**Computer vision-based analysis of tumor cell invasion and adhesion**

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**Abstract:**

**Innovation and Impact Statement:**

This is a new collaborative project between the Bear and Gomez groups that brings together expertise in the cell biology of tumor cell behavior and computer vision-based analysis of massive image data sets. With the advent of multicolor live-cell imaging and gene manipulation, there is an increasing need to find new methods to extract quantitative information from images and movies. The cellular structures that are the focus of this project, invadopodia and focal adhesions, are critical for tumor cell metastasis. Yet the standard in the field for analyzing these structures is to hand select a small number (<10) and perform limited measurements of area and some dynamic properties. The method under development here will allow unbiased analysis of *all* the invadopodia and/or focal adhesions (~50-1000 per cell) over 12-16 hours. This level of data analysis is required to identify subtle or combinatorial effects of genetic and/or pharmacological manipulation. Beyond method development, this approach will allow us to understand the effects of genes known to be involved in tumor metastasis such as PTEN and LKB1 that have never been studied at this level. Furthermore, we will evaluate the effects of B-Raf inhibition on these structures using the new Plexicon compound in a B-Raf mutant melanoma cell line. Thus, this project will have a significant impact on the field of cancer metastasis by allowing the inter-related dynamics of extracellular matrix degradation and cellular adhesion to be studied at an unprecedented level.

**Specific Aims:**

In this proposal, we seek to develop methods to analyze tumor cell behavior using computer vision-based techniques. We will use these methods to understand the role of known pro-invasive genes and test the role of metastasis-implicated genes such as PTEN and LKB1 whose role in matrix degradation and adhesion control is unknown. To accomplish this, we propose two specific aims:

**Aim 1. Computer vision-based analysis of invadopodia:** In this aim, we will develop an automated, computer vision-based assay to quantify invadopodia population statistics and dynamics from live-cell movies. Using this assay, we will evaluate gene knockdowns of candidate metastasis genes such as PTEN and LKB1. In addition, we will evaluate the use of this tool with pharmacological inhibitors such as the B-Raf inhibitor.

**Aim 2. Multiplex analysis of invadopodia and focal adhesions:** In this aim, we will combine or multiplex the established method for quantification of focal adhesion dynamics with the new method of invadopodia quantification from Aim 1 using 3-color live-cell imaging. By simultaneously and quantitatively examining these structures, we can begin to ask questions about how cells manage to dynamically balance adhesion and matrix degradation to achieve invasive motility.

**Background:**

Advanced melanoma is one of the most feared human cancers {Sharpless, 2003 #5122; Chin, 1998 #5123}. Although curable through surgery when diagnosed at early stage, melanoma is characterized by its therapeutic resistance, aggressive clinical behavior, and proclivity for early metastasis. Metastasis is the process by which tumor cells leave the primary lesion and move to other parts of the body, thereby initiating tumor formation at multiple sites. During this process, cells detach from their adhesive contacts, degrade barriers such as the basement membrane, migrate to other locations within the body, invade new organs, and regain adherence to re-initiate growth {Kopfstein, 2006 #6767}. It is therefore not surprising that metastatic tumor cells have high *in vitro* cell motility and invasive capabilities.

Through the combined work of many labs, a conceptual framework for understanding cell migration has emerged {Lauffenburger, 1996 #304}. Motile cells display a characteristic cycle of steps leading to translocation. First, cells protrude a structure such as lamellipodia in the direction of migration. Second, after the protrusion has extended forward, it must become stabilized by attachment to the underlying substratum. These attachment points are known as focal contacts or focal adhesions and contain clustered integrin receptors that create a bridge between the extracellular matrix and the actin cytoskeleton. Third, the cell body is then squeezed forward from the rear in an actomyosin-based contraction event. Finally, cells must lose substratum attachment at the rear or trailing edge. In the case of tumor cells, these general steps of migration must be preceded by the local degradation of extracellular matrix using spot-like structures called invadopodia that are heavily enriched with matrix metaloproteases (MMPs). The coordination of matrix degradation by invadopodia and adhesion to matrix required for movement is poorly understood.

Computer vision approaches have been applied in cellular biology settings to analyze large collections of both still and time lapse microscopy to quantitatively assess cellular phenotypes (Peng 2008, Bioinformatics). The applications of computer vision approaches to analyzing microscopy images have ranged from large siRNA-based screens designed to discover proteins involved in mitosis (Neumann 2010 Nature) to tracking the effect of camptothecin on protein localization in individual cells (Cohen 2008 Nature). In the field of cellular motility, studies have demonstrated, using computer vision techniques, the subtle connection between cell edge movement and the Rho GTPases (Machacek 2009 Nature). These applications of computer vision techniques demonstrate the power of combining computational image processing techniques to determine the connection between perturbations cells and phenotype. By applying computer vision methods to examine Invadopodia and the interplay between Invadopodia and focal adhesions, this proposal seeks to determine the effects of several candidate genes on metastasis and develop sets of image processing tools that can be widely applied.

**Preliminary Studies:**

In collaboration with the Hahn lab at UNC, the Gomez lab has developed a software suite that automates the analysis of time-lapse movies of fluorescently tagged focal adhesions. This work is in revision for PLOS

Biology after a positive first round of reviews. This manuscript describes a set of programs that automatically finds focal adhesions in each frame of a time-lapse movie, tracks those focal adhesions through time and then collects various sets of properties that describe the focal adhesions (Fig. 1). These properties fall into three categories: static, dynamic and spatial. The static properties of the adhesions include area, intensity and eccentricity. The dynamic properties, such as the assembly and disassembly rates, are determined by automatically analyzing the properties of the adhesions through time (Fig. 2). These automated methods are comparable to prior methods used to assess focal adhesion properties (Webb 2004), but the computer vision approaches allow all the detectable adhesions to be analyzed. Thus, while manual methods are able to quantify small number of adhesions (10-25) per experimental condition, the automated methods we have developed can scale to collect hundreds of data points (Fig. 2). Finally, the software also tracks the position of each focal adhesion through time in comparison to the nearest cell edge, providing a normalized spatial measurement (data not shown). The software suite was designed as a series of modules to make expansion of capabilities easier. We expect that several of the key software components developed to analyze focal adhesions will be able to be reused with little or no modification in the development of the invadopodia analysis.

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| graphic_workflow.png  **Figure 1:** Summary of Focal Adhesion Analysis Methods showing a small region of a migrating fibroblast, the puncta identified in this region and how a single puncta is tracked. | kinetics.png  **Figure 2:** (A) Sample tracking of single adhesion (green), surrounding adhesions (blue) and the cell edge (red) (B) The intensity of the adhesion in A over time with the assembly phase (green), the stability phase (yellow) and the disassmbly phase (red) indicated. (C) Summaries of the assembly and disassembly rates. |

**Research Plan:**

**Aim 1. Quantitative analysis of invadopodial dynamics:**

*Rationale*: Invadopodia are highly dynamic structures, which form and disassemble as cancer cells degrade the extracellular matrix. Their role in cancer cell metastasis has been known for many years, but the methods used to quantify them have been limited to relatively qualitative measurements. We propose to develop computer vision software designed to quantitatively assess the properties currently collected by hand and develop new quantitative measures of invadopodial properties to evaluate genetic and pharmacological manipulations.

*Experimental plan*: This aim focuses on using the well-characterized human melanoma cell line WM2664 to assess the properties of invadopodia. We have extensive expertise manipulating and imaging this cell line both *in vitro* and *in vivo*. The basic experimental protocol is to plate cells onto a thin layer of fluorescently labeled gelatin (red channel) and image for 12-16 hrs. As the cells attach to the gel, many begin forming invadopodia, resulting in regions where the fluorescent signal from the gelatin gradually disappears. In addition, a fluorescent tag for F-actin (Lifeact-GFP) expressed in these cells is also imaged. This results in two sets of movies: one of the gelatin and the other of the actin tag. To determine the role of various proteins in influencing the properties of invadopodia, a set of candidate genes have been selected for gene knockdown using lentiviral shRNAs. These genes have either been implicated in invadopodia function or have known effects on tumor cell metastasis, but have never been tested for a role in invadopodia function (Table 1). Knockdown constructs for most of these genes already exist in our lab or published studies. These shRNAs will be combined with an expression cassette for LifeAct-GFP in our Knockdown-marker lentivirus published previously. As WM2664 cells harbor the V600E B-raf mutation common in melanoma, we will use this approach to test the role of B-Raf in invadopodia formation using the newly available B-Raf inhibitor from Plexicon.

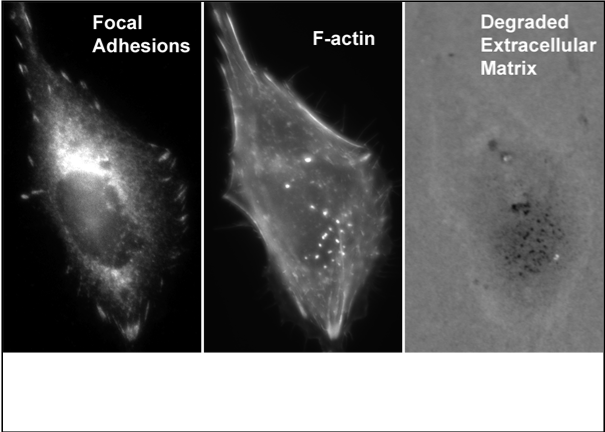
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| **Table 1: Candidate Genes** | **Reference** |
| Pten |  |
| LKB1 |  |
| Cortactin |  |
| Coronin 1C/1B |  |
| FAK |  |
| Fascin |  |
| Exo70 |  |

The first stage of the image processing pipeline is to identify the regions of high F-actin concentration (actin puncta) in each frame of the movie. This is accomplished using a thresholding algorithm based on the focal adhesion identification methods described above. With each of the puncta identified, they are then tracked through the entire movie and various properties, such as size and longevity are extracted from the raw image data. With the puncta identified and tracked, the next stage of the analysis involves determining which of the long-lived puncta are actual invadopodia. Our method uses the intensity of the gelatin in the region near the puncta as a filter for identifying invadopodia (Fig. 3). Specifically, our current method uses the difference in average value of the region immediately beneath the actin puncta and the region in a five-pixel border around the puncta as a filtering method. If this local difference value is consistently below zero, then we know that the region of gel immediately underneath the puncta has lost fluorescence relative to the surrounding region, indicating degradation of the gelatin. We also look to the frame immediately before the actin puncta was born to determine if the gelatin appeared degraded, perhaps due to a previous invadopodia or an uneven gel coating. If there are significant differences in the degradation before and during the puncta’s life, we classify the puncta as an invadopodia (Fig. 4). In addition, we are developing a population method to scan an entire field of cells to quantify the percentage of cells that form invadopodia meeting the outlined criteria.

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| local_diff_filtering.png  **Figure 3:** Overall filtering strategy for identifying Invadopodia is based around the mean values of the gelatin intensity underneath and adjacent to the actin puncta extent. The punta overlay rows indicate the puncta extent in green and the adjacent region in purple. | sm_data_plot.png  **Figure 4:** (A) Sample Invadopodia (green), the surrounding region (purple), other puncta (blue), frames immediately before birth and after death are included (B) the full set of difference measurements from Invadopodia in A, the error bars are 95% confidence intervals on the mean |

*Potential pitfalls*: Although the focal adhesion methods are quite robust, they may not be perfectly applicable to invadopodia and could require optimization such as changes in the local difference approach.

**Aim 2. Combined imaging of invadopodia and focal adhesions:**



**Figure 5:** WM2664 cell plated on fluorescent gelantin and stained for focal adhesion (Vinculin) and F-actin

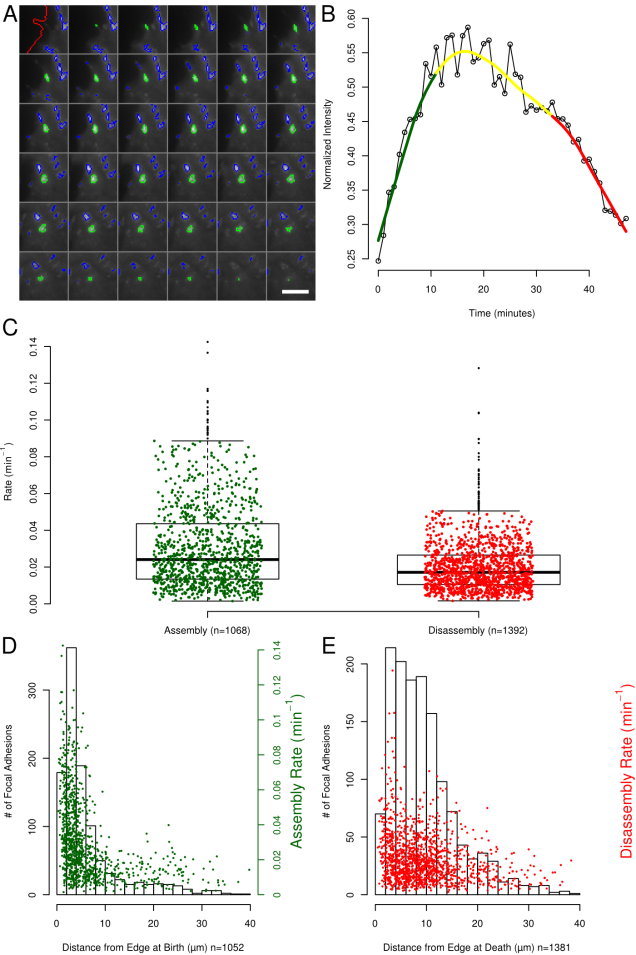
Rationale: Tumor cells can simultaneously contain both focal adhesions that anchor the cells to the extracellular matrix and invadopodia that are degrading it (Fig. 5). How these two processes are coordinated and integrated is completely unknown. By combining the existing focal adhesion analysis method with the newly developed invadopodia analysis method, we can begin to ask questions about this coordinate regulation that is required for maximum invasion.

Experimental Plan: We propose to develop a 3-color imaging method that will allow us to quantify invadopodia and focal adhesions simultaneously using GFP-Paxillin to mark focal adhesions, LifeAct-mCherry and Cy5-gelatin to monitor invadopodia. With simultaneous image sets collected, we will then coordinate the results of the invadopodia and focal adhesion analysis methods.

With sets of movies that show the position of Invadopodia, Focal Adhesions and gelatin degradation, we will apply both of the software suites to independently determine the properties of Invadopodia and Focal Adhesions in single cells. From these data sets, additional software will be written that combines these data sets. While difficult to predict the types of relationships we will observe in this combined data, we expect several areas to be of particular interest. Comparisons between the rate of focal adhesion turnover and the formation of successful Invadopodia will indicate the relative timing between these events crucial for a successful metastasis event. For example, we may observe that adhesion turn over rates increase, a necessary condition for rapid cell motility, following several Invadopodia formation events. We will also be developing tools needed to compare the spacial characteristics of the Invadopodia and Focal Adhesion populations. These tools will make it possible to quantify how formation of Focal Adhesion and Invadopodia affect one another in local regions of the cell, suggesting how these two structures interact and negotiate sharing similar components. The combination of a novel imaging setup and sophisticated computer vision algorithms will provide the first analysis of how Focal Adhesions and Invadopodia interact in cancer cells.

Potential pitfalls:

Alternative Figures



A more complicated version of Figure 2, I think the simpler figure works though.