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Multi-scale Time-lapse Intravital Imaging of Soft Tissues to Map Single Cell Behavior

Jessica Pastoriza[6,2,3], Yarong Wang[1,2,3], Maria Sosa[7], Julio Aguirre-Ghiso[4], John Condeelis[1,2,3], Maja Oktay[1,5], David Entenberg[1,2,3]

[1.] Department of Anatomy and Structural Biology, Albert Einstein College of Medicine, New York, New York.
[2.] Integrated Imaging Program, Albert Einstein College of Medicine, New York, New York.
[3.] Gruss-Lipper Biophotonics Center, Albert Einstein College of Medicine, New York, New York.
[4.] Departments of Medicine, Division of Hematology and Oncology, Otolaryngology and Oncological Sciences, Head and Neck Cancer Basic Research, Departments of Otolaryngology, NCI-Designated Tisch Cancer Institute at Mount Sinai, Icahn School of Medicine at Mount Sinai New York, NY, USA.
[5.] Department of Pathology, Albert Einstein College of Medicine, New York, New York.
[6.] Department of Surgery, Montefiore Medical Center, Bronx, New York.
[7.] Department of Pharmacological Sciences, Ichan School of Medicine at Mount Sinai, New York.

After more than 10 years of research it has become increasingly clear that the tumor microenvironment (TME) plays a dominant role in determining tumor phenotype. This research has led to a new understanding of the TME's impact upon tumor heterogeneity and all steps of cancer progression including tumor cell proliferation, metastasis, survival, and dormancy. A full understanding of this heterogeneity, both temporally and spatially can therefore elucidate mechanisms for these steps as well as their response to therapeutic interventions. Studies which have examined the complexity of tumors have highlighted the need to understand them as integrated systems of genes, gene networks, cell-cell and cell-stroma interactions. These latter two can best be observed through the use of multiphoton intravital microscopy (MIVM) where the interplay between cells and their immediate microenvironment can be directly visualized live and in real time.

We have developed a new technology in the form of large volume MIVM where many images, covering large tumor volumes, are stitched together to form a comprehensive record of the cellular interactions that occur throughout the heterogeneous tumor. This technique, not commonly utilized in intravital imaging, is similar to that used by pathologists in the diagnosis of disease states in tissues and organs. It consists of the utilization of both high-resolution, high-magnification images (to identify the staining and morphology of individual cells), and low-magnification overview images (to give their context and location).

Here, we report a protocol for obtaining large area high-resolution mosaic time-lapse imaging in living animals. The protocol is composed of 1) surgical techniques for the stabilization of soft tissues, including lymphatics[1, 2], lymph nodes[1], lung[3], liver, and the mammary fat-pad[4], and 2) techniques for the acquisition of multiple high-magnification tiles can be stitched together to form a large-area low-magnification. In addition to protocols for the surgery we have also developed tools such as a mammary imaging window (Fig. 1A) and microscope stage plate (Fig. 1B) which enables tissue stabilization during skin flap surgeries (Fig. 1C and 1D). Having stabilized tissue enables the acquisition and stitching of the images to generate very large MVIM data sets (Fig. 2A). From this, a subarea of interest can be selected (Fig. 2B) and high magnification high resolution time lapse imaging can be conducted to visualize individual cell dynamics (Fig. 2C).

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Acquisition of these very large MVIM image data sets enables multiparametric analyses using support vector machine (SVM) classification[5] and other systems analysis techniques that utilize arbitrary distributions and/or non-linear parameter correlations. This level of analysis can then define the combinations of microenvironmental parameters where tumor cell phenotypes of interest are likely to occur. The application of this approach at widely varying temporal and spatial scales (from minutes to weeks and from sub-cellular to tissue wide) and at different stages (early carcinoma on to metastasis), can result in a catalog of interactions between microenvironmental conditions and tumor cell phenotypes that lead to metastasis, potentially providing new insights into the mechanisms of metastasis.

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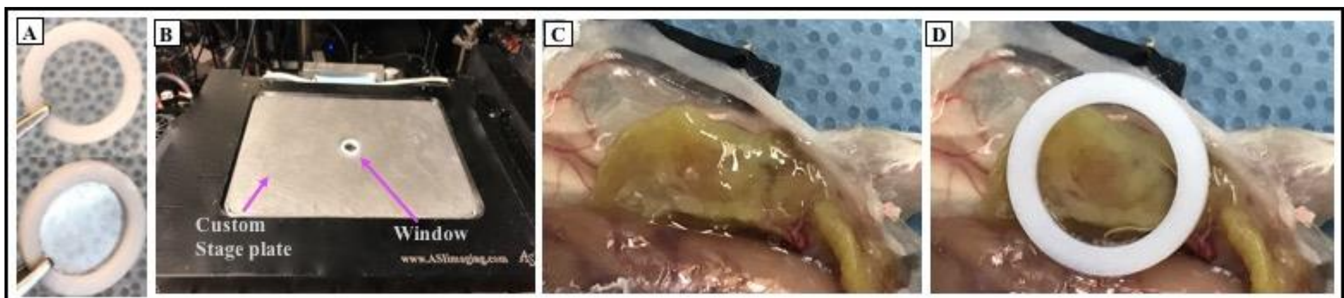

Figure 1. Overview of Surgical Technique. **A)** Stabilization imaging windows **B)** Custom made stage plate and stabilization imaging windows. **C)** Surgically flapped mouse with exposed primary tumor and viable vasculature. **D)** Stabilization imaging window adhered to tumor tissue. Adhesive is placed under the window frame, but away from the glass, to immobilize the tissue relative to the frame. Window frame is then inserted into stage plate.

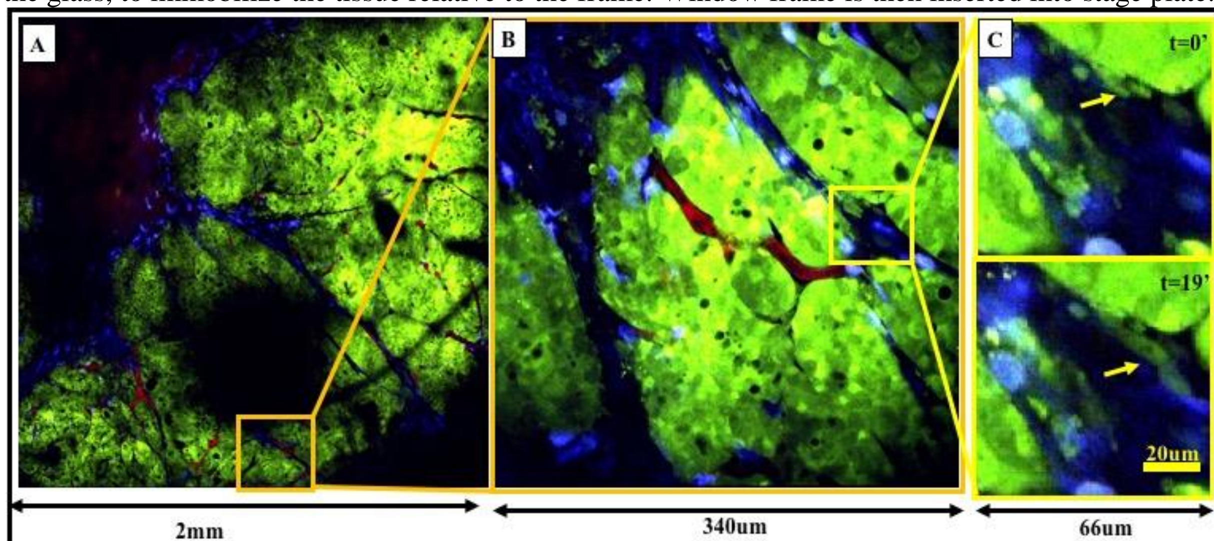

Figure 2. Mosaic time lapse images taken with MIVM in transgenic mouse in real time. **A)** 10x10 mosaic, late stage carcinoma. Green=Dendra2 expressing tumor cells, Red=Fluorescent dextran labeled vasculature, Blue=CFP expressing macrophages. **B)** Selected sub-region of interest showing an area of inflammation. **C)** Stills from a high resolution time lapse movie showing tumor cells invading from lobule (yellow arrow). Single cells can be tracked at 0.4 um resolution.

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