Quantitative phase imaging reveals matrix stiffness-dependent growth and migration of cancer cells

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Cancer progression involves complex signals within the tumor microenvironment that orchestrate proliferation and invasive processes. The mechanical properties of the extracellular matrix (ECM) within this microenvironment has been demonstrated to influence growth and the migratory phenotype that precedes invasion. Here we present the integration of a label-free quantitative phase imaging technique, spatial light interference microscopy (SLIM)—with protein-conjugated hydrogel substrates—to explore how the stiffness of the ECM influences melanoma cells of varying metastatic potential. Melanoma cells of high metastatic potential demonstrate increased growth and velocity characteristics relative to cells of low metastatic potential. Cell velocity in the highly metastatic population shows a relative stability at higher matrix stiffness suggesting adoption of migratory routines that are independent of mechanics to facilitate invasion. The use of SLIM and engineered substrates provides a new approach to characterize the invasive properties of live cells as a function of microenvironment parameters. This work provides fundamental insight into the relationship between growth, migration and metastatic potential, and provides a new tool for profiling cancer cells for clinical grading and development of patient-specific therapeutic regimens.

The mechanical properties of the tumor microenvironment plays a role in guiding cancer development, transformation and invasive processes¹. The extracellular matrix (ECM) is an important component of the microenvironment and consists of proteins, glycoproteins, proteoglycans, polysaccharides, and other biochemically distinct components²,³. This ordered structure contains unique chemical, physical, and mechanical properties which are essential in numerous physiological processes including homeostasis⁴, differentiation⁵,⁶ and migration⁷,⁸. The ECM proteins also bind to soluble growth factors to regulate their activation and distribution in order to pass signals into the cell⁹. The biomechanical properties of the ECM, such as its viscoelasticity, can also influence disease development and progression¹⁰,¹¹.

The ECM is a dynamic system that is constantly being remodeled by the cells that inhabit it. This in turn influences adjacent cells to modify their behavior¹². In the tumor microenvironment, abnormal ECM dynamics are common and contribute to the process of progression, transformation, and dissemination. For instance, a hallmark of cancer is the excess production of ECM proteins including collagen I, II, III, V, and IX, which leads to tissue fibrosis¹³⁻¹⁷. This in turn increases the stiffness of the tumor microenvironment as compared to the surrounding tissue, which then further enhances cancer progression via reducing levels of tumor suppressors PTEN and HOXA9 in cancer cells¹⁷,¹⁸. Weaver and colleagues demonstrated how breast adenocarcinoma cells will secrete lysyl oxidase which crosslinks ECM proteins, leading to additional stiffening to facilitate invasion¹⁹. This increase in stiffness also impacts surrounding cells including creation of cancer-associated fibroblasts²⁰ and tumor-activated macrophages²¹.

Cancer metastasis is a multistep process which involves the intravasation from the tumor, survival in the circulatory and/or lymphatic system, extravasation and colonization at a distant site²²,²³. In order to intravasate or extravasate from solid tumors, cancer cells will generally undergo transformations between epithelial phenotypes.
and invasive mesenchymal or amoeboid phenotypes\textsuperscript{24}. Several groups have identified key roles for the ECM in facilitating these transformations, including a pronounced role for the mechanics of the surrounding matrix\textsuperscript{25}. For example, during epithelial to mesenchymal transition (EMT) where polarized epithelial cells transition to more mobile mesenchymal cells during biological processes such as embryogenesis and cancer progression\textsuperscript{24}, laminin-rich ECM can suppress EMT, whereas fibronectin-rich ECM can promote it\textsuperscript{26}. Stiffening of the microenvironment has also been shown to drive EMT of breast tumor cells, increasing its invasion potential and metastasis\textsuperscript{27} and tissue polarity aids death resistance of mammary tumor cells\textsuperscript{28}. In histopathology, recent work has revealed that tumor microenvironment carries prognosis information\textsuperscript{29–33}. While considerable work has led to the identification of processes underlying cancer cell invasiveness, no technique can simultaneously probe the interdependence of matrix parameters on multiple complex functions, i.e. migration and growth, which are critical aspects of invasion.

In this paper, we use spatial light interference microscopy (SLIM) as a label-free quantitative phase imaging (QPI)\textsuperscript{34} technique to explore how matrix stiffness influences cancer cell growth and migration in real time (Fig. 1A). Quantitative phase imaging is a method that can measure nanometer scale pathlength scale changes in a biological specimen. Typical quantitative phase methods, however, use coherent light sources that compromise the contrast of the images with speckles. SLIM overcomes this drawback with the use of a broadband field, and measures nanoscale details and dynamics in live cells via interferometry\textsuperscript{35}. SLIM couples Zernike's phase contrast microscope, which produces high contrast images of transparent samples, with Gabor's holography, which records the sample's phase information. The result is a quantitative optical pathlength map across the specimen. Here we use malignant melanoma as a model metastatic cancer—subclones of varying metastatic potency, including a putative cancer stem cell isolated through matrix engineering\textsuperscript{36,37}. The B16 melanoma cells are ideal

![Figure 1. Schematic setup for SLIM. (A) The SLIM module is attached to a commercial phase contrast microscope (Axio Observer Z1, Zeiss). (B) Experimental well plate setup: 10 kPa, 40 kPa and 100 kPa polyacrylamide hydrogels were prepared in different wells. (C) Decay rate vs. spatial mode associated with phase images generated with (A).](image-url)
as model cancer cell lines when studying metastasis due to the same parental tumor background with different degrees of metastatic potential. We show that metastatic potential is underpinned by specific growth and migratory characteristics that are dependent on the stiffness of the matrix.

**Methods**

Unless otherwise noted, all materials were purchased from Sigma-Aldrich. Tissue culture plastic ware was purchased from VWR. Glass coverslips were purchased from Fisher Scientific. Cell culture media and reagents were purchased from Gibco.

**Cell Culture.** B16 F0 and B16 F10 (ATCC), mouse melanoma cell lines were cultured according to the recommended protocols. B16 F0 cells exhibit less metastatic potential and B16 F10 cells have higher metastatic potential. Cells were passaged at ~80% confluency with 0.25% Trypsin:EDTA and media was changed every 3–4 days. For imaging, cells were seeded at ~50,000 cells/cm² in a 6 well glass bottom plate (P06-20-1.5-N) and were imaged for a period of 24 hours at 30-minute intervals and a capture speed of 6 frames/s. The cells were imaged at incubator conditions. Several frames were selected in each well for time-lapse SLIM measurements (Fig. 1B). For immunofluorescence, cells were seeded on patterned polyacrylamide hydrogels at ~50,000 cells/cm² and cultured for 5 days before fixation.

**Immunocytochemistry.** B16 F0 and B16 F10 cells on surfaces were fixed with 4% paraformaldehyde (Alfa Aesar) for 20 minutes at room temperature. 0.1% Triton X-100 in PBS was added for 30 minutes to permeabilize cells and blocked with 1% bovine serum albumin (BSA) for 15 minutes. Cells were labeled with mouse anti-αS51 (1:200 dilution, Emd Millipore) primary antibody in 1% BSA/PBS at 4°C overnight. Goat 647-anti-mouse (1:200 dilution) along with Hoechst 33342 (1:3000 dilution) was used for secondary labeling and were incubated with cells for 20 minutes in a humid chamber (37°C). Immunofluorescence microscopy was conducted with a Leica Microsystems DMi8 confocal microscope.

**Gel Preparation.** Polydimethylsiloxane (PDMS, Polysciences, Inc.) was polymerized on top of SU-8 patterned silicon masters fabricated via conventional photolithography to create PDMS stamps. 25 μg/ml fibronectin was incubated with Sodium Periodate for 45 minutes and pooled on top of the patterned PDMS stamps for 30 minutes. Stamps were then dried under air for 30 seconds and applied to the surface of hydrazine treated hydrogels that were dried at room temperature for one hour to form desired patterns.

**Gel Patterning.** Polymethylsiloxane (PDMS, Polysciences, Inc) was polymerized on top of SU-8 patterned silicon masters fabricated via conventional photolithography to create PDMS stamps. 25 μg/ml fibronectin was incubated with Sodium Periodate for 45 minutes and pooled on top of the patterned PDMS stamps for 30 minutes. Stamps were then dried under air for 30 seconds and applied to the surface of hydrazine treated hydrogels that were dried at room temperature for one hour to form desired patterns.

**SLIM.** Measurements were made using the SLIM system, comprising an inverted phase contrast microscope (Axio Observer Z1, Zeiss, in this case) and an add-on module (CellVista SLIM Pro, Phi Optics, Inc.). SLIM gener-

A) Dry mass: The dry mass surface density (\( \rho \)) of cellular matter was obtained from SLIM phase images using the following relationship,

\[
\rho(x, y) = \frac{\lambda}{2\pi\eta} \varphi(x, y),
\]

where \( \lambda \) is the center wavelength of the optical source, \( \eta = 0.2 \text{ ml/g} \), corresponding to an average of reported values, and \( \varphi \) is the phase values of the cells. The total dry mass of a cell was computed by integrating \( \rho \) over all cellular areas and was used to quantify cell growth in a noninvasive fashion.

B) Dispersion-relation phase spectroscopy DPS: To study the dynamics of cellular mass transport, we employed the dispersion phase spectroscopy (DPS) method. This computational technique enables the extraction of spatiotemporal intracellular mass transport from a series of time-lapse phase images. The dry mass density dynamics is governed by a advection-diffusion equation,

\[
D \nabla^2 \rho(r, t) - v(t) \nabla \rho(r, t) - \frac{\partial}{\partial t} \rho(r, t) = 0,
\]

where \( D \) is the average diffusion coefficient and \( v(t) \) is the advection velocity. The temporal autocorrelation function at each spatial frequency, \( q \), with temporal delay, \( \tau \), is

\[
\gamma(q, \tau) = e^{\nu_0 \tau} \Psi^\prime e^{-q \Delta x - \Delta q^2 \tau},
\]

where \( \nu_0 \) is the mean and \( \Delta x \) the standard deviation of the velocity distribution. By taking the azimuthal average of the spatial power spectrum, we obtained the 1D decay rate, the dispersion relation, \( \Gamma(q) = \Delta \nu q + D q^2 \).
Thus, the $Dq^2$ term contains the random (passive, equilibrium) component of cellular transport, while $\Delta vq$ the deterministic (active, out-of-equilibrium) one. The relationship between decay rate and spatial frequency was thus used to obtain information about the velocity distribution of mass transport. Since it is calculated over the entire field of view, DPS is highly conducive to automated and high-throughput analysis. And because the calculation is based on whole frame analysis, it generates comprehensive information on cellular distribution on a range of relevant spatial scales.

Results

Relationships between the degree of metastatic potency and growth responsivity to matrix stiffness. To explore how matrix stiffness affects invasiveness of melanoma cancer cells, focusing on fibronectin rich environments, we cultured two types of B16 melanoma cell lines of varying metastatic potential on hydrogel matrices of varying stiffness approximating cancerous tissue and other stiffer sites of common metastasis47,48. To do this, we used the well-established material polyacrylamide that can be formulated to span the wide range of all physiologically relevant moduli49,50, with a covalent protein conjugation method involving hydrazine activation of acrylamide, oxidation of protein and deposition through contact printing36,37. Cells cultured on fibronectin coated polyacrylamide can freely migrate and proliferate with no ill effects within the imaged time period. B16 F0s, cells of lower metastatic potential, and B16 F10s, cells of higher metastatic potential, were seeded on hydrogels and imaged under the SLIM system in order to investigate cellular response upon first contact with a new stiffness. SLIM imaging has the benefit of allowing label-free measurement of cell growth by quantifying the dry mass of the cell instead of overall volume. The dry mass of the cell indicates the amount of total protein within the cell and is a better measurement of cell growth. Previous research has shown that melanoma exhibit higher proliferation at higher stiffness51, however previous research often looks at the total volume of cells when comparing growth rates. Cell volume can change in response to external cues such as stiffness due to water efflux52. B16 F0 and B16 F10 were seeded onto polyacrylamide hydrogels of 10 kPa, 40 kPa, and 100 kPa and cells attached onto surfaces freely and exhibited healthy morphology (Fig. 2A). Once cells were fully attached, we performed SLIM imaging for 24 hours. The statistical method used to interpret the significance of the results is the student's t-test.
Conclusions

Cancer cell growth and migration are critical aspects underlying oncogenesis, with clear roles during all stages of progression and metastasis. In this paper, we uncover differences in cancer cell behavior as a function of metastatic potential and the mechanics of the underlying matrix through the combination of engineered extracellular matrices and quantitative phase imaging. Cells with higher metastatic potential exhibited greater growth rate than their less metastatic counterpart on soft matrices, and comparable growth rates on stiff matrices. In addition, high
metastatic potential corresponds with higher migration profiles, as determined by the velocity width distribution, which did not increase with increasing stiffness. This is in contrast to the cells of lower metastatic potential, which demonstrated a stiffness dependence in migratory behavior, consistent with previous studies. This is important because it suggests that invasive processes underlying metastasis correspond to a cell’s ability to proliferate and migrate irrespective of matrix stiffness. To supplement these results, we primed the cells of lower metastatic potential to a highly aggressive metastatic phenotype through a matrix engineering approach, and demonstrated that these cells adopt characteristics closely aligned with the cells of higher metastatic potential. Interestingly, this stem cell-like population shows non-linear growth characteristics more akin to proliferation of cancer cells within a growing tumor. In conclusion, we have demonstrated how combining quantitative phase imaging with engineered extracellular matrices can reveal changes in growth and velocity during culture that may prove useful as a label free approach to classifying invasiveness and metastatic potential. Future scope includes using these tools to study patient cells from biopsy or resection towards new diagnostic and prognostic assays to guide cancer management and therapeutic intervention.

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K.A.K. and G.P. conceived the ideas. Y.L. and M.J.F. designed and performed the experiments. Y.L., M.J.F., K.A.K. and G.P. analyzed the data and wrote the manuscript.

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