Study the Sub-nanometer Membrane Fluctuations in Single Cells Using a Plasmonic Imaging Microscope

A Thesis

Presented to

The Faculty of the Department of Electrical Engineering

University of Houston

In Partial Fulfillment

Of the Requirements for the Degree

Master of Science in Electrical Engineering

By

Suraj Deepak Khochare

December 2018

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Abstract

Plasma membrane of live cells undergo active membrane fluctuations. Scrupulous study of single cell membrane fluctuations can provide better insight into the physiological processes like metabolism, mitosis and cell motility which are very important while developing drug assays for diseases like cancer. Therefore, it is crucial to study the localized membrane fluctuations which pose a technical challenge due to their small yet swift movement. Here, a plasmonic microscope has been used to study membrane fluctuations in HeLa cancer cells. Sub-nanometer level membrane movement has been imaged with a spatial resolution of 0.5 μm in real time. A study of membrane fluctuation amplitude is made to discuss about active membrane movement, cell heterogeneity and interdependence of different cell regions within a single cell. These results could be helpful to study the cell's mechanoresponse, its viscoelastic properties and analyze the activity of biomarkers when cancer cells are exposed to different drug treatments.

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1 CHAPTER ONE: INTRODUCTION

1.1 THE ORIGIN AND SIGNIFICANCE OF MEMBRANE FLUCTUATIONS

Live cell imaging is very essential to study fundamental biological functions in cells. The live cells exhibit active membrane fluctuations corresponding to different activities taking place inside the cell. This activity is primarily classified as one due to the thermal energy exchange relating to the surrounding environment while active processes like ATP-driven processes could be another reason[1].

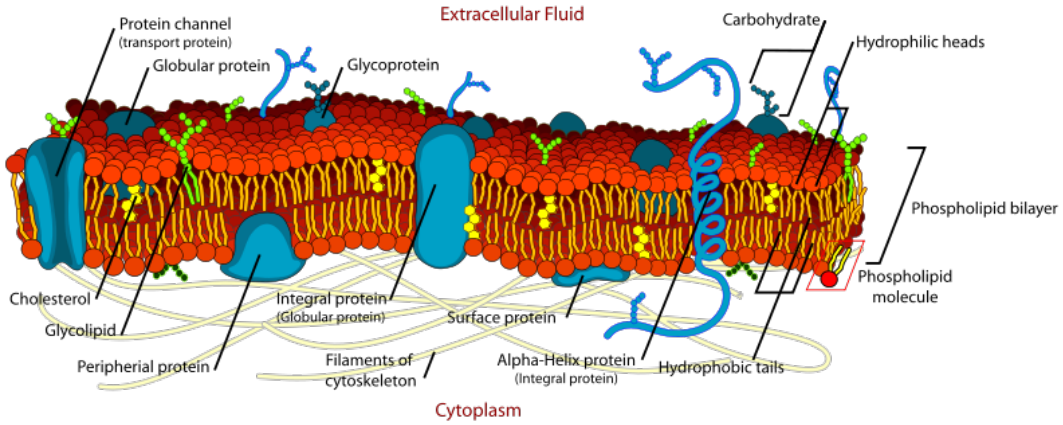

Figure 1. ***Detailed Diagram of Cell Membrane Cross-section (Courtesy: Mariana Ruiz, Wikipedia page).*** The diagram presents different primary components of a cell membrane like phospholipid bilayer, the cytoskeleton and various proteins surrounding the membrane.

Though the thermal fluctuations determine the entropy of the system, the active fluctuations arise from out of the equilibrium activity of the proteins within the cell[2, 3]. All these leading to cell membrane undulations makes it a very important aspect to reveal the fundamental properties of this biological system. One of the main activity is active transport of molecules across the cell membrane. It is challenging to develop drug assays because of the selectively permeable nature of the cell membrane. This keeps one away from understanding the interactions of a specific drug with the cell membrane. Studying these interactions may reveal the natural response of a cell or its membrane to the

applied drug. Thus, the study of a single cell membrane is important to explain the physiology of the cell in a better way.

1.2 Review of Existing Imaging Techniques

Imaging the membrane fluctuations and analyzing them has several experimental challenges. Some techniques exert minimal forces which itself may change the impact of fluctuations. Some other techniques use fluorescent dyes to enhance contrast but might end up altering the properties of the cell themselves. Also, it is very important to measure the sub-nanometer level fluctuations which take place in a very short time duration. In all these techniques, light-based imaging serves a good purpose provided they have a good contrast along with high spatial and temporal resolution. Recently, there have been techniques like quantitative phase imaging[4] which can observe translucent objects unlike ordinary light microscopes. In this technique, the generation of a phase shift image improves the image quality compared to other phase contrast methods. It helps to measure collective information of the entire cell. Reflection interference microscope is another promising technique that can measure the bottom membrane of a cell[5]. However, there is some scattering of light by small cell organelles which can deter the image quality.

1.3 Surface Plasmon Resonance for Imaging the Membrane Fluctuations

In this paper we use Surface Plasmon Resonance (SPR) to image the sub-nanometer fluctuations of a single cell. SPR is more sensitive to small movements near the plasmonic surface because of the evanescent wave which penetrates up to 200 nm in the vertical direction[6]. We can only image the cell bottom membrane using SPR and interference

because of small features like cell organelles is less likely. Firstly, we demonstrate how we convert the intensity image obtained from SPR to a 3-dimensional cell bottom membrane morphology. Secondly, we show the sub- nanometer membrane fluctuations and prove that they are active cell membrane fluctuations using cell fixation process with two distinct concentration. The first part includes cell fixation with high concentration of paraformaldehyde by cross-linking the proteins[7] and making the cell membrane more rigid. Thus, we have no active membrane fluctuations. In the second part, we add a very low concentration of paraformaldehyde which is not enough to form covalent bonds and cross-link the proteins and hence we see some active membrane fluctuations initially. Thirdly, we demonstrate cell heterogeneity by analyzing the membrane fluctuations for individual cells. We also show the non-linear characteristics of cell membrane fluctuations against cell area which explains difference in metabolic activity and/or cell status difference. Lastly, we compare the cell membrane movement with sub-nanometer membrane fluctuations to find that they correlate at cell regions which have more active movement.

2 CHAPTER TWO: EXPERIMENTAL PROCEDURES AND TECHNIQUES

2.1 CELL SUBCULTURE

Hela cells are grown in Dulbecco's Eagle Modified Medium (Corning) supplemented with 10% Fetal Bovine Serum (Corning) and 1% Penicillin/Streptomycin (Corning) and incubated at 37°C with 5% CO_2. We aspirate the old culture medium to start with the subculture. The cells are then thoroughly rinsed with 1X Phosphate Buffered Saline (Corning). The cells are re-suspended from the cell culture flask using 1 ml of 0.25% Trypsin (2.21mM EDTA, 1X sodium bicarbonate) and incubating them at 37°C, 5% CO_2 for 1 minute. 3 ml of culture medium is added to the culture flask to prevent the further effect of trypsin. The re-suspended cells are collected inside a centrifuge tube and centrifuged for 3-4 minutes at 125 x g. After centrifuge, the cells are recovered by carefully aspirating the supernatant. We then add appropriate aliquots of cells to new cell culture vessels.

2.2 CELL FIXATION

A chemical fixative is used to distinguish the membrane fluctuations due to active processes from the thermally triggered fluctuations. Paraformaldehyde (4% in PBS) is used as a chemical fixative for cell fixation. Initially, the cells were sub-cultured as per the procedure mentioned in Section 2.1 above. Appropriate aliquot was added on a plasmonic substrate (22x22 mm BK-7 glass chips with 2 nm NiCr adhesion layer and 42 nm Au coating). The substrate with cells was incubated at 37°C and 5% CO_2 for 48 hours to allow the cells to adhere, grow and form a monolayer. The sample was removed from the incubator and loaded on the SPR setup to carry out the cell fixation experiment. The SPR

angle and objective focus were adjusted to get the SPR image before adding any fixatives. Firstly, a high concentration of Paraformaldehyde was added which kills the cells by cross-linking all the proteins to make sure there are no active membrane fluctuations. The highly concentrated fixative was prepared by mixing 100 µl Paraformaldehyde in 200 µl of culture medium. Similarly, a new fresh sample with a well-grown cell monolayer was loaded on the SPR setup to be treated with less concentrated Paraformaldehyde. This is to make sure that not all proteins are cross-linked and there exists some amount of active membrane fluctuations. The experiment procedure is same as the one for highly concentrated chemical fixation. The low concentration fixative was prepared by mixing 1 µl Paraformaldehyde in 300 µl of culture medium. Both these experiments were carried out separately and a fresh sample was used each time.

2.3 PLASMONIC IMAGING

This imaging technique is developed based on total internal reflection microscopy. An incident light with wavelength 670 nm is used to focus on the plasmonic surface using an oil immersed objective (Olympus Apo N 60X/1.49). When the incident angle is greater than the critical angle, an evanescent wave propagates almost parallel to the substrate and excites the surface plasmons. These resonating surface plasmons absorb most of the incident light and thus we have 'Surface Plasmon Resonance'. However, the critical angle is dependent upon the refractive index of the refracting medium. In that case, if there are cells adhered at certain locations on the substrate, the critical angle for those locations is much less and hence we observe reflectance of light. This refractive index keeps changing as the cell membrane moves up and down exhibiting active membrane fluctuations. The change in refractive index meaning membrane fluctuations is detected as change is reflectance which is used to analyze the data. The reflectance from the sample is imaged

by a CCD camera Pike (Allied Vision Technologies, Exton, PA, USA) and we collect the raw data for further quantitative analysis.

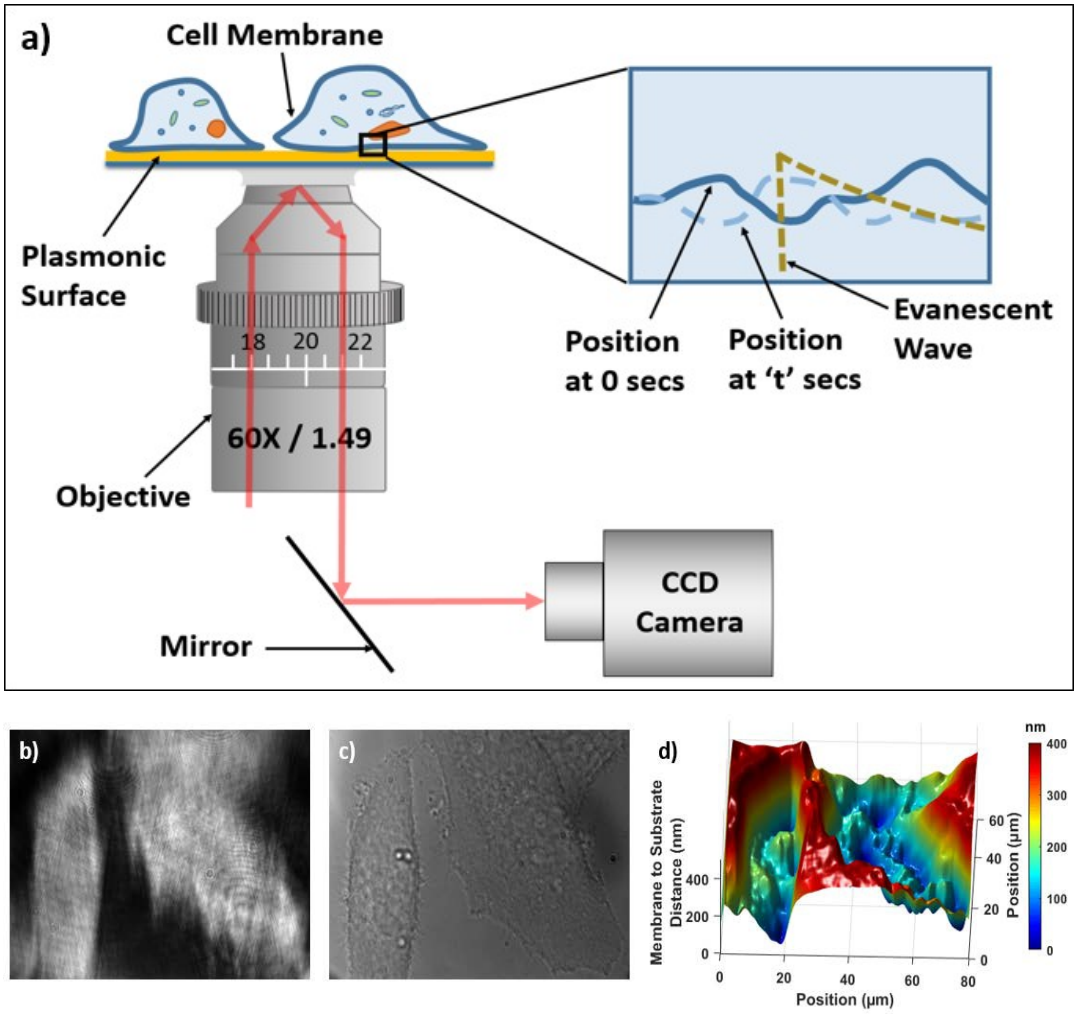

Figure 2. ***Principle of Surface Plasmon Resonance Imaging.*** (a) SPR imaging setup and zoom-in view of bottom cell membrane. (b) SPR image for two distinct cells (HeLa cell line) and (c) bright field image for the respective SPR image. (d) 3D view of cell bottom membrane variations.

3 CHAPTER 3: RESULTS AND DISCUSSION

3.1 3D CELL BOTTOM MEMBRANE MORPHOLOGY

Surface Plasmon Resonance can obtain images with high spatial resolution (0.5 μm) in real time. Also, the limited penetration depth of the evanescent wave brings up the advantage of high resolution in z-direction. Thus, we can plot morphology of the bottom cell membrane with high resolution in x, y and z directions at a given instance in real time. Figure 2b and 2c show the SPR image and bright field image of HeLa cancer cells respectively. The SPR image is a high contrast image showing the bottom cell membranes of respective HeLa cells. This data is collected at a fixed angle of incidence (71.47°) for the incident light with wavelength 670 nm. The background goes completely dark because of the light absorbed by plasmonic resonance while the change in refractive index avoids the same at the cell-substrate interface. We model this setup in a simulation software and use it as a reference to convert the intensity values to distance. Thus, we create a 3-dimensional plot showing membrane to substrate distance for the entire location shown in Fig. 2d. Additional details related to this calibration are mentioned in the supporting material (section 1). As seen from Fig. 2d, some portions of the cell membrane in either of the cells are close to the substrate while some portions are away from the substrate. These positions can relate to multiple functions carried out by the cell membrane. It includes passive transport of ions and gases like carbon dioxide (CO_2) and oxygen (O_2) during osmosis. Transport of substances like sugars or amino acids into the cell and export of metabolism products are led by various transmembrane protein channels which stimulate the membrane activity. The changing position or activity of the cell membrane is primarily due to the processes like endocytosis and exocytosis which are termed as

actively driven processes by the cell which lead to deformations in the cell membrane. A 3-dimensional view of this bottom cell membrane morphology is displayed as a movie in supplementary material (Movie 1).

3.2 SUB-NANOMETER MEMBRANE FLUCTUATIONS

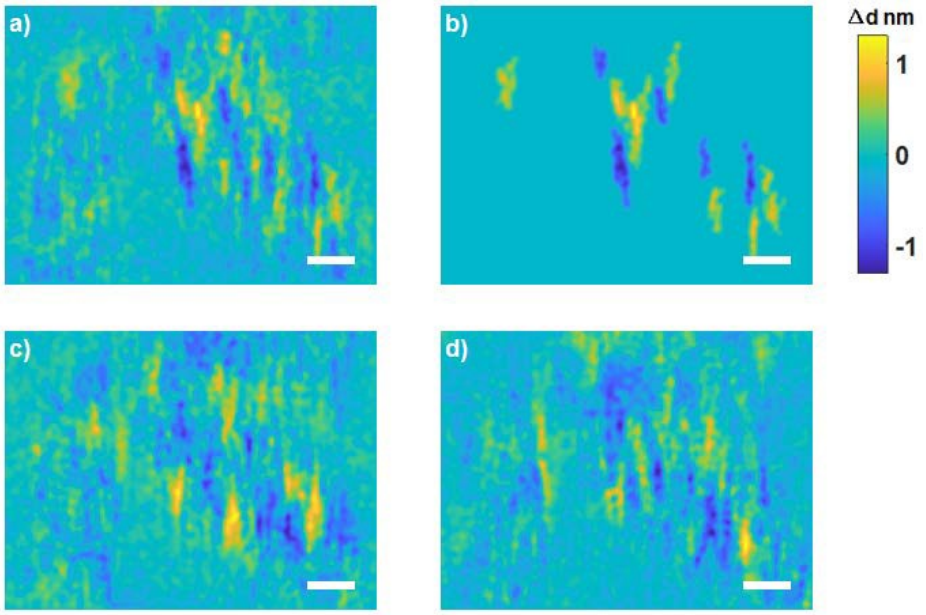

Figure 3. ***Sub-nanometer Cell Membrane Fluctuations.*** (a), (c) and (d) snapshots of sub-nanometer fluctuations at different points in time. (b) An image processing algorithm is used to detect significant patches. A demonstration of patch detection is shown in Movie 2.

The live cells have active membrane fluctuations because of multiple physiological processes like endocytosis, exocytosis, cell adherence, motility, etc. Such biologically relevant membrane fluctuations are partly because of ATP-driven processes and partly powered by thermal energy. Many functionalities like cell motility, mitosis, cell organelle movement, structure of the cytoskeleton, viscoelastic properties, cell mechanoresponse and metabolism can be explained by analyzing the cell membrane fluctuations[8, 9]. It is challenging to observe these membrane fluctuations because of their small movement. Because of the high sensitivity of SPR in z-direction, we can image these fluctuations at a sub-nanometer level. Figure 3a, 3c and 3d show the membrane fluctuations in HeLa cells

as we record the data over a continuous period of time. Since the intensity baseline is higher in an SPR image as shown in Fig. 2b, we subtract the baseline (first image in the dataset) and then subtract each image at a fixed interval from the previous one to detect the change in reflectance (ΔI) over half a second. We go back to use the same simulation model to convert this ΔI to change in distance (Δd) in nanometers. After calculating the change in distance for the entire image, we detect significant movement using image processing algorithms which use data segmentation and morphology based operators and discard all other data as noise. We call these significant movements as patches. This helps us to get a sophisticated estimate of the amplitude and size of membrane fluctuations.

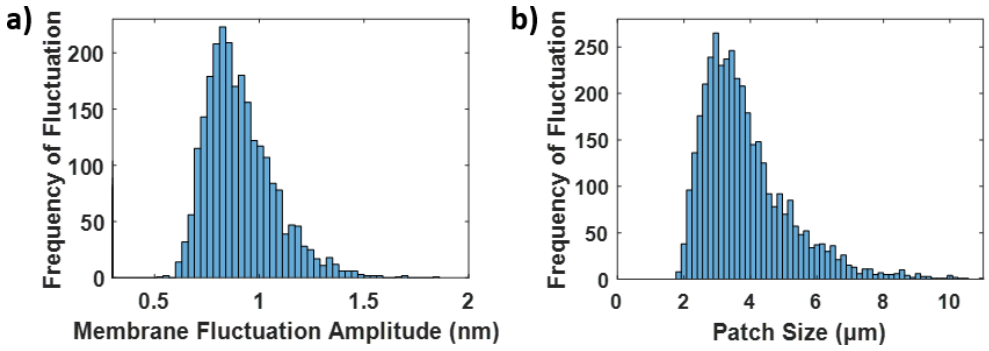

Figure 4. ***Histogram Based Analysis of Membrane Fluctuations.*** Shown in Fig. 2b, significant patches detected for data collected over 2 min and displayed in the form of a histogram. (a) Distribution of fluctuation amplitudes and (b) Distribution of size of fluctuations.

We put this data together in form of a histogram as shown in Fig. 4a and Fig. 4b. We observe that the membrane fluctuation amplitude (Δd) mostly lies in the range 0.6 – 1.4 nm. Maintaining the previous statement, it would be more useful to understand that the average amplitude of these fluctuations over half a second is in the range 0.7 – 1.2 μm. The histogram of patch size shows that the lateral size of these membrane fluctuations lies in the range 2 – 6 μm. We measure the patch size in the horizontal direction as the patches are elongated in the vertical direction because of light scattering.

3.3 CONFIRMATION OF ACTIVE MEMBRANE MOVEMENT

The active cells have two different kinds of movement namely: membrane fluctuations driven by active processes and Brownian motion. We use chemical fixation of cells using paraformaldehyde to test the active membrane movement against Brownian motion. Paraformaldehyde gives better penetration depth compared to fixatives like formaldehyde. We perform two separate experiments for cell fixation with high concentration and low concentration of paraformaldehyde respectively. The high concentration (30 %) of paraformaldehyde will fix the cells by cross-linking of proteins through covalent bonds. This will block the lateral diffusion of molecules across the cell membrane. It will also anchor soluble proteins to the cytoskeleton making the cell membrane more rigid. Hence, we expect no active membrane fluctuations after chemical fixation using paraformaldehyde. We see the same in our results as shown in Fig. 5a where we do the histogram- based analysis before and after cell fixation. We observe minimum membrane fluctuations after 5 minutes of adding paraformaldehyde. Similarly, we test another fresh sample with low concentration (0.3 %) of paraformaldehyde for cell fixation. Adding such a low concentration is not enough to cross-link all the proteins and increase cell rigidity. Hence, we expect some active membrane fluctuations and we see the same in our result in Fig. 5b. This also proves to be a test of our plasmonic imaging technique to detect active cell membrane fluctuations.

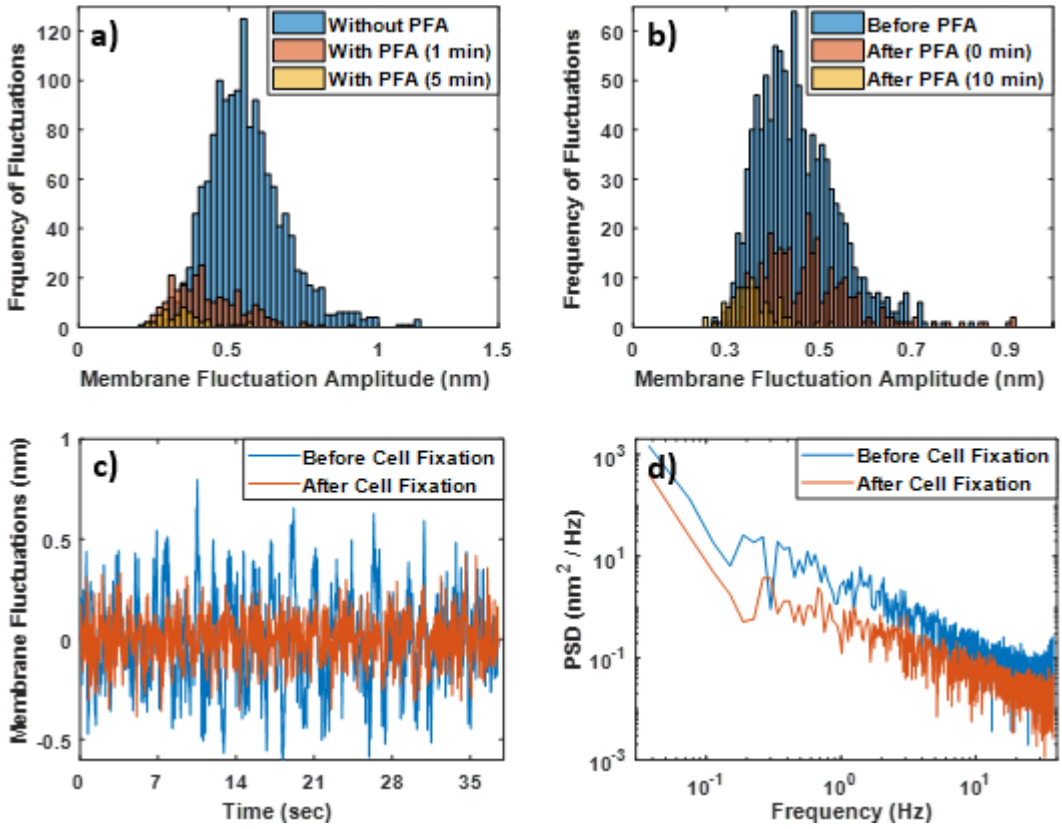

Figure 5. ***Confirm Detection of Active Membrane Fluctuations.*** (a) Distribution of fluctuation amplitudes during high concentrated cell fixation. (b) Distribution of fluctuation amplitudes during low concentrated cell fixation. (c) Difference in fluctuation amplitudes. (d) PSD plots for membrane fluctuations.

The difference in membrane activity before and after cell fixation can also be observed through a temporal plot. We select a small portion (1 x 1 µm) of the cell membrane before cell fixation and plot the fluctuation amplitude of this portion over time. We select the same portion of the cell membrane after cell fixation and plot the fluctuation amplitude over time. These two plots are plotted together in Fig. 5c to show the difference in membrane fluctuation amplitude before and after cell fixation. We can see a clear difference in the membrane fluctuations for these two conditions and which is maintained over time. We also see the drop in the power spectral density plot for fixed cells compared to normal cells as shown in fig. 5d. Also, Fig. 6 shows the cell fixation process where we observe the dampening of total cell movement after adding

paraformaldehyde.

The entire fixation process is plotted in Fig. 6 where the chemical fixative was added around 35th second and we can see an immediate effect of the same on the total cell movement.

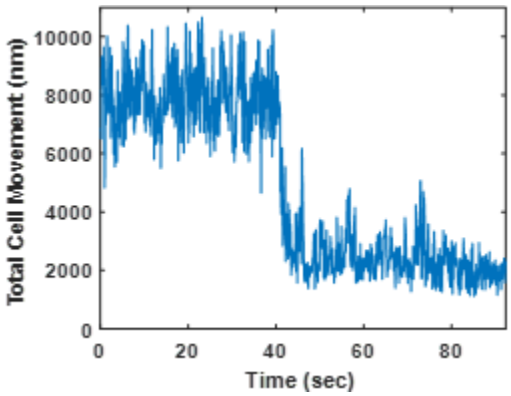

Figure 6. ***Cell Fixation Process.*** The membrane fluctuations over the entire cell fixation process as a function of time. A high concentration chemical fixative was added approximately around 35 seconds and the fluctuations are seen to dampen very instantly.

3.4 CELL HETEROGENEITY

In the previous part, we analyzed sub-nanometer membrane fluctuations for a finite population of HeLa cells and discussed about the range of fluctuation amplitude and patch size of a given population. However, the characteristics of a population may not reflect the behavior of individual cells because of the cell-cell heterogeneity. Cell heterogeneity can be intertumoural and intratumoural[10]. Tumor heterogeneity between two patients having the same kind of tumor is termed as intertumoral heterogeneity. This can be caused due to genetic variations and differences in environmental conditions. Intratumoral heterogeneity is another type of heterogeneity in tumor cells within a single patient. This can be due to differences in interaction of cell to local environment at different sites. It can also be due to DNA replication errors while cell division. This type of heterogeneity is important to study as it drives the evolution of

cancers and promote drug resistance. Hence, it is important to study the membrane fluctuations of multiple single cells in a given population to evaluate cell heterogeneity. We observed a difference in membrane fluctuations amongst a cell population because of some phenotypic differences like cell shape, cell status and metabolism. To study this, we choose five individual cells at a given location to analyze the cell membrane fluctuations as shown in Fig. 7a. As we already have patch detection results for the entire location shown in Fig. 7a, simply applying binary mask corresponding to a particular cell can give results for membrane fluctuations in a single cell. To evaluate this result at a particular instance of time, we sum up the fluctuation amplitude (Δd) from patches detected for that particular cell for each image frame. We label this sum at a given point of time as 'total cell movement'. Fig. 7b and Fig. 7c shows the corresponding total cell movement for the individual cells as mentioned above. Fig. 6b shows that the Cell 2 is more active compared to other cells in the surrounding. However, the area of that particular cell and amount of reflectance at that location plays a very important role in deciding Δd. Hence, we normalize the fluctuation amplitude with respect to intensity and area and plot the same in Fig. 7c. Now we can see that Cell 2, Cell 4 and Cell 5 have higher total cell movement per unit area compared to other 2 cells. It is interesting to observe that a couple of active cells like Cell 4 and Cell 5 have less area compared to the two non-active cells. In fact, Cell 5 has the highest total cell movement being the smallest cell in size amongst other cells. The area of the most active Cell 5 is 189.35 μm^2 as opposed to 224.27 μm^2 of Cell 4 if we take two cells with relatively less area into consideration. This kind of cell-cell variation can be due to different stages of development in the lifecycle of an individual cell. At some of the crucial stages like mitosis, the cell membrane is more active which could possibly be a case for Cell 2, Cell 4 and Cell 5 looking at the bright field

image (Fig. 7a). Another factor can be genetic discordance with the parent cell due to segregation errors during cell division. Interaction with the surrounding cells can also be an important factor leading to cell heterogeneity. These results also show that there is significant variation in the amount of membrane undulations for different cells in the same local environment.

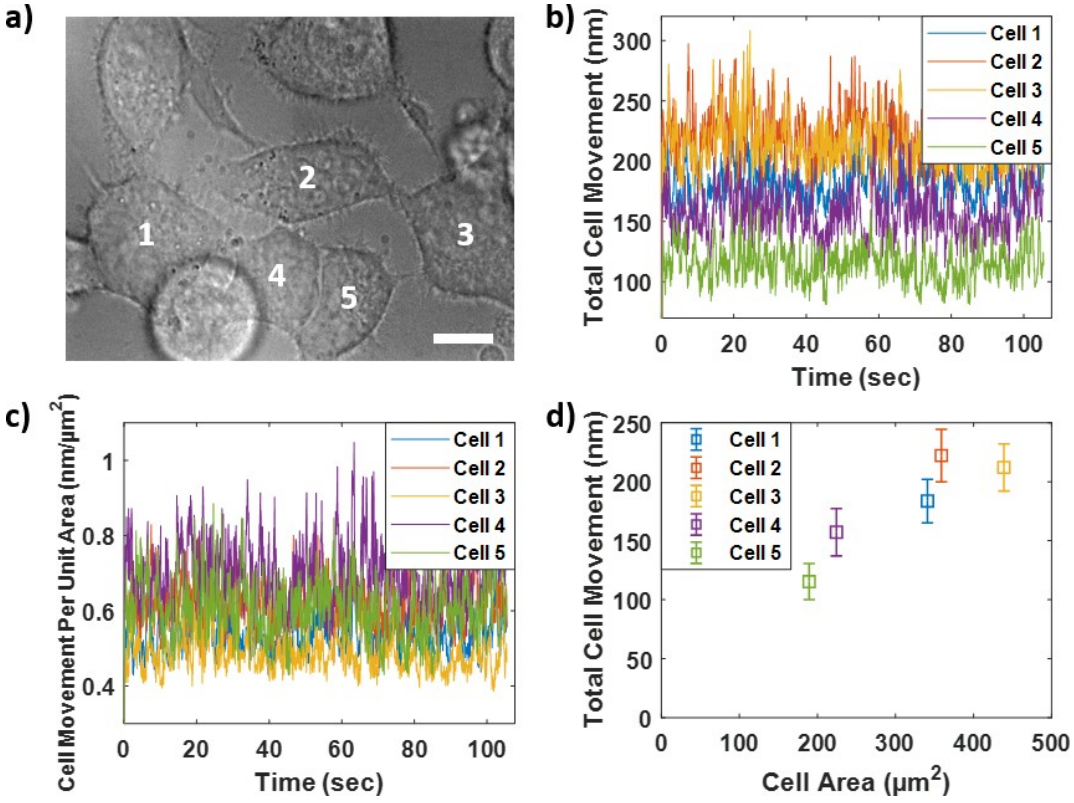

Figure 7. ***Cell-Cell Variations in Membrane Fluctuations.*** (a) 5 cells picked-up from a population for data analysis. (b) Cell heterogeneity in respective 5 cells. (c) Cell heterogeneity after area normalization. (d) Plot of total cell movement v/s cell area.

If all the cells are identical, we should expect linear characteristics for total cell movement against cell area. But predominant cancer cells at different sites can have a distinct molecular makeup. Some cancer cells relocate to a different location away from the parent site and are exposed to distinct environmental conditions. Thus, they may end up as genetically diverse from their parents due to interactions with other cells in the local environment. Fig. 7d shows a similar plot for individual cells selected above and we

observe non-linear characteristics. This can be due to the metabolic activity difference between cells or due to the cell status difference. Thus, the membrane activity varies from cell to cell because of their phenotypic differences.

3.5 Intra-cell correlation and differences

In Section 3.4, heterogeneity between physically distinct cells of the same population is discussed. We further utilize our good lateral resolution along with the resolution in z-direction to analyze the activity of different regions within a single cell. Such analysis enables us to calculate the energy expenditure of a cell at a given location. While some studies mention that the membrane activity is ATP-regulated[1], these results may be helpful to analyze the effect of anti-cancer drugs used for ATP-depletion. The ability to analyze membrane fluctuations at multiple regions within a single cell is essential to analyze the activity of biomarkers used for cancer treatment. In this case, a plot of cell region movement against time shows correlation and differences from region to region. This explains the coordinated activity at a given cell region for systematic functioning of a cell.

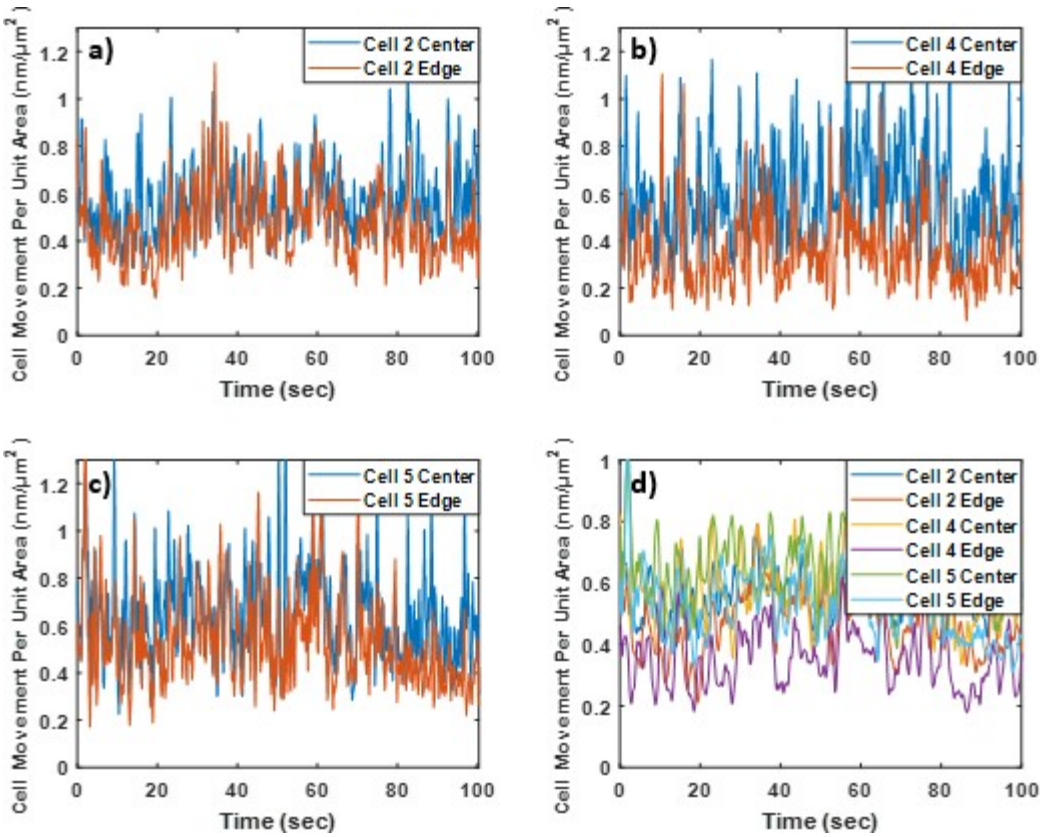

Figure 8. ***Comparison Between Center Region and Distal Regions in a Single Cell.***
Analysis of two distinct regions within a single cell: (a) Cell 2, (b) Cell 4 and (c) Cell 5 as mentioned in Fig. 6a. (d) Combined plot of (a), (b) and (c).

A comparison of cell movement per unit area between the center region of the cell and edge of a cell is shown for three different HeLa cells in Fig. 8. The calculation of cell movement per unit area is the same as mentioned in Section 3.4 except that a binary image mask with region of interest is applied to extract data for a particular region. It was observed that the membrane activity in the center region is comparatively higher compared to the membrane activity at the edge. It was also observed that the rise or fall in membrane activity shows correlation for two different at a given time point. Both these observations may be related to the uniformity and/or non-uniformity in the amount of ATP expenditure by a cell along the plasma membrane. Plotting the results of all three cells together in Fig. 8d shows that the fluctuations at center regions of most of the cells are

more active compared edges of the all other cells. Although these results show comparison between two regions within a cell, the SPR resolution enables us to analyze multiple small regions in a given cell which is discussed below.

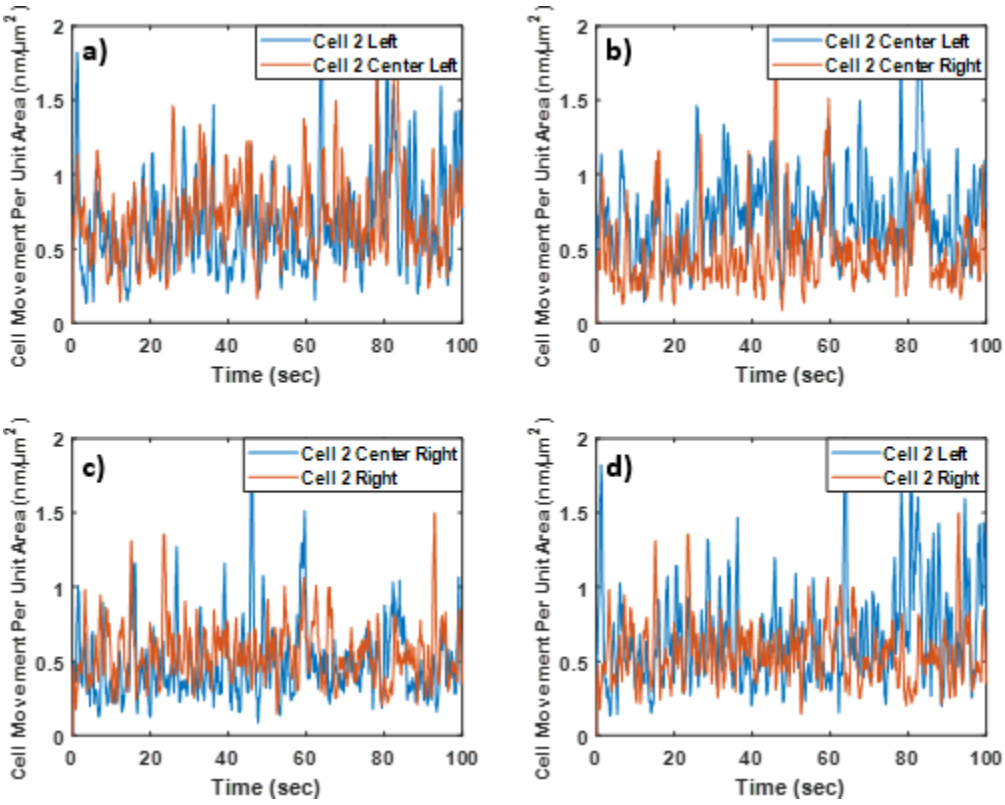

Figure 9. ***Intra-cell Correlation and Variations in Cell 2.*** Comparison between different regions (2 x 2 μm in size): (a) left v/s center-left, (b) center-left v/s center right, (c) center-right v/s right. The comparison of non-adjacent regions is plotted as (d) left v/s right.

We further discuss about the membrane activity of intra-cell regions with no specific boundary conditions like the cell center or cell edge. Figure 9 shows comparison of adjacent and non- adjacent cell regions in Cell 2. It can be observed that the adjacent regions plotted in Fig. 8a and Fig. 9c show good correlation in membrane activity over a given period of time. At the same time, the results in Fig. 8b show difference in membrane movement as a function of time. This provides a distinct insight into intra-cellular heterogeneity compared to the one shown in Fig. 8. Another distinct observation is seen in Fig. 9d where the non-adjacent regions show good correlation in fluctuation

amplitudes over a period of time except that they display transient heterogeneities around 80 seconds and 100 seconds respectively.

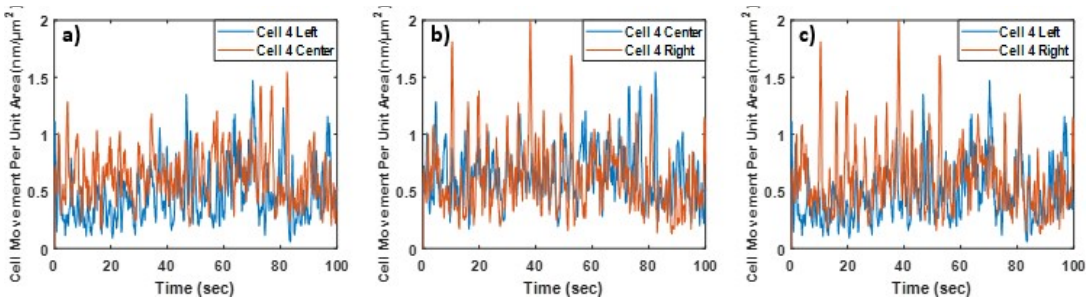

Figure 10. ***Intra-cell Correlation and Variations in Cell 4.*** Comparison between different regions (2 x 2 μm in size): (a) left v/s center, (b) center v/s right. The comparison of non-adjacent regions is plotted as (d) left v/s right.

The plots in Fig. 10 give yet distinct example of intra-cell heterogeneity. While the adjacent regions plotted in Fig. 10a show less correlation in cell membrane movement, the adjacent regions plotted in Fig. 10b show good correlation. The non-adjacent regions plotted in Fig. 10c show good correlation except that they display transient heterogeneity around 5 seconds. This behavior is similar to the one mentioned in case of Cell 2. Cell 4 could be divided into only 3 distinct regions of size 2 x 2 μm because the area of this cell is less compared to Cell 2.

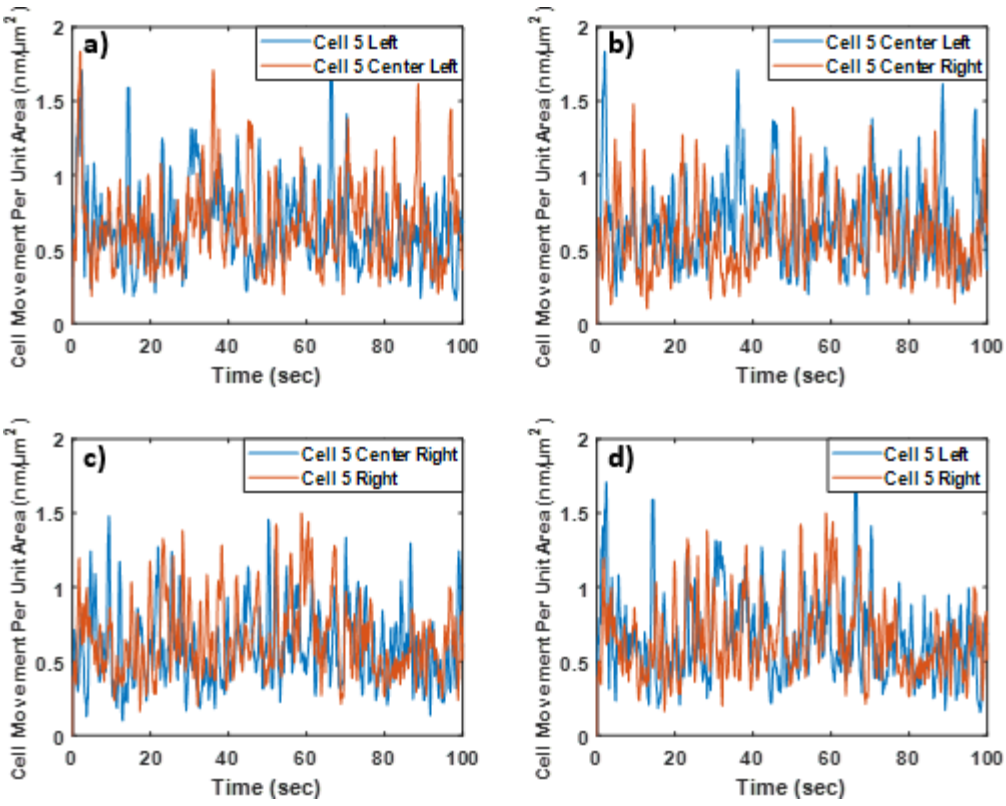

Figure 11. ***Intra-cell Correlation and Variations in Cell 5.*** Comparison between different regions (2 x 2 µm in size): (a) left v/s center-left, (b) center-left v/s center right, (c) center-right v/s right. The comparison of non-adjacent regions is plotted as (d) left v/s right.

The analysis of different regions in Cell 5 is shown in Figure 11. In this case, most of the cell regions, adjacent or non-adjacent show good correlation over a period of time except a couple of transient heterogeneities at some time points.

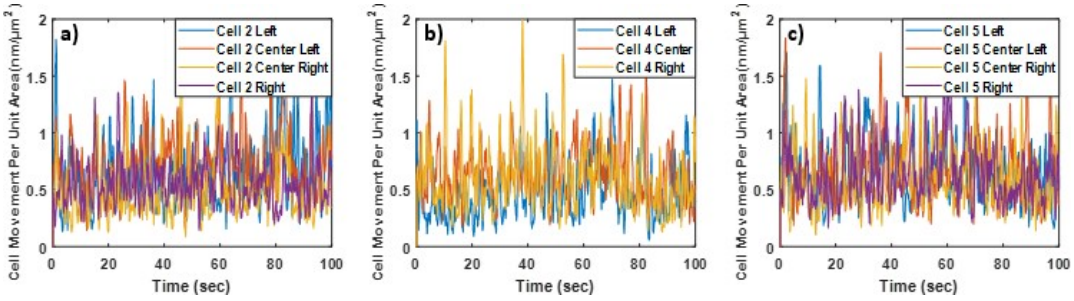

Figure 12. ***Combined Intra-Cell Correlation and Variations for Each Cell.*** Combined plots of different cell regions in (a) Cell2, (b) Cell 4 and (c) Cell 5.

The comparison between different cell regions of single cells is put together in Figure 12 which shows that the average fluctuation amplitude for a given period of time is more or less similar for all these regions.

3.6 CELL MEMBRANE MOVEMENT AND ITS RELATION TO SUB-NANOMETER MEMBRANE FLUCTUATIONS

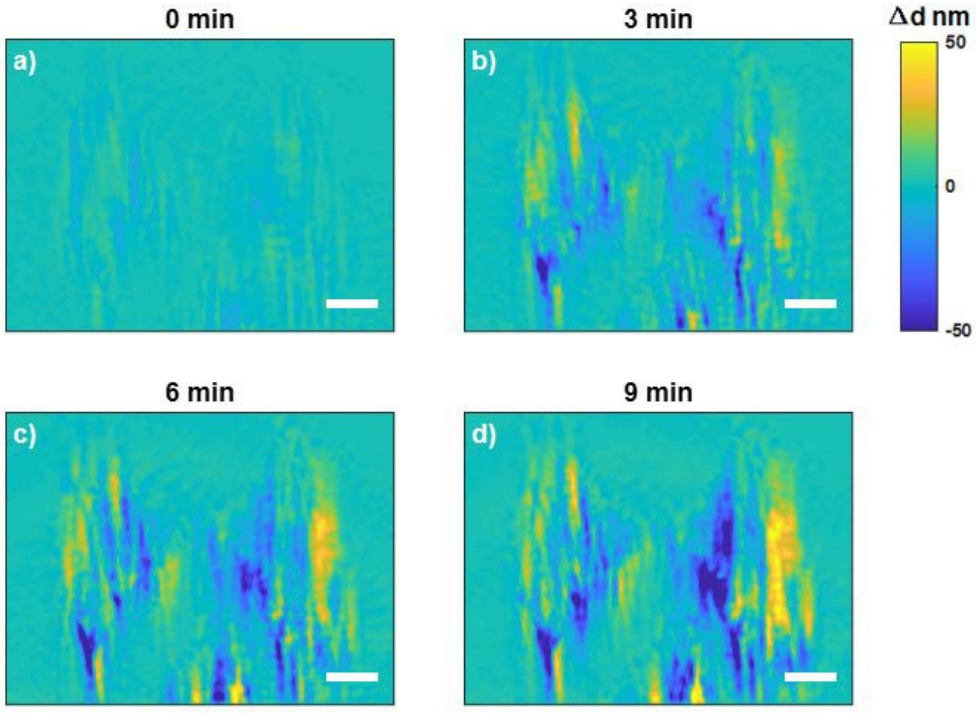

Figure 13. ***Correlation in Cell Membrane Movement Over Longer Time Duration.*** Membrane movement at (a) 0 min, (b) 3 min, (c) 6 min and (d) 9 min. A 3-dimensional demonstration of this data can be found in Movie 3.

Apart from the small membrane fluctuations that we discussed so far, we also see a collective movement of a large region within the cell as we extend the time of observation. We recorded a 10 minutes video to image the membrane movement of the cell bottom membrane with SPR. We show this movement at different points in time in Fig. 13a – 13d. After 6 minutes, we clearly see the cell on the right having a significantly huge movement. A region of this cell is seen to move 10's of nanometers towards the

substrate while the other region has moved similar distance away from the substrate. This shows the interdependence of cell regions within a single cell while carrying out different physiological processes. We tried to correlate these membrane movements with sub-nanometer membrane fluctuations. Fig. 14 shows the SPR image, sub-nanometer fluctuation map and a membrane movement map for two cells shown in Fig. 13a – 13d.

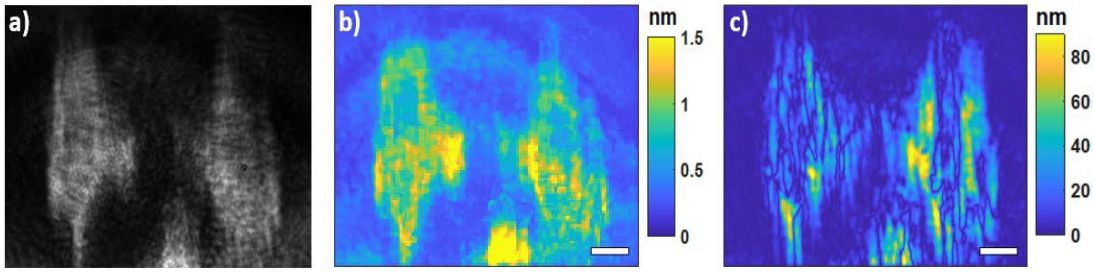

Figure 14. ***Correlation Between Sub-Nanometer Fluctuations and Membrane Movement.*** (a) SPR image of two cells mentioned in Fig. 13a – 13d, (b) sub-nanometer fluctuation map and (c) membrane movement map for the two cells mentioned in (a).

We get the sub-nanometer fluctuation map by taking the standard deviation of the small fluctuations for every 5x5 pixel region over time. Additional details for this calculation can be found in the supporting material (section 3). The absolute value of Fig. 13d gives us the membrane movement map. We observe that these two map correlate in the regions where the cell membrane is more active compared to other cell regions. These maps are not meant to correlate 100% as both the movements are driven by independent active processes some of which can be common to both.

3.7 Cell Metastasis

Cancer cells are distinguished from normal cells mainly due to malfunctioning of genes that regularize processes like DNA replication and cell division. Tumor is formed at sites where such a rapid division takes place. The rapidly advancing technology for tumor surgery would make this a

trivial issue. However, some cancer cells gain the ability to relocate from primary tumor sites to secondary sites by penetrating through the tissues at the primary site. This movement of cell is termed as cell metastasis and is mainly responsible for producing malignant cancer inside the body. The motility of a cell is enabled due to the alterations in plasma membrane guiding the cell on its way to the most favorable secondary site for invasion. These invasions are mainly guided by actin-rich invaginations or protrusions in the plasma membrane like filopodia, lamellipodia, invadopodia, podosomes, phagocytic cups, uropods and blebs[11]. The cell membrane is exposed to various mechanical forces to undergo such alterations responsible for cell metastasis. The analysis of membrane fluctuations can be correlated to cell motility as a measure of cell's mechanoresponse. HeLa is a human cervical cancer cell line found in the epithelial tissue. It is classified as a human carcinoma type of cancer cell line and is less known for formation of metastases. Hence, we use HT-1080 which is a fibrosarcoma cell line derived from the connective tissues with softer cell membrane and widely known for cell motility assays. This cell line was used to look for a correlation between cell motility and fluctuations of the plasma membrane. It is found that the area of displacement during cell motility shares a linear relationship with the total cell movement in terms of the amplitude of membrane fluctuations. The HT-1080 cell line was cultured in a way similar to HeLa cell culture process as mentioned in Section 2.1. These cells were cultured on a plasmonic substrate and were incubated for around 48 hours at 37°C and 5% CO_2 to form a monolayer. A popular scratch assay has been used to test the motility of these cells. A soft PDMS knife was used to scratch away some of the cells and the supernatant was replaced with a fresh culture medium already incubated for about 15 minutes. The sample with remaining cells is loaded on the SPR setup to carry out the experiment. Cell motility is observed for

multiple cells located near the boundary of the scratch with a 60X objective lens. Firstly, a video with bright field images was recorded to measure area of displacement over a fixed time interval. Then we switch to SPR mode for the same cells as in the bright field recording. Now the membrane fluctuations are recorded to calculate the total cell movement over a fixed interval of time. The same process is repeated for multiple cells to record the area of displacement and total cell movement for each cell. The cells have distinct displacement and membrane fluctuations because of cell heterogeneity as discussed in Section 3.4. Hence, we observe differences in these two factors for multiple cells observed for a fixed time interval. However, the plot of the area of displacement v/s total cell movement for these cells turns out to be a linear relationship as shown in Fig. 15 below.

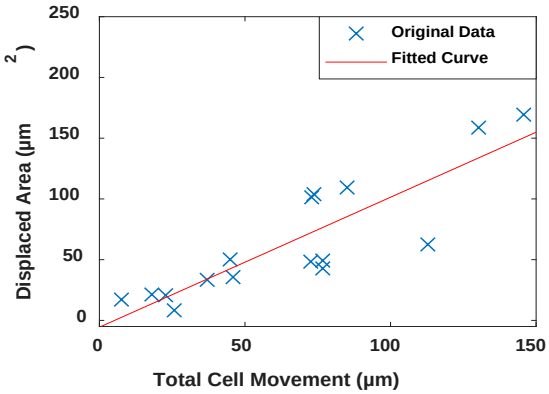

Figure 15. ***Correlation Between Cell Motility and Membrane Fluctuations.*** Total cell movement extracted from the membrane movement map as mentioned in Section 3.6 shows linear relationship against the area displaced during cell motility.

It can be seen that more the displacement of a cell, the higher is the total cell movement. This finds more relevance to the fact that these adherent cells try to counteract on their innate adherence property in order to move from one place to the other. As seen earlier, the alterations in cell membrane play a key role in metastasis. Hence, it is quite logical to observe the highly active cell membrane for larger displacement during cell motility.

REFERENCES

1. Biswas, A., A. Alex, and B. Sinha, *Mapping Cell Membrane Fluctuations Reveals Their Active Regulation and Transient Heterogeneities.* Biophys J, 2017. **113**(8): p. 1768-1781.

2. Monzel, C. and K. Sengupta, *Measuring shape fluctuations in biological membranes.* Journal of Physics D: Applied Physics, 2016. **49**(24).

3. Ramaswamy, S., J. Toner, and J. Prost, *Nonequilibrium noise and instabilities in membranes with active pumps.* Pramana-Journal of Physics, 1999. **53**(1): p. 237-242.

4. Rylander, C.G., *Quantitative phase-contrast imaging of cells with phase-sensitive optical coherence microscopy.* Optics Letters, 2004. **29**(13): p. 1509-1511.

5. Limozin, L. and K. Sengupta, *Quantitative Reflection Interference Contrast Microscopy (RICM) in Soft Matter and Cell Adhesion.* Chemphyschem, 2009. **10**(16): p. 2752-2768.

6. Wang, W., *Single cells and intracellular processes studied by a plasmonic-based electrochemical impedance microscopy.* Nature Chemistry, 2011. **3**(3): p. 249-255.

7. Reipert, S., *Rapid microwave fixation of cell monolayers preserves microtubule- associated cell structures.* Journal of Histochemistry & Cytochemistry, 2008. **56**(7): p. 697-709.

8. Zimmerberg, J. and M.M. Kozlov, *How proteins produce cellular membrane curvature.*

Nature Reviews Molecular Cell Biology, 2006. **7**(1): p. 9-19.

9. McMahon, H.T. and J.L. Gallop, *Membrane curvature and mechanisms of dynamiccell membrane remodelling.* Nature, 2005. **438**(7068): p. 590-596.

10. Dagogo-Jack, I. and A.T. Shaw, *Tumour heterogeneity and resistance to cancer therapies.*

Nature Reviews Clinical Oncology, 2018. **15**(2): p. 81-94.

11. Fackler, O.T. and R. Grosse, *Cell motility through plasma membrane blebbing.*

Journal of Cell Biology, 2008. **181**(6): p. 879-884.

Supplementary Material

A. SPR CALIBRATION FOR CONVERSION FROM REFLECTANCE TO DISTANCE

We present a much detailed and sophisticated process to convert originally obtained reflectance image from SPR to distance and change in distance of the cell membrane from the plasmonic substrate. Initially the angle of incidence is aligned perpendicular to the substrate and appropriate objective focus is adjusted. As the angle of incidence is increased with constant speed, we record the entire process to obtain the SPR curve as shown in Fig. S1a. This reflectance as a function of angle of incidence data is recorded and termed as an angle scan. The minimum reflectance or maximum absorption angle is different for cell adhered surface compared to vacant surfaces. This has been already shown in Fig. 2b. It is a key point to note that the angle with best contrast does not mean it the best angle to observe membrane fluctuations. If the angle with highest contrast is chosen, it will most probably lead to saturation of reflected light for anything close to the plasmonic substrate. On the other hand, having a good dynamic range is also important to distinguish very small movements in the z-direction. A good dynamic range can be achieved if an angle is selected somewhere on the slope of the SPR curve while saturation can be avoided by selecting an angle on the lower end of this slope. In our case, we choose the angle where reflectance is 30% of the maximum reflectance. This 30% value is with respect to the cell region as we are interested in studying very small fluctuations of the bottom cell membrane. The incident light is parked at this angle as shown with a vertical green line in Fig. S1a.

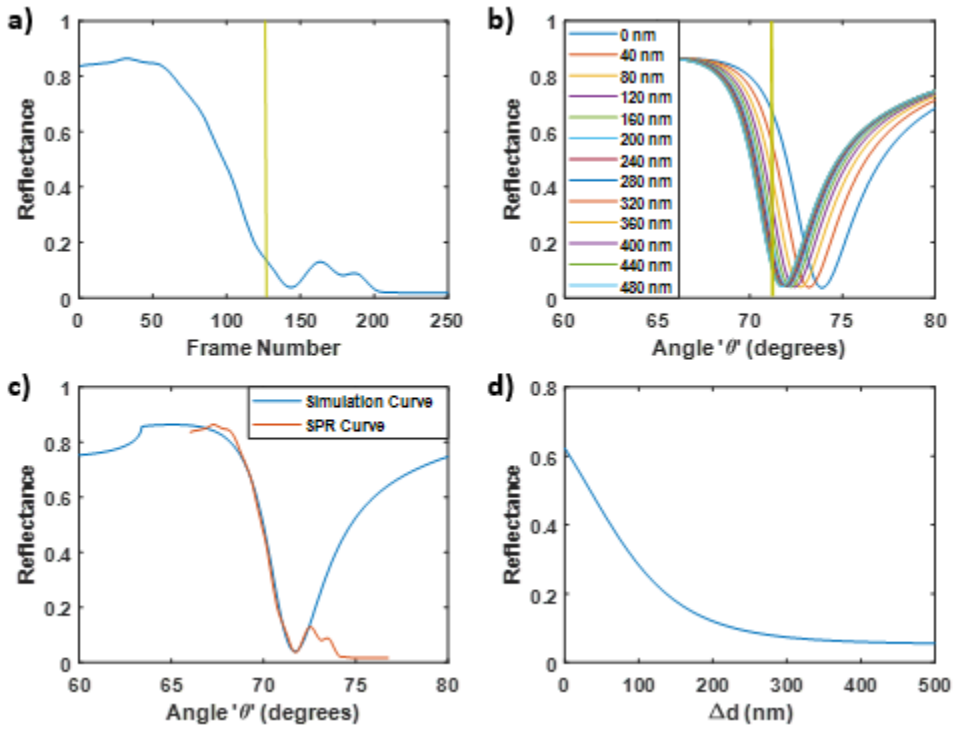

Figure S1. ***Mapping SPR Angle Scan and Simulation to Derive a Calibration Curve.*** (a) SPR angle scan plot for a non-cell region, (b) Simulation of optical path, (c) Mapping the angle scan plot of SPR to the plot from the simulation data and (d) calibration curve to show the relation between reflectance and change in distance.

By parking the angle of incidence as mentioned above, we record a movie to observe the bottom cell membrane movement over time. Evaluation of this membrane movement needs a robust calibration to convert reflectance to distance or change in reflectance to change in distance. For this purpose, a simulation software is used to model the system under test. The system includes materials with distinct refractive indices like glass substrate, plasmonic surface, surface to membrane gap filled with culture medium, cell membrane and cytoplasm. The gap between plasmonic surface to cell membrane is the salient feature of this system/model as the change in reflectance if primarily due to change in the gap thickness. Thus, we simulate this gap from 0 – 500 nm to obtain a plot of reflectance v/s angle of incidence with multiple thickness curves as shown in Fig. S1b. We use this plot as a reference to convert reflectance signal from SPR into distance. If we cut a vertical line on this simulation data at the parked angle, we get a calibration

curve for reflectance v/s distance as shown in Fig. S1d. The purpose of this calibration curve will be comprehended as we go through Fig. S2.

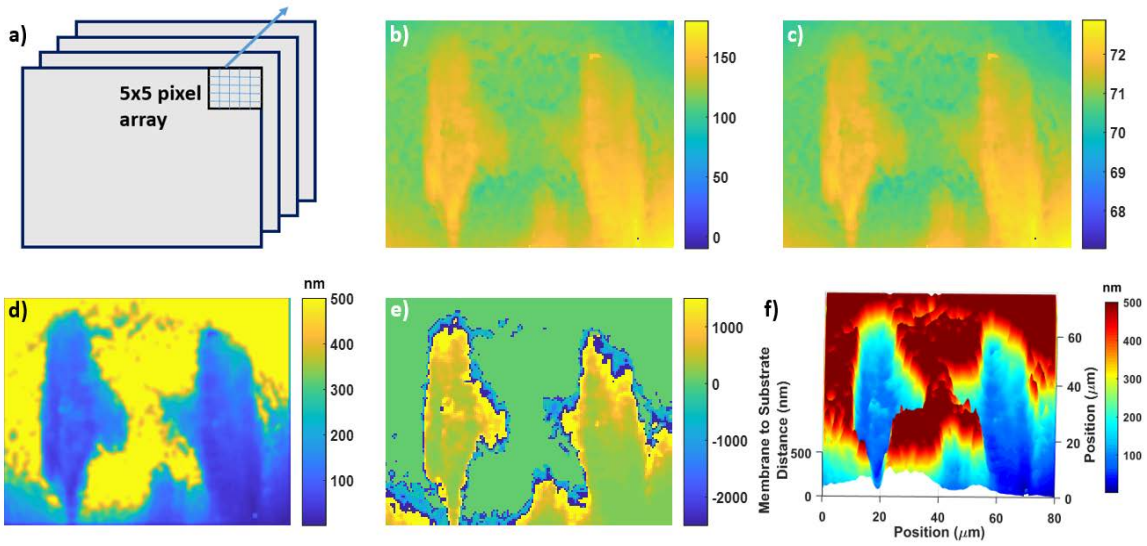

Figure S2. ***Mapping Reflectance to Distance in nm.*** (a) Implement sliding window. (b) Frame Number Map. (c) Angle Map. (d) Distance Map. (e) Slope Map. (f) 3-dimensional plot of the distance map mentioned in (d).

To start with the calibration, let's first note that the SPR data gives us angle scan recording and intensity as a function of frame numbers while the simulation curve contains reflectance as a function of angle of incidence. Our end goal is to relate the reflectance signal from SPR with thickness curves with the help of angle of incidence. Let's begin with converting those frame numbers from SPR angle scan data to angle of incidence's. For this purpose, we select the no-cell/vacant surface from SPR angle scan data and we select a 500 nm thickness curve from the simulation data which also corresponds to a no-cell region. Normalizing the SPR no-cell curve and fitting its x-axis (frame numbers) with the simulation curve as shown in Fig. S1c, gives us an equation to convert all frame number values to corresponding angle of incidence values. The primary purpose of collecting angle scan data was to lead the way to this very equation. The decisive bottom membrane movements are recorded as a movie by parking the angle of incidence at a fixed angle with 30% reflectance. To correlate this data to the simulation plot, we iterate over

the angle scan image by selecting a 5x5 pixel region at a time as shown in Fig S2a. For every such 5x5 region, reflectance is plotted v/s frame number and the frame number for reflectance with 30% of maximum reflectance value is recorded in an entire 5x5 region of a new 2D image. Similarly, frame numbers corresponding to 30% reflectance values are recorded for all other 5x5 regions and a frame number map is created as shown in Fig. S2b. Now, using the above equation, this frame number map is converted to an angle map as shown in Fig. S2c. As we already know that all these angles correspond to 30% reflectance values, we find the corresponding thickness curves at 30% reflectance values for these angles in the simulation plot. Detecting these thickness curves is equal to finding the distance map corresponding to the 30% reflectance values. This distance map can be seen in Fig. S2e which also gives a 3D bottom membrane morphology as shown in Fig S2f. To make the data analysis easier, we use the calibration curve to find the slope map ($\Delta d/\Delta I$) corresponding to the distances in the distance map. Now, if we multiply the image for change in reflectance over time (ΔI) from movie data with the slope map, we convert the entire reflectance image into a distance image. We can simply multiply the slope map to a stack of such images to convert a whole stack of SPR reflectance images into distance images. This step makes the quantitative analysis of membrane fluctuations like patch detection, evaluating fluctuation amplitude, patch size, total cell movement, confirmation of active membrane fluctuations and cell-cell variations much easier.

B. Membrane Fluctuations over Different Time Intervals

The plasma membrane exhibits continuous active membrane fluctuations which can be perceived as distinct over different time intervals.

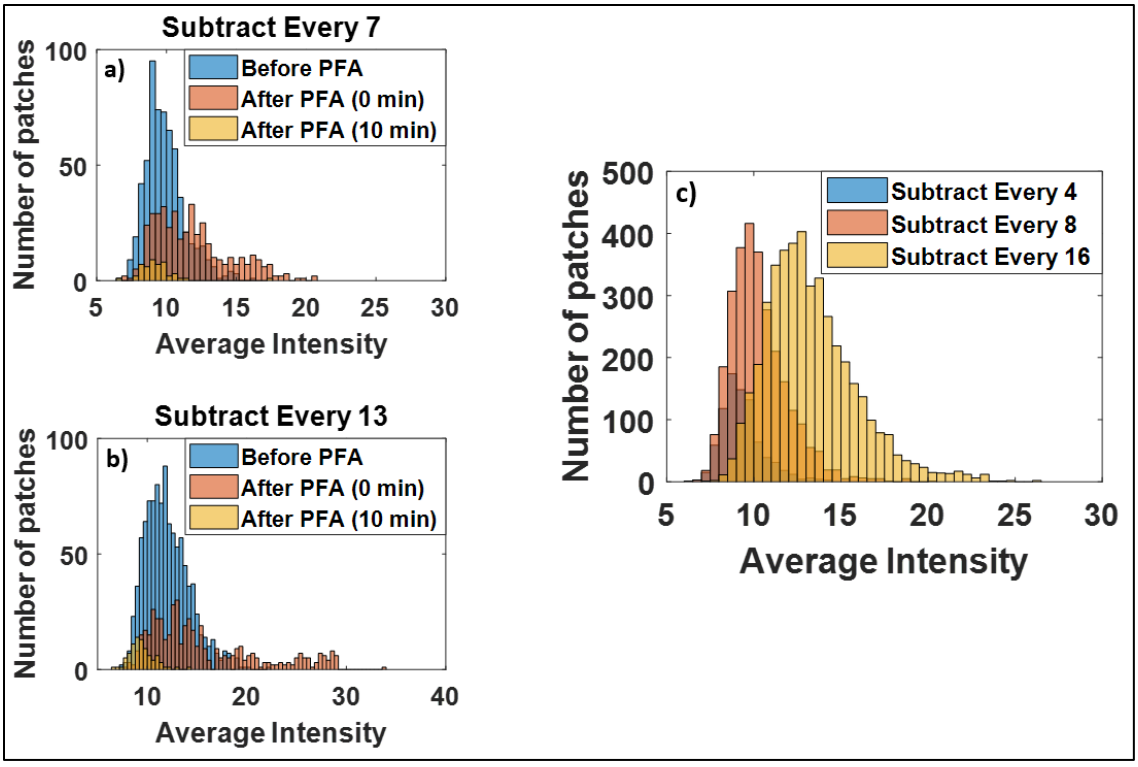

Figure S3. ***Membrane Fluctuations as a Function of Time.*** (a) Cell fixation process analysed using membrane fluctuations over half a second. (b) Cell fixation process analysed using membrane fluctuations over one second intervals. (c) Histogram of membrane fluctuations over different time intervals.

C. Analyze Temporal Membrane Fluctuations Before and After Cell Fixation

The time domain membrane fluctuations shown in Fig. 5c are extracted by the process explained in Figure S4 as shown below.

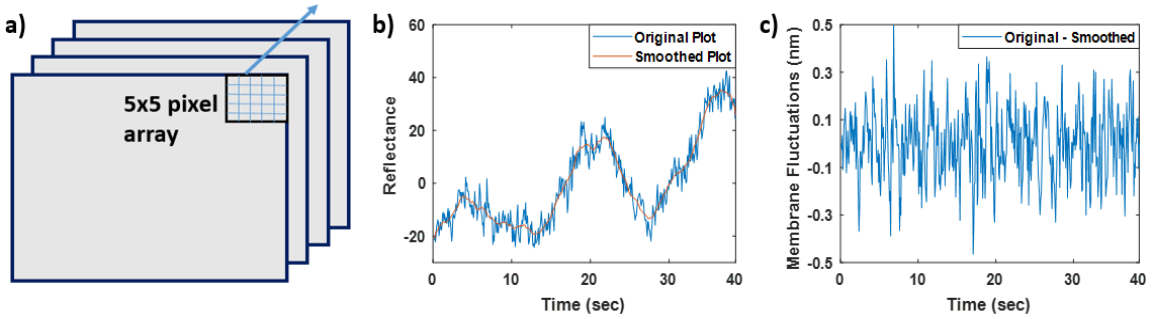

Figure S4. ***Extracting Sub-nanometer Membrane Fluctuations.*** (a) Choose a 5x5 pixel location from an image stack. (b) Plot the reflectance v/s time curve for the corresponding location (c) Subtract the smoothed version from original plot to get sub-nanometer membrane fluctuations.

Select a 5x5 pixel data from the one of the cell regions in the SPR image. Then collect an image stack for the same 5x5 pixel location in the same SPR data. The plot of this location versus time is the one shown as original plot in Fig. S4b. Although this plot shows big membrane fluctuations over a longer period of time, we are interested in the sub-nanometer membrane fluctuations to analyse cell fixation response. Thus, smooth the original plot in time to obtain a smoothed plot as shown in Fig. S4b. Subtract the smoothed plot from the original plot to get the sub-nanometer membrane movement as shown in Fig. S4c. The same process is carried out for SPR data before cell fixation and data after cell fixation. The results for the same are mentioned in Fig. 5c.

Similar process is again used in Fig. 8b to create a sub-nanometer fluctuation map. It is distinct from the above process in the sense that the algorithm iterates over the entire image to get plot shown in Fig. S3c for every 5x5 location. The standard deviation values of these plots are replaced at these locations in a new image which is termed as 'Sub-nanometer Fluctuation Map'.

D. SPR AND BRIGHT-FIELD DATA BEFORE AND AFTER CHEMICAL FIXATION

We have already seen the temporal plots showing reduced membrane fluctuations after cell fixation. Fig. S5 shows the corresponding change in bright-field and SPR data after fixation.

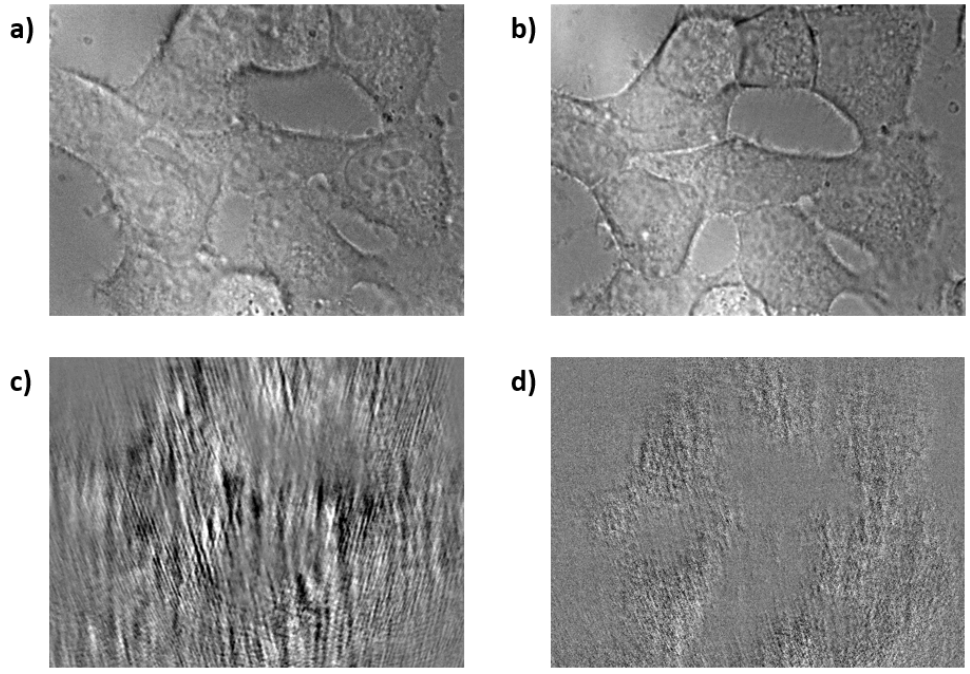

Figure S5. ***Difference in Cell Status After Cell Fixation.*** (a) Bright-field image of multiple cells before cell fixation. (b) Bright-field image of multiple cells after cell fixation. (c) Membrane fluctuations from SPR data before cell fixation. (d) Membrane fluctuations from SPR data after cell fixation.

Although we can observe no change in the bright field images before and after cell fixation in a 2-dimensional data, we clearly see inhibition of organelle movement in the movie for data after fixation. At the same time, we can see a significant change in the membrane fluctuations before and after cell fixation. The same is explained in Fig. 5a – 5e in the main article.