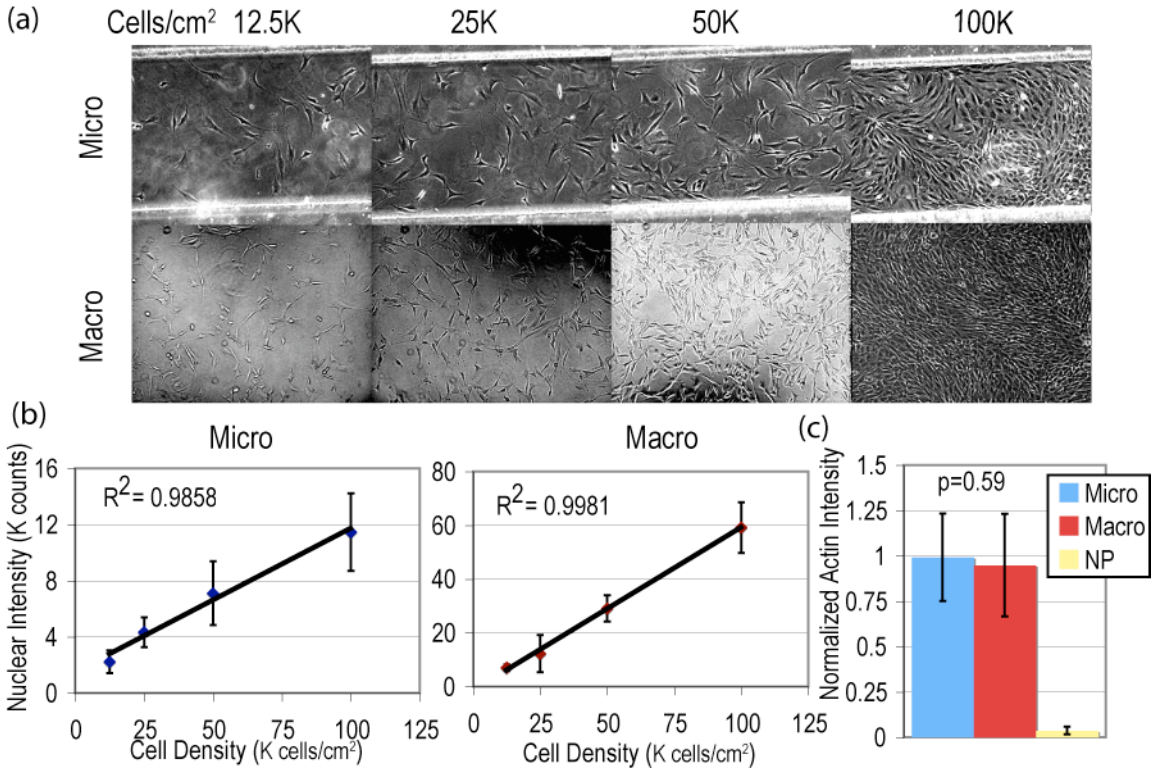

Figure S1: Phase images of MMFs (a), linearity assays (b), and actin ICWs (c). These results demonstrate that the fixation and analysis technique used for proliferation and ICW assays will provide accurate results regardless of culture scale, and that ICW results regardless of scale provide comparable results. Similar results were obtained for another mammary gland epithelial cell line (data not shown), suggesting that these data are not specific to this cell type.

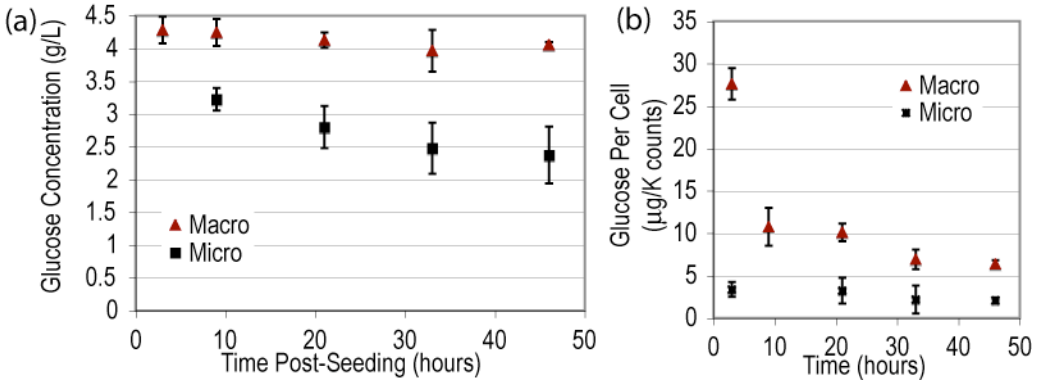

Figure S2: The glucose concentration over time was measured in micro and macroscale cultures (a) and the glucose per cell at each timepoint calculated (b). These data were used in conjunction with proliferation data to calculate the average per cell-hour glucose consumption rates cited in the main text.

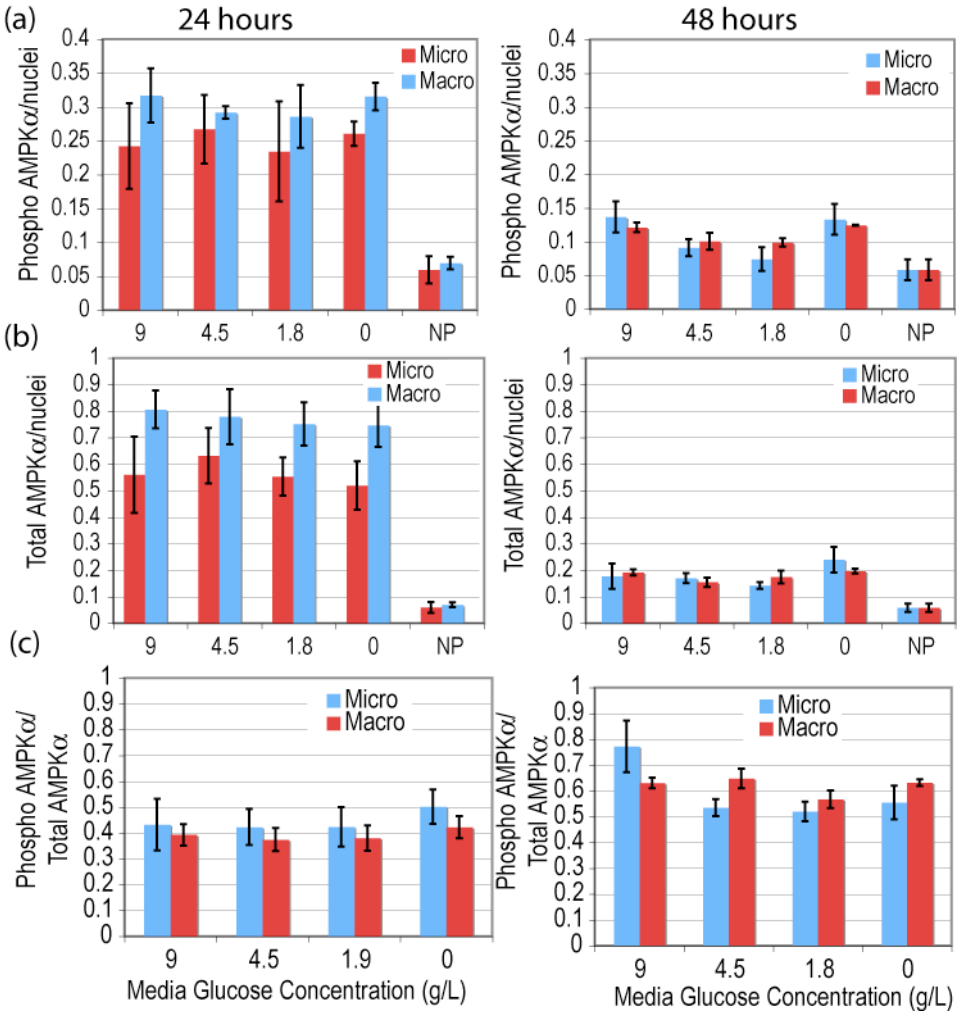

Figure S3: Raw AMPK data for phospho (a) and total (b) AMPKα normalized to nuclei for microcutlures and macrocultures in dilutions of media glucose concentrations at 24 hours and 48 hours after seeding. These data were collected using different antibodies, thus the ratio of phospho to total (c and in main text), is not necessarily a percentage of the total AMPK, but can be compared between scales and timepoints.

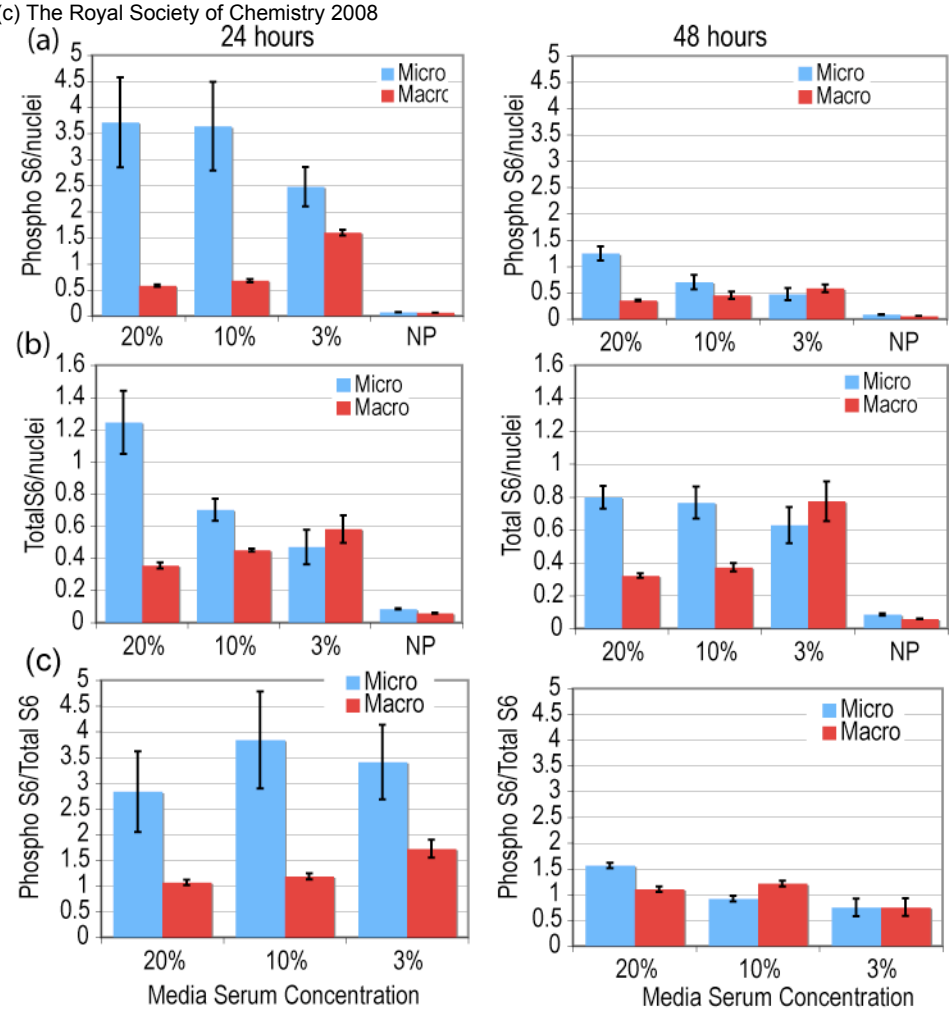

Figure S4: Raw S6 data for phospho (a) and total (b) S6 normalized to nuclei for microcutlures and macrocultures in dilutions of media glucose concentrations at 24 hours and 48 hours after seeding. These data were collected using different antibodies, thus the ratio of phospho to total (c and in main text), is not necessarily a percentage of the total S6, but can be compared between scales and timepoints.

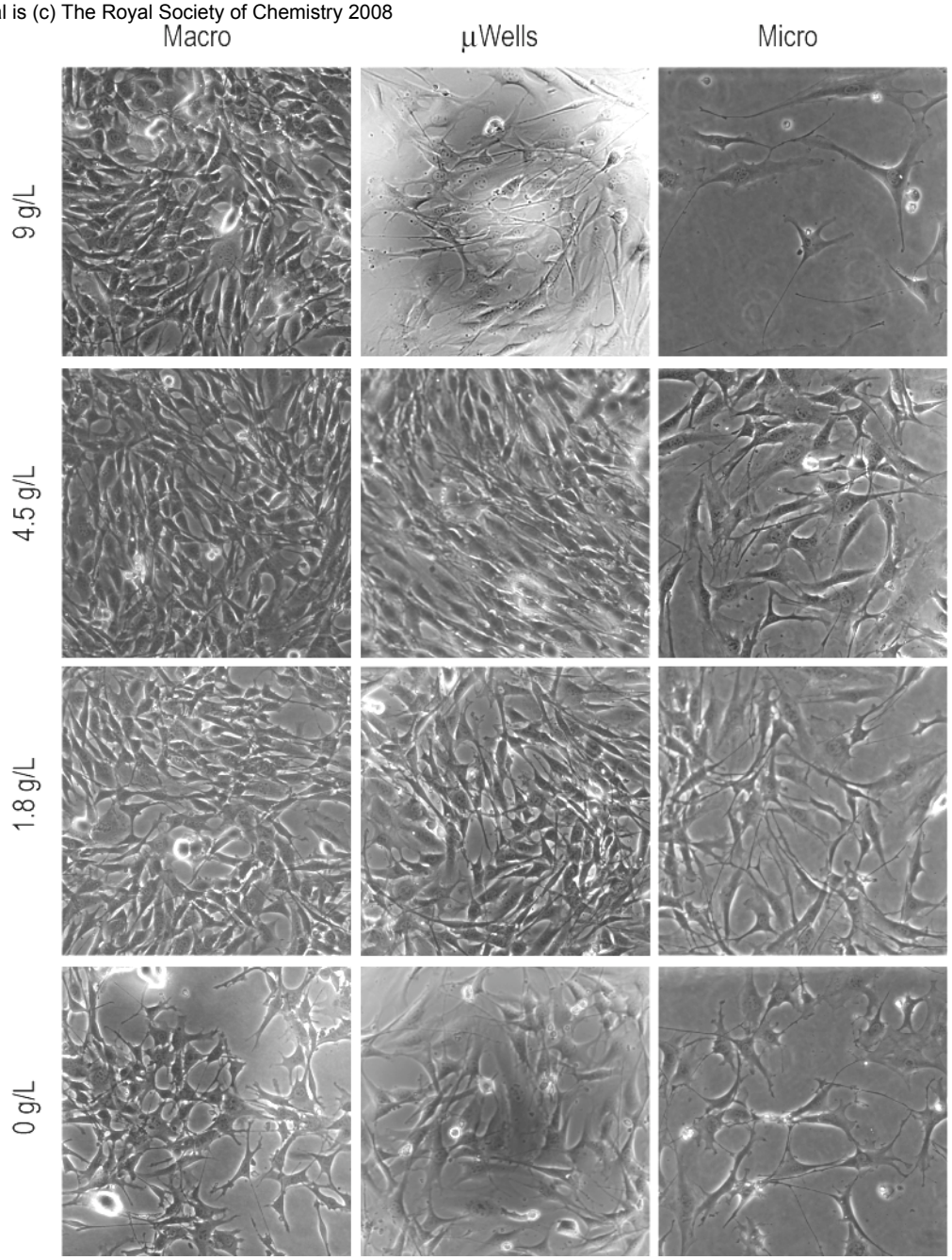

Figure S5: Phase images of microchannels, microwells and macroscale cultures at 48 hours post seeding for each glucose concentration. The morphology of cells in no glucose media in all culture types is altered, and it is clear that this cell type does not survive well in no glucose medium regardless of scale (similar results were seen in 3% FBS medium, data not shown). The density of microchannel cultures in any of the media is consistently lower than either of the two culture types, even microwell cultures, however the degrees of confluency at 3 hours post seeding were indistinguishable in all three culture types. The confluence at 48 hours post seeding seen in these conditions were consistent with the proliferation rates observed.

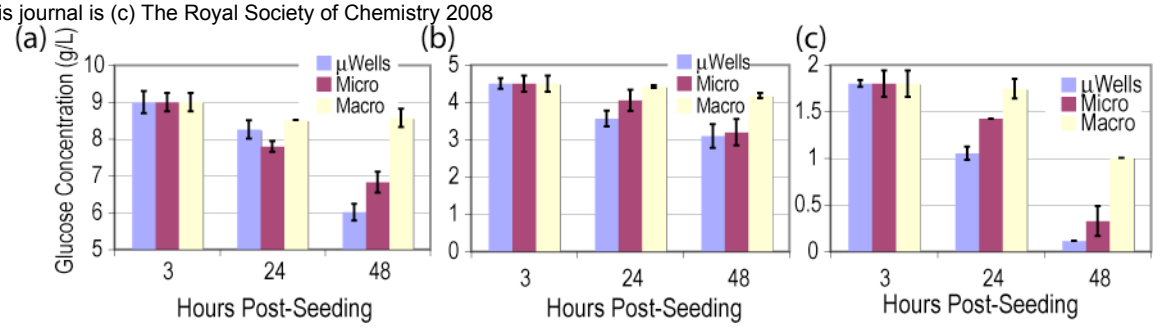

Figure S6: Cells seeded in macroculture (Macro), microchannels (Micro), and microwells (uWells) in media with a range of glucose concentrations were tracked over time for glucose concentration. (a) 9 g/L glucose media at 3, 24 and 48 hours after seeding, (b) 4.5 g/L media, and (c) 1.8 g/L media. The glucose concentration in the media at 3 timepoints for each culture type is shown in Figure S2 for 9 g/L (a), 4.5 g/L (b), and 1.8 g/L (c) glucose medium. A small amount of glucose is present in 0 g/L glucose medium contributed by the serum, but the amount was below the limit of detection of the assay. These data were used in conjunction with the proliferation data obtained at each timepoint to calculate the per cell-hour glucose consumption rates cited in the text.

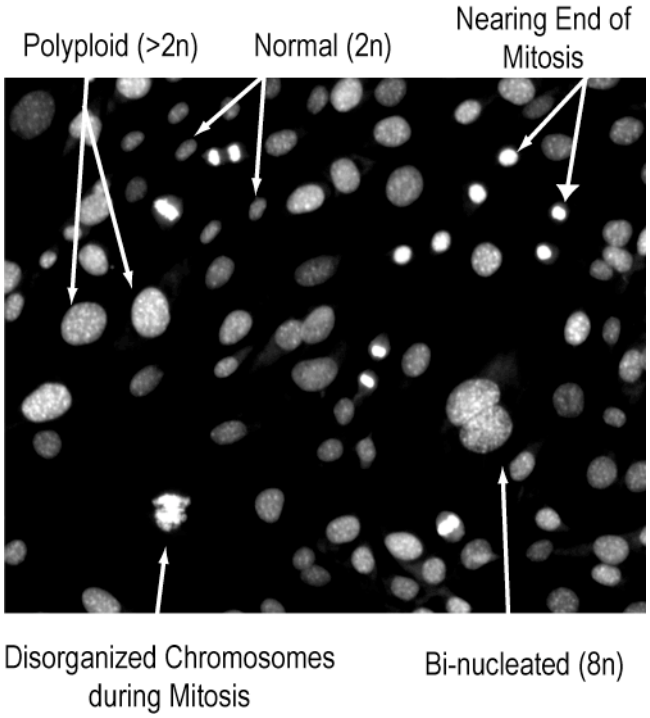

Figure S7: Defects in cell division and ploidy were common in microchannel cultures, but could not be identified in other culture types.

Many large polyploid nuclei could be found in microchannels that were verified to have more than the typical 2n or 4n amount of DNA via quantification of integrated intensity. These nuclei were noticeably larger and have higher intensities than the nuclei in macroscale cultures or microwells, which were typically the same size as those labeled "Normal" in Figure S7 (for a reference, images in Figure 5 show all three culture types with the same magnification). It is possible that many of the 4n nuclei were arrested in S or G2 phase of the cell cycle, but the large numbers of nuclei with DNA contents higher than 4n would not be explained by a G2/S arrest (e.g., the equivalent of 6n or more).

Examples of more extreme defects such as disorganized chromosomes in mitotic cells such as the cell labeled were fairly common. The sample cell in Figure S7 (labeled "Disorganized Chromosomes during Mitosis") in particular likely is polyploid as well (approximately 8n when the intensity is compared to the normal nuclei), and the extra chromosomes cannot be properly aligned during mitosis resulting in what looks like several axes forming and more DNA than the other examples of dividing cells in M phase in the image (verified to have the expected 4n amount of DNA or 2n per individual nuclei for those that were in later phases of separation). This cell, had it been able to continue to try to divide, could potentially have ended up bi-nucleated such as the one labeled. This bi-nucleated cell not only has two distinct nuclei (verified via phase microscopy to be one continuous cell), but each nuclei contains twice the normal amount of DNA, resulting in the total integrated intensity of the total nucleus being 4 times higher than the average value from macroscale cultures (which would presumably be 8n).

Stress Assays

The success of the ICW technique relies upon good antibody specificity, but also the ability of the antibodies to recognize the antigen in the non-reduced, non-denatured conformation, but instead the modified (by paraformaldehyde and triton) conformation found in fixed and permeabilized cells. Recognition of the fixed conformation can be verified by immnocytochemistry and microscopy. Changes in the levels of a specific protein in response to a control treatment known to cause changes in expression or localization can provide an idea of the specificity of the antibody beyond just verification of the expected localization in untreated cells.

Because ICWs do not provide any molecular weight information like a Western blot can, non-specific staining cannot be eliminated by only analyzing bands of the expected molecular weight. Because of this, the signal that is expected from an ICW would likely be more similar to the total intensity of all the bands in a Western blot than just the specific band. Despite the differences in protein conformation in a Western blot, antibody specificity can be evaluated by showing only one band in a traditional Western. However, it does not necessarily guarantee specificity in ICWs, but when combined with ICC data with appropriate controls, this data can be useful in validating the accuracy of an antibody.

For ICWs that provided somewhat small changes in protein levels, or (as in the case for BiP), unusual results were obtained in ICWs, the corresponding ICC and Western blots were performed to get a better idea of how the antibodies were functioning. The following figures includes ICC and Western blot data for many of the readouts listed as validation for the responses seen. Each set of ICC images (between images for phospho-proteins with positive and negative controls, and also between those and the no primary controls) were taken with all the same exposure and magnification parameters and all further image processing steps (to convert the formats suitable for publication) were kept the same to maintain a semi-quantitative comparison in the images. Also, all ICC data were done in macroscale cultures. In all of the Western blot images, after scanning on a laser scanner, the contrast was increased in order to see the nonspecific staining as well as the band of interest to better understand how specific the antibodies were in Western format. In each, the entire lanes are shown from approximately 15kDa (at the bottom), to 150kDa (at the top).

Note also that levels of phospho-proteins and total protein for the following readouts were obtained using two different antibodies, thus ratios of phospho/total do not necessarily represent the percentage of the total protein that is phosphorylated, but is an accurate measurement that can be

compared between scales and timepoints.

AMP Kinase activated protein kinase and S6 Ribosomal protein

Results for ICWs for both AMPKα and S6 are shown in Figure S8. Positive controls for phosphorylated AMPKα (the catalytic subunit of AMPK) were MMFs incubated with 25mM 2DG (2-deoxy-D-glucose) in 10% FBS medium, while negative controls were incubated with 10g/L glucose in 10% FBS medium each for 30 minutes, then fixed and stained. For S6 ICWs, positive and negative controls were incubated with 1% serum for 1 hour, with the addition of 200nM Rapamycin for negative controls. Media was then replaced in both positive and negative controls with 5µg/mL insulin in serum free medium for 15 minutes, then fixed and stained. AMPK and S6 ICW results were consistent with both the Western blot data from macroscale cultures, and also the ICC images taken after the same positive and negative control treatments (Fig. S9).

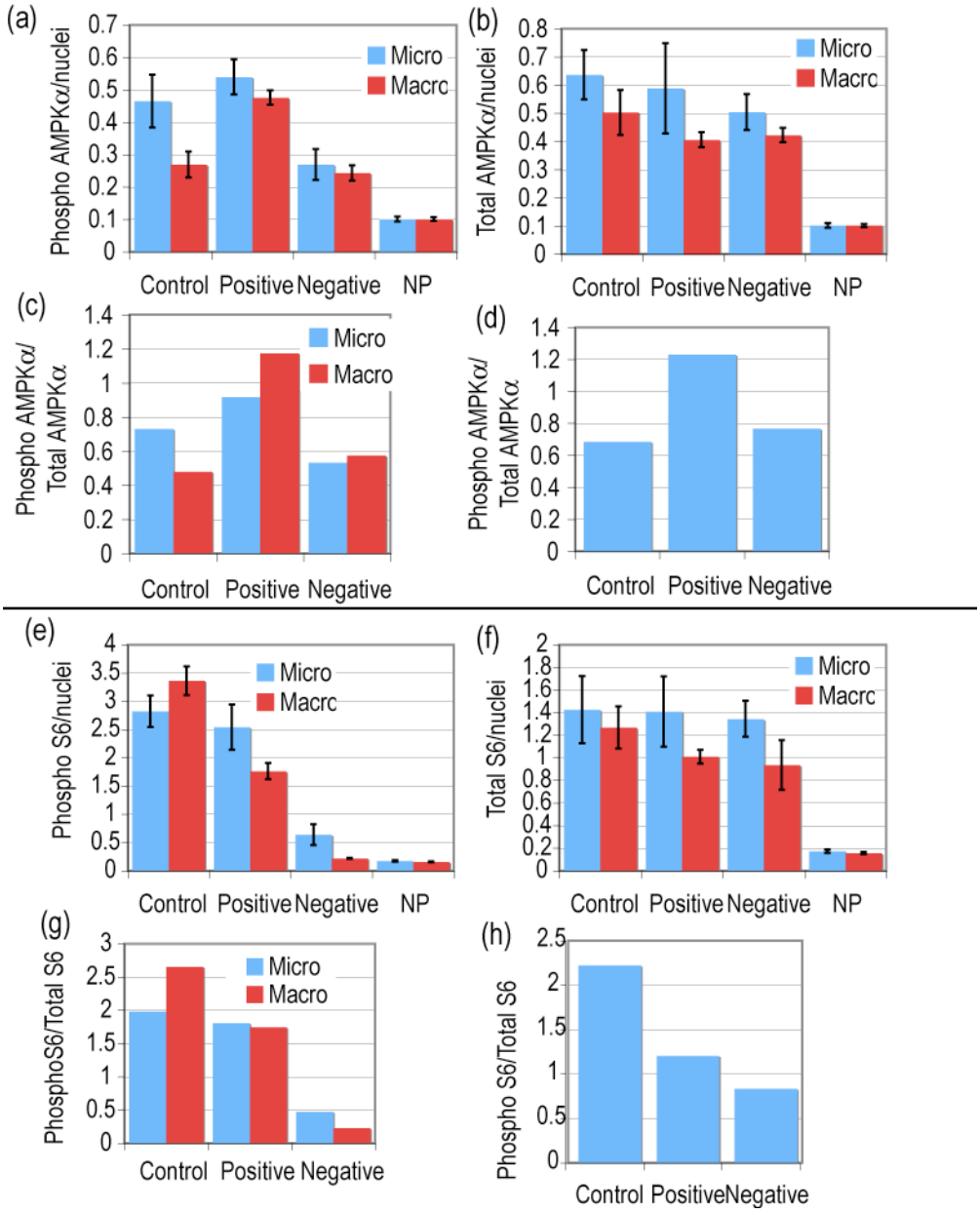

Figure S8: Integrated intensities of either phospho-AMPKα (a) or total-AMPKα (b) were normalized to nuclear intensities for each condition in each scale. The values for phospho AMPKα were then normalized to total AMPKα (c). Results from macroscale cultures via traditional Western blots gave similar results to that of the ICWs (d). A similar analysis was performed for S6, with phospho-S6 (e) or total-S6 (f) normalized to nuclear intensities for each condition in each scale, the values for phospho S6 normalized to total S6 (g) and finally, results from macroscale cultures via traditional Western blots (h). Error bars are one standard deviation for which macro cultures have an n of 3, and micro an n between 4 and 6. NP (No Primary) is the level of background due to nonspecific staining.

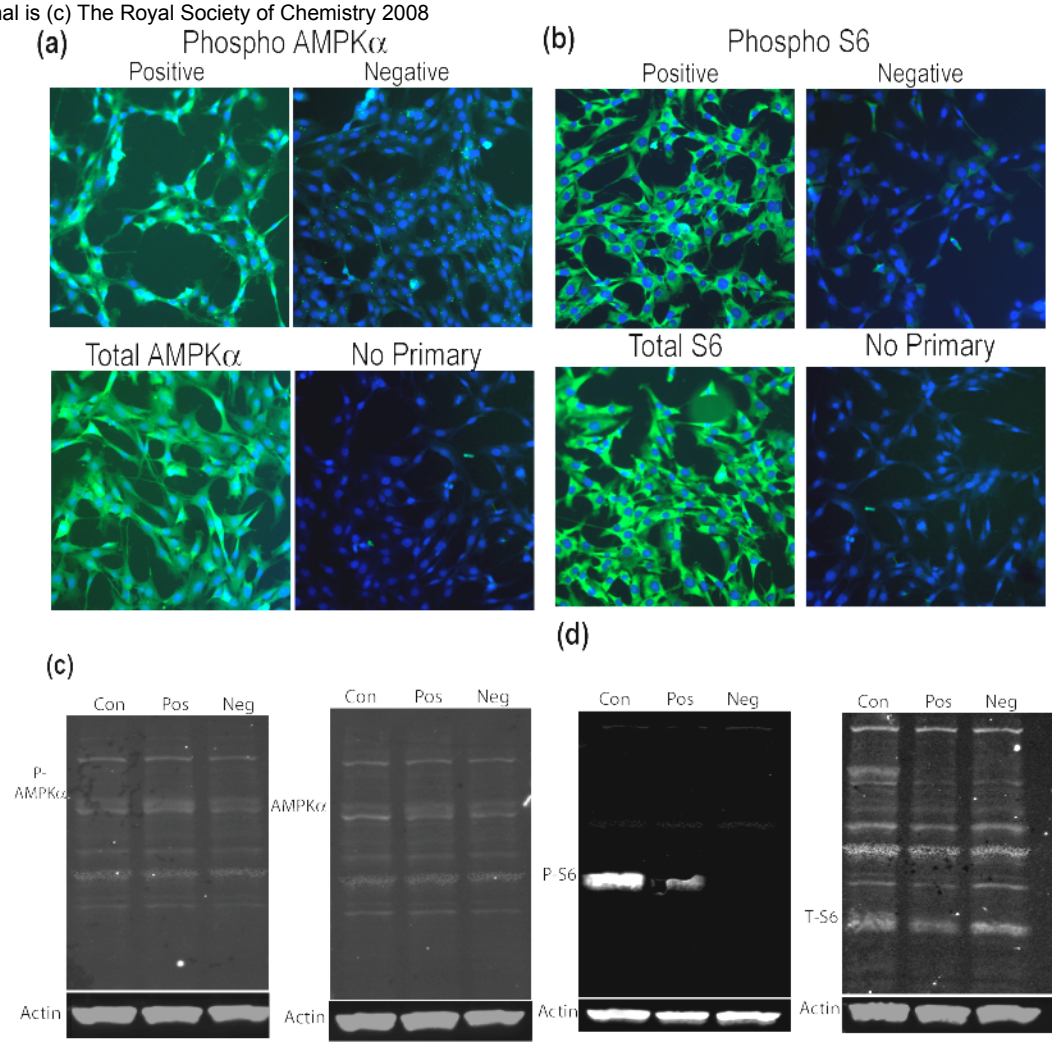

Figure S9: ICW results were checked by the corresponding immunocytochemistry (a, AMPK and b, S6). Western blots were done from macroscale cultures (c, AMPK and d, S6) as well. The responses were very clearly different in the ICC results, although the Western blots seemed less specific.

Hsp70 and BiP

Data for both HSP70 and BiP are included in Figure S10 and S11. The positive control for Hsp70 ICWs was heat shock at 42°C for 30 minutes then 45 minutes at 37°C as a recovery, (with no negative control as this is a ubiquitously expressed protein). The positive control used for BiP ICWs was the addition of thapsigargin at the specified concentrations for 16 hours (with no negative control as this is a ubiquitously expressed protein as well). Thapsigargin causes ER stress by inhibiting the pumping of calcium into the ER, thus raising the intracellular calcium concentration. Two doses were used as the MMFs used in this experiment tended to adhere very poorly to the surface after Thapsigargin treatment, and could be easily washed off during fixation and staining. Figure S10b, shows the reduction in cell number remaining after fixation in macro and microscale cultures, indicating that macroscale cultures suffered the most cell loss due to more flow during fluid exchange (though confluence in macroscale cultures treated with thapsigargin was similar to untreated cells prior to fixation).

The Western blot data from macroscale cultures (Fig. S11) showed a much larger increase in BiP expression than seen in ICWs, which indicates that the ICW result in macroscale cultured treated with thapsigargin are likely lower than the true value due to cell loss during fixation (which is not in issue for traditional Western blots). Additionally, cells were fixed very carefully in macroscale cultures for

immunocytochemistry and proved to have a visible increase in BiP fluorescence after Thapsigargin treatment (Fig. S11). Thus, likely the cells that are remaining in the macroscale cultures during ICW assay are those that are less sensitive to Thapsigargin induced ER stress, causing the values to be lower than expected. However, the control conditions can be compared between macro and microscale cultures as no cell loss occurred without the addition of Thapsigargin.

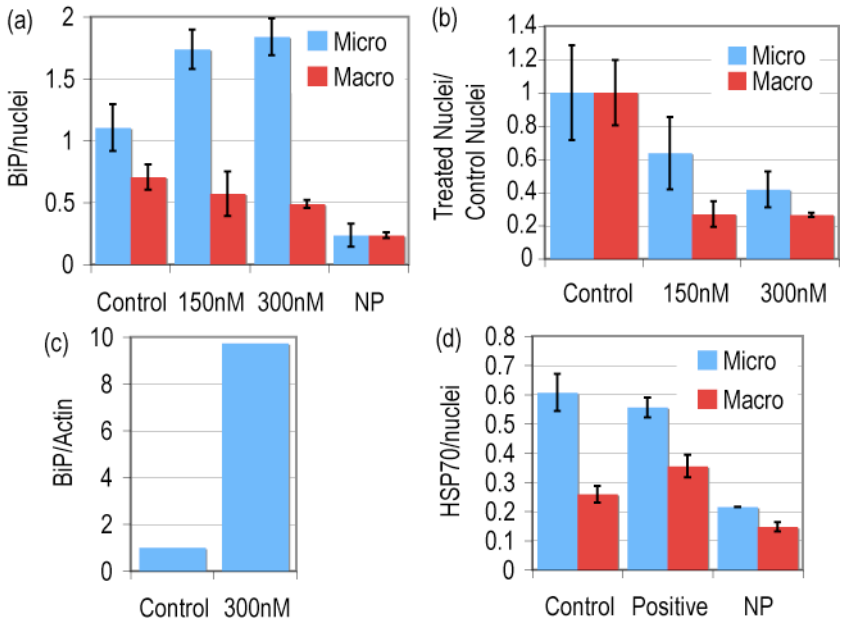

Figure S10: Integrated intensities of BiP were normalized to nuclear intensities for each condition in each scale, with two different doses of thapsigargin for 16 hours (a). Due to the differences in fluid handling between the culture scales, cells treated with thapsigargin in macroscale cultures did not remain attached to the substrate during fixation and staining. The quantification of the signal from the nuclei alone normalized to the levels in control conditions is shown in (b). Results from macroscale cultures via traditional Western blots showed significant upregulation of BiP, indicating that the ICWs treated with thapsigargin likely underestimate the degree of upregulation due to cell detachment (c), however the untreated conditions are still accurate. Heat shock protein 70 ICW results showed a significant increase (over two-fold) in HSP70 levels in microcultures than in macrocultures (d). Error bars are one standard deviation for which macro cultures have an n of 3, and micro an n between 4 and 6. NP (No Primary) is the level of background due to nonspecific staining.

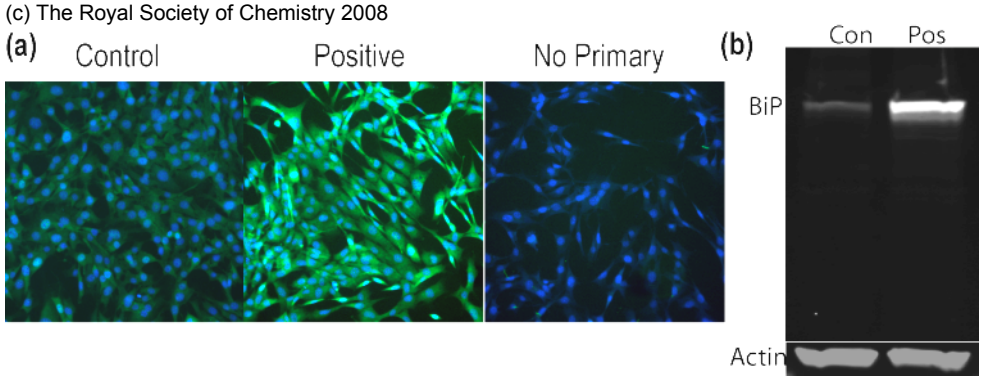

Figure S11: BiP ICC was done in macroscale cultures with the positive control having 300nM Thapsigargin for 16 hours prior to fixing. Careful fixing was done in macroscale cultures to prevent washing the cells off the surfaces, as occurred in the ICWs. Also, the Western blot showed a very large upregulation in BiP, and proved to be very specific. These data suggest that despite the cell lift off issue in macroscale cultures, the BiP ICW for control conditions comparing cultures in either scale are likely still accurate, as the antibodies function as expected.

ERK1/2

The ICW results for ERK1/2 are shown in Figure S12. For controls, media was replaced with 1% FBS DMEM with the addition of a cell permeable ERK activation inhibitor peptide (Calbiochem, #328005) at 30 μM for 1 hour for the negative controls. Media with 20% serum was then added to both positive and negative control cultures for 20 minutes prior to fixation and staining. Western blots and ICC for total ERK and Westerns for phosphorylated ERK are included in Figure S13.

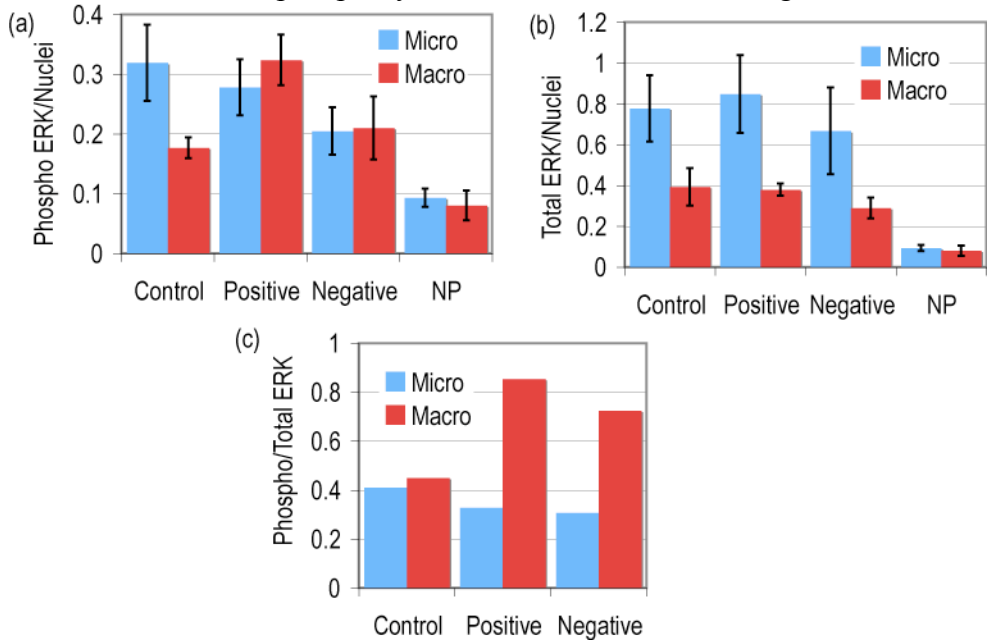

Figure S12: ICWs for phospho (a) and total (b) ERK1/2 showed consistent upregulation in microcultures, while the normalized amounts were consistent (c). Error bars are one standard deviation for which macro cultures have an n of 3, and micro an n between 4 and 6. NP (No Primary) is the level of background due to nonspecific staining.

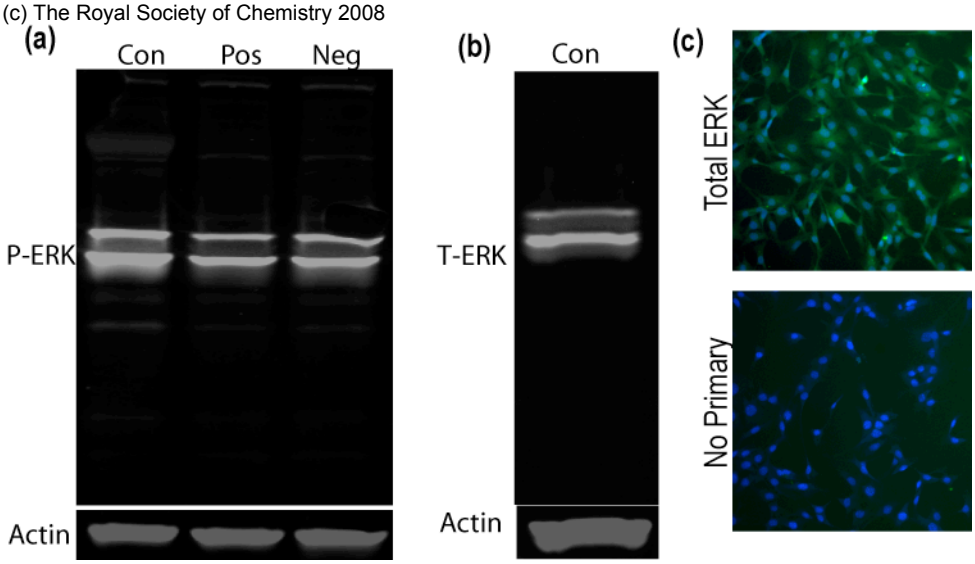

Figure S13: ERK Westerns including phosphorylated ERK with positive and negative controls (a). Due to the serum starve done for the positive and negative controls, the levels of phospho-ERK in these actually are lower than in the control cultures, but this matches what was seen in the ICWs. Blots for total ERK were done as well (b) just to check the specificity of the antibody. ICC for total ERK (c) was done to verify that the somewhat smaller values seen in ICWs were accurate. In fact the ICC results showed weaker staining with the same protocol than what was observed for many other readouts, though results were always significantly above background levels.

γH2A.x

Figure S14 shows results of ICWs for γH2A.x, indicating that no significant difference is seen between macro- and microcultures. The positive control used for γH2A.x was 30 minutes of exposure to UV in a laminar flow hood, followed by 15 minutes of recovery at 37°C in the incubator. The same fixing and staining protocol used for all other ICWs was followed except for the fixation step. Fixing was done with 2% PFA in PBS at room temperature for 10 minutes, without phosphatase inhibitor cocktail then the standard protocol was followed. ICC and Western blot validation was done as well and is shown in Figure S15.

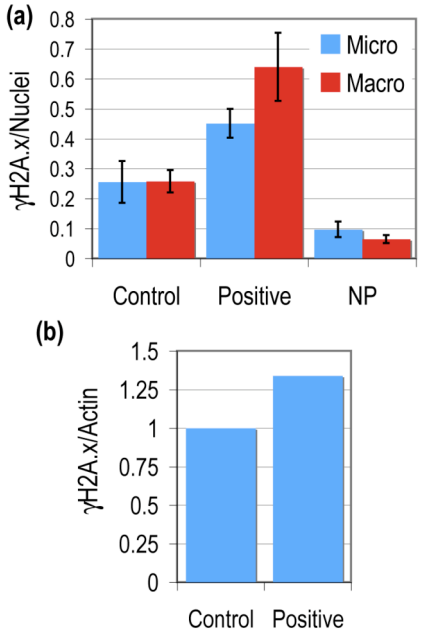

Figure S14: Integrated intensities of γH2A.x were normalized to nuclear intensities for each condition in each scale (a). Results from macroscale cultures via traditional Western blots showed similar results (b). Immunocytochemistry for each condition also verified that localization and treatments result in significant differences in levels of γH2A.x (c). Error bars are one standard deviation for which macro cultures have an n of 3, and micro an n between 4 and 6. NP (No Primary) is the level of background due to nonspecific staining.

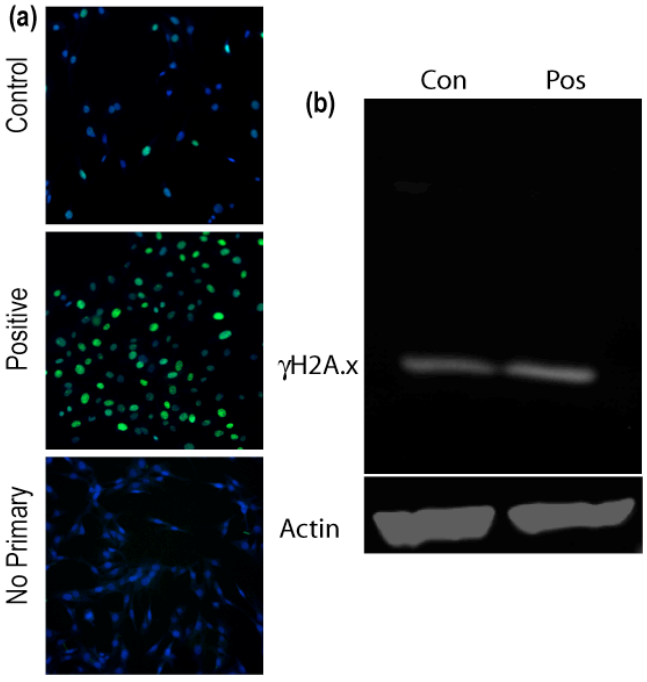

Figure S15: H2a.x staining was done (a) along with the Western blot (b) to ensure that localization was correct in the ICC results. An ICC verification for this readout was important because this was the only nuclear protein included in the panel, and was also a phosphorylated protein, both which may cause ICC/ICW methods to fail due to the antigens not being very accessible or easily degraded.