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14. ABSTRACT We report the achievements of the first year of our project. These achievements include the successful building of a super-resolution microscope that is capable of performing 2D and 3D Stochastic Optical Reconstruction Microscopy (STORM) and Photoactivated Localization Microscopy (PALM). With this microscope, we have imaged the spatial organization or clustering of the CCR7 receptor on the surface of MCF-7 breast cancer cells that had been cultured on gelatin substrates. This imaging is the first of its kind for breast cancer. We intend to correlate expression of this receptor and others with the extracellular matrix proteins within which we culture breast cancer cells. We have also begun work on an elegant method of a dynamically controllable synthetic matrix that is based on light-modulated proteins. We anticipate that in 2-3 months we will have the capability to synthesize, reversibly and controllably, 3D cell-culture environments whose mechanical and chemical properties can be modulated using near-IR light. This would be a significant accomplishment, as most synthetic matrix environments that are being developed are static and 2D. Ultimately, we will use this dynamically controllable synthetic matrix to study how the cellular microenvironment maintains and controls the hypothesized CD44+CD24-ALDH1+ cancer stem cell phenotype.					
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Introduction

Several markers, $CD44^+$ and $CD24^-$, and more recently $ALDH1^+$, have recently been found to be indicative of breast CaSCs. In order to increase our understanding of cancer stem cells and their role in cancer progression and metastasis, our goal has been to study *how the cellular microenvironment maintains and controls this cellular phenotype*. To realize this goal, we proposed to use 3D super-resolution microscopy to visualize how individual breast CaSCs and tumor cells interact with each other and their microenvironment. Knowledge of the spatial patterning of these intercellular connections would enable us to understand what maintains and controls the cancer stem-cell phenotype. Equally important, with 3D super-resolution imaging, we would be able to view changes in cell-cell junctions and surface-marker expression in response to extracellular perturbations (e.g. mechanical and chemical). In so doing, we would be able to address outstanding questions about the $CD44^+CD24^-$ phenotype: Is the phenotype stable throughout all stages of tumor growth? Is it a temporary state that is dynamically switched by chemical and mechanical properties of the microenvironment? Do these cells undergo asymmetric division? What conditions are required to maintain quiescence? Finally, we proposed to create small controllable microenvironments, in which we could simultaneously culture small groups of cells, and monitor their response to mechanical and chemical stimuli. As will be described in the Body of this Annual Report, we have accomplished a significant number of objectives outlined in our original proposal: the building of our super-resolution microscope, the super-resolution imaging of surface markers of cells, and developing a synthetic microenvironment. Importantly, we have made a number of improvements to simplify and increase the accuracy of our measurements, and in the case of the synthetic microenvironment, devised an elegant and simple alternative to what we had originally proposed.

Body

We have accomplished a tremendous amount toward our stated goals in our proposal.

Specific Aim 1: To determine the properties of the breast CaSC niche

•We have completed building our super-resolution microscope. This microscope has the capability of performing both two-dimensional (2D) and three-dimensional (3D) Stochastic Optical Reconstruction Microscopy (STORM) and Photoactivated Localization Microscopy (PALM). Currently, we are utilizing our microscope to perform 2D STORM (to be described below); however with a cylindrical lens (which we acquired with funds from this grant) in the imaging path, we can also perform astigmatic 3D STORM or PALM. This latter method is

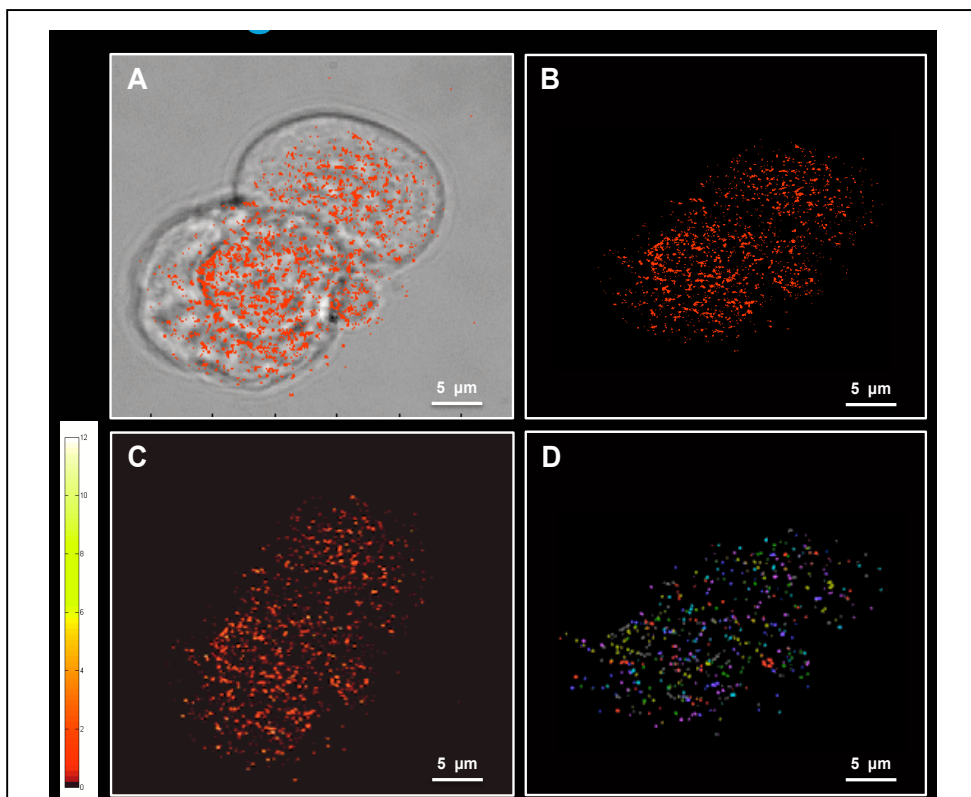


Figure 1: 2D STORM image of the CCR7 receptor on the surface of MCF-7 breast cancer cells cultured on a gelatin substrate. (A) Bright-field image of two cells with 2D STORM image overlaid on top. Each red “dot” corresponds to an individual CCR7 receptor. Clustering of the receptor is apparent. (B) 2D STORM image of the two cells in (A). (C) Density of localizations or receptors on the surface of the cells. Red corresponds to low density, yellow to high density. (D) Individual clusters of CCR7 receptors on the cells’ surface.

far more simple and cost-effective than what we had originally described (i.e. iPALM) in our grant. In addition, we have recently purchased a 658 nm diode laser that will allow us to perform 2-color STORM or PALM.

•As a first step toward imaging the native breast CaSC microenvironment, we have begun to image specific markers on the surface of MCF-7 breast cancer cells using 2D STORM. We are currently focusing on the marker CCR7, whose high expression has been correlated with poor survival and whose expression may be regulated by the tumor microenvironment [1]. MCF7 cells were cultured in DMEM with 10% FBS, 0.1 mM MEM non-essential amino acids, and 1 mM sodium pyruvate at 37 °C and 5% CO₂. Cleaned coverslips were coated with gelatin attachment factor from Invitrogen and incubated at RT for 2 hours or 37C for 30-45 min, and washed with media. The MCF7 cells were dissociated from their culture plate using enzyme-free dissociation buffer from Invitrogen, and transferred to culture wells with the coverslips sitting in the bottom. The cells were subsequently cultured overnight before they were fixed and stained with Alexa Fluor 568- (the reporter dye) and Alexa Fluor 405- (the activator dye) conjugated anti-CD197 antibody to thus tag the CCR7 marker on the cell surface. Fig. 1A is bright-field image of two MCF-7 cells overlaid with the 2D STORM image of the CCR7 marker on the surface of these cells. Fig. 1B is solely the 2D STORM image—it is the *first super-resolution image of a breast cancer cell to our knowledge*. Since clustering or spatial

organization of surface markers can influence signaling, we have performed an initial analysis of our 2D STORM images. We have performed a preliminary analysis of our images. Utilizing a tree-clustering algorithm [2] that estimates the number of clusters followed by a Gaussian Mixture model that then fits the data based on the estimated number of clusters, we are able to identify each cluster of CCR7 on a cell surface. Fig. 1C shows the density of localizations (i.e. the density of markers in region, with red to yellow corresponding to low to high density) and Fig. 1D identifies each cluster.

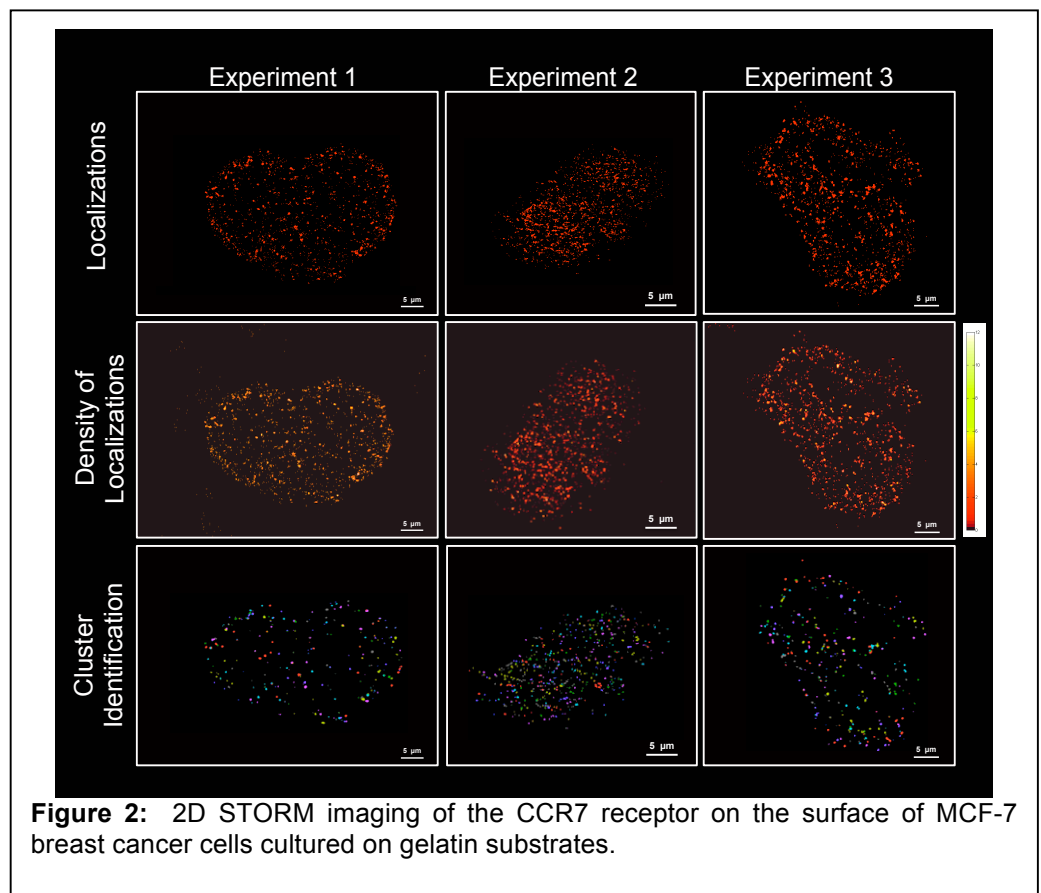


Figure 2: 2D STORM imaging of the CCR7 receptor on the surface of MCF-7 breast cancer cells cultured on gelatin substrates.

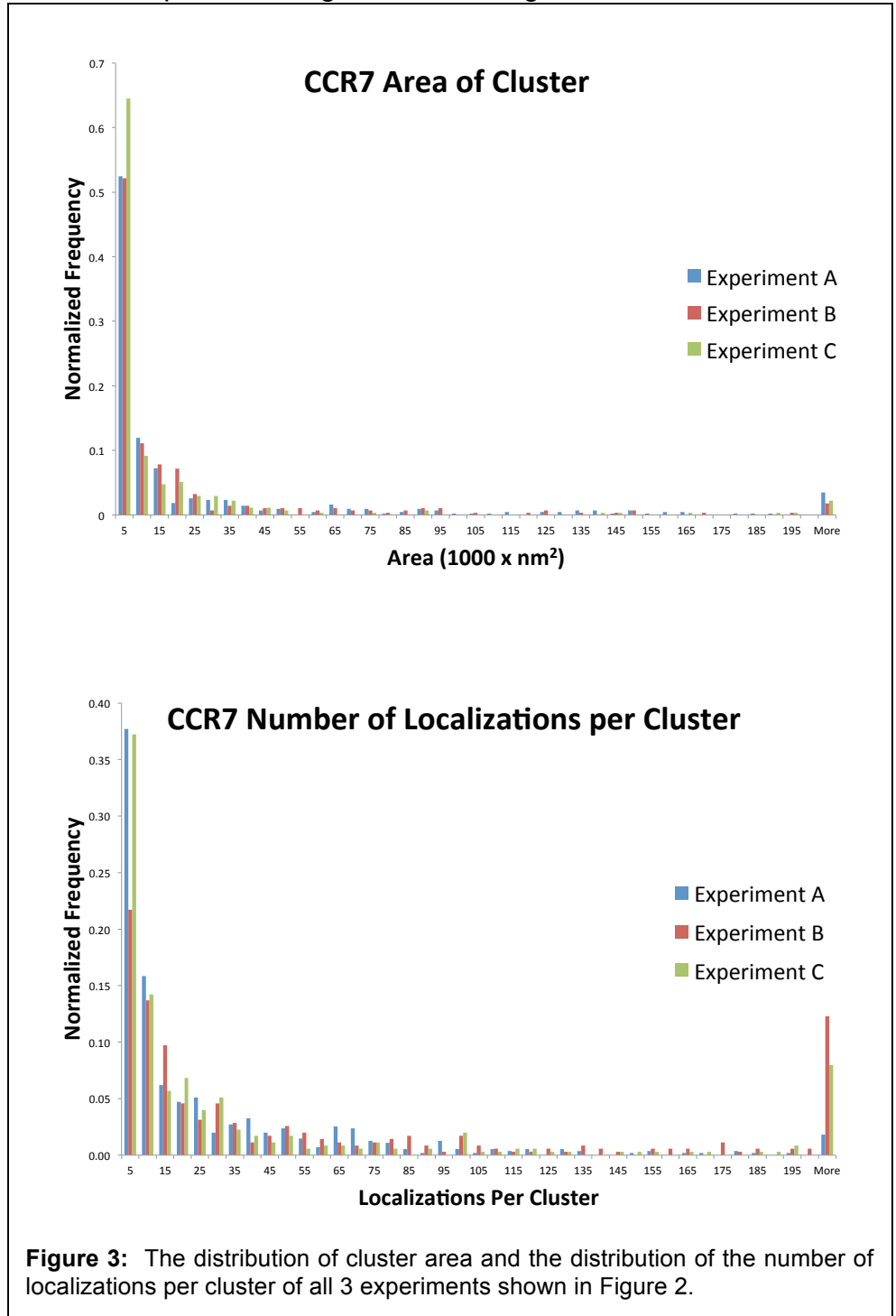
Figure 2 shows the 2D STORM images and analyzed results of three different experiments. Fig. 3 shows the distribution of cluster area and the distribution of the number of localizations per cluster of all 3 experiments.

Currently, we are repeating these experiments and developing a clustering algorithm that may be more appropriate for our surface-marker data. As well, in the coming weeks, we intend to characterize and compare the spatial organization of CCR7 on MCF-7 cells cultured on different extracellular matrix (ECM) protein-coated substrates (e.g. collagen and fibronectin). To improve upon these initial studies (Figs. 1-3), we will employ a silicone-based gel (Qgel 920 from Quantum Silicones) on top of the coverslips as the substrate upon which collagen or fibronectin will be coated. Since cells can sense hard surfaces as far as 10-15 μ m below the surface of a soft 1 kpa gel, the

coverslips will be coated with a soft 1-5 kpa silicone gel before being coated with various ECM proteins using EDC, a carbodiimide cross linker from Pierce Protein. We will thus be able to obtain information about how surface receptors cluster under conditions that are more similar to native tissue. For a control, we will also be imaging MCF10A cells, a non-cancerous cell line.

We will next characterize the organization of other tyrosine kinase receptors and adhesion molecules relative to each other using multi-color STORM. This should lay the foundation for imaging the native breast CaSC microenvironment.

•We have just begun setting up our magnetic particle force spectroscopy. Our major breakthrough here is how we will be measuring the displacements of the magnetic particles that are adhered to specific ECM units and subject to a magnetic field. We will be using *in-line digital holographic* microscopy [3, 4]. With *in-line* holographic microscopy, the 3D position of the magnetic particles is determined by reconstructing their position based on the image of the interface between light scattered off the beads and the incident reference wave. Holographic microscopy is the ideal optical tracking method for our force spectroscopy because it has: 1) high-localization nanometer precision; and 2) high-speed image acquisition that is 2-3 orders of magnitude faster than conventional 3D optical-tracking techniques such as confocal microscopy. Furthermore, holographic microscopy relies on scattered light vs. fluorescent labels, which ultimately mean that the magnetic particles only need to be functionalized with antibodies and not fluorescent labels as well. Finally, there is unlimited signal because we are measuring scattered laser light. We have just completed creating our *in-line* digital holographic microscope.



Specific Aim 2: To fabricate small controllable microenvironments, in which we can simultaneously culture small groups of cells and monitor their response to external perturbation

Our initial plan to create the small controllable microenvironments had been to use an appropriate composition of extracellular proteins and proteoglycans (e.g. collagen gels, matrigel, hyaluronic acid gels, and polyacrylamide gels functionalized with various ECM proteins) in a microfluidic reservoir. We would alter dynamically the mechanical properties of this microfluidic culture environment either through the application of stress or strain via mechanical actuators. We have since come up with an elegant method of creating a dynamically controllable microenvironment that does not require mechanical actuators.

Our method relies upon creating a polyvinyl alcohol (PVA) gel whose polymer crosslinking and functionalization can be achieved via a light-dependent protein-protein interaction. Specifically, Phytochrome interaction factor (PIF) and Phytochrome B (PhyB) associate in a light-dependent manner [5]. When exposed to 650nm light, phycocyanobilin (PCB), a ligand of phytochrome B, undergoes a photoisomerization that induces an allosteric transition and consequently promotes binding between PhyB and PIF. Subsequent exposure to 750nm light causes a *reversible dissociation* of the two proteins. Using *p*-Maleimidophenyl isocyanate, we can functionalize PVA polymers with PIF and PhyB, thereby allowing us to synthesize, reversibly and controllably, 3D cell-culture environments whose mechanical and chemical properties can be modulated using near-IR light. Because our intended gel can quickly decompose using light that is not phototoxic to the cells, we can perform an immediate analysis of cells.

Currently, we have we have expressed both PIF and PhyB in BL21 competent *E. coli* cells using a T7 expression vector. We have incorporated a SUMO cleavage sequence followed by a histidine tag to promote the expression, solubility, and stability of our proteins while also providing a means for purification. We are attempting to purify our proteins using a histidine affinity column, followed by cleavage of the SUMO sequence, and finally purification via size-exclusion chromatography. We are monitoring each step using Western blots, and plan to confirm isolation of the final product by mass spectroscopy.

Execution of our dynamically active protein matrix requires a custom optical setup, as the whole gel must be exposed to 650nm light in order to maintain integrity. In particular, a two-path optical system is required, and thus, we have added an upright beam path, which projects the appropriately patterned 650 and 750 nm light on a sample, to our existing super-resolution microscope. The light in the upright beam path will be patterned using two digital micro-mirror devices (DMDs). Using a combination of shutters, DMDs, and light emitting diodes (LEDs)—all of which we have acquired—we will be able to alter rapidly (on the order of milliseconds) the pattern, intensity, and wavelength of the light to which the sample is exposed, thereby controllably modulating the properties of the PVA hydrogel.

We intend to quantify the stresses and strain we can induce using our light-modulated gel with the magnetic-particle force spectroscopy technique we are developing in Specific Aim 1. Once we have parameterized the gel, we will then begin culturing cells in the gel.

Key Research Accomplishments

- Completed building of a super-resolution microscope that is capable of performing both 2D and 3D Stochastic Optical Reconstruction Microscopy (STORM) and Photoactivated Localization Microscopy (PALM)
- Acquired the first known super-resolution (2D STORM) images of the surface receptor CCR7 on single MCF-7 breast cancer cells
- Analyzed the clustering of CCR7 on MCF-7 breast cancer cells cultured on gelatin substrates
- Built an in-line digital holographic microscope to track accurately the displacement of magnetic particles
- Expressed and have begun purifying the light-modulated proteins Phytochrome interaction factor (PIF) and Phytochrome B (PhyB) in BL21 competent *E. coli* cells—these proteins are necessary to create a dynamically controllable synthetic microenvironment

•Created a custom optical setup to pattern 650 and 750 nm light onto a microfluidic reservoir that would control the binding and unbinding of PIF and PhyB once they were functionalized in a polyvinyl alcohol (PVA) gel

Reportable Outcomes

Meeting Abstracts

1. Evan Lyall, Matthew Chapman, and Lydia L. Sohn, "Characterizing Spatial Organization of Cell-Surface Receptors in Human Breast Cancer with STORM," Oral Presentation at the Annual American Physical Society March Meeting, Boston, Massachusetts, 2 March 2012.
2. Matthew Chapman, Bo Qing, and Lydia L. Sohn, "Using a Light-Activated Culture Matrix to Determine the Microenvironmental Cues that Initiate Breast-Cancer Tumor Metastasis," Oral Presentation to be given at the 243rd American Chemical Society National Meeting (BIOT Division), San Diego, California, 27 March 2012.

Press Conferences and Interviews

1. Lydia L. Sohn in "The Physics of Cancer" Press Conference, American Physical Society Press Conference, Boston, Massachusetts, 28 February 2012.
2. Lydia L. Sohn interviewed by Calla Cofield for APS News, 12 March 2012.

List of Personnel Receiving Pay from Research Effort

1. Lydia L. Sohn, PI
2. Matthew R. Chapman, Graduate Student Researcher
3. Bo Qing, Undergraduate Researcher

Conclusion

We are well underway with our funded project. The super-resolution images of the CCR7 receptor on the surface of MCF-7 breast cancer cells we have achieved are the first of their kind in the scientific or medical community to our knowledge. In the next few weeks, we will be correlating the clustering and spatial patterning of CCR7 on the surface of breast cancer cells with specific ECM proteins. Thus, we should be able to determine how expression of CCR7 is controlled by the microenvironment of tumors. This is of extreme importance given the fact that high expression of CCR7 is correlated with poor survival outcomes and metastases. We have also begun work on an elegant method of a dynamically controllable synthetic matrix that is based on light-modulated proteins. We anticipate that in 2-3 months we will have the capability to synthesize, reversibly and controllably, 3D cell-culture environments whose mechanical and chemical properties can be modulated using near-IR light. This would be a significant accomplishment, as most synthetic matrix environments that are being developed are static and 2D. Finally, we will apply all of the technologies we have been developing to characterize breast-cancer tumors and investigate the microenvironment of the CD44⁺ CD24⁻ALDH1⁺ phenotype that is thought to represent a CaSC.

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Appendix

Abstract for the American Physical Society Annual March Meeting, Boston, MA March 2012

Session Y41: Focus Session: Physics of Cancer III -- Imaging

8:00 AM–10:12 AM, Friday, March 2, 2012 Abstract: Y41.00004 : Characterizing Spatial Organization of Cell Surface Receptors in Human Breast Cancer with STORM

Authors:

Evan Lyall

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(Biophysics Graduate Group, University of California, Berkeley)

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Regulation and control of complex biological functions are dependent upon spatial organization of biological structures at many different length scales. For instance Eph receptors and their ephrin ligands bind when opposing cells come into contact during development, resulting in spatial organizational changes on the nanometer scale that lead to changes on the macro scale, in a process known as organ morphogenesis. One technique able to probe this important spatial organization at both the nanometer and micrometer length scales, including at cell-cell junctions, is stochastic optical reconstruction microscopy (STORM). STORM is a technique that localizes individual fluorophores based on the centroids of their point spread functions and then reconstructs a composite image to produce super resolved structure. We have applied STORM to study spatial organization of the cell surface of human breast cancer cells, specifically the organization of tyrosine kinase receptors and chemokine receptors. A better characterization of spatial organization of breast cancer cell surface proteins is necessary to fully understand the tumorigenesis pathways in the most common malignancy in United States women.

Abstract for the 243rd American Chemical Society National Meeting (BIOT Division), San Diego, California, 27 March 2012.

186 - Using a light-activated culture matrix to determine the microenvironmental cues that initiate breast-cancer tumor metastasis

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Many types of gels (e.g. collagen, and polyacrylamide) are currently employed to study how cells interact with their environment. The mechanical properties of these gels are established by the degree of polymer crosslinking. Once synthesized, these gels have static mechanical and chemical properties that cannot be changed. Thus, they cannot be used to study how evolving microenvironmental conditions affect cell behavior and signaling. Here, we describe a light-activated culture matrix for studying how cells adapt to dynamic microenvironmental conditions. Using a reversible, light-mediated interaction to crosslink biocompatible polymers, we can synthesize a 3D-culture environment whose mechanical properties can be modulated with near-IR light. We are employing this dynamically controllable culture matrix to investigate how microenvironmental cues facilitate tumor progression in breast cancer. In particular we are investigating the role of the

microenvironment in controlling the behavior (e.g. quiescence vs proliferation) of CD24⁻/CD44⁺ tumor-initiating cells.