Depth-resolved cellular microrheology using HiLo microscopy

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Abstract: It is increasingly important to measure cell mechanical properties in three-dimensional environments. Particle tracking microrheology (PTM) can measure cellular viscoelastic properties; however, out-of-plane data can introduce artifacts into these measurements. We developed a technique that employs HiLo microscopy to reduce out-of-plane contributions. This method eliminated signals from 90% of probes 0.5 µm or further from the focal plane, while retaining all in-plane probes. We used this technique to characterize live-cell bilayers and found that there were significant, frequency-dependent changes to the extracted cell moduli when compared to conventional analysis. Our results indicate that removal of out-of-plane information is vital for accurate assessments of cell mechanical properties.

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References and links
1. J. Lammerding and R. T. Lee, “The nuclear membrane and mechanotransduction: impaired nuclear mechanics and mechanotransduction in lamin A/C deficient cells,” in Nuclear Organization in Development and Disease, Novartis Foundation Symposium Vol. 264 (Wiley, 2005), pp. 264–273.
2. T. P. Kole, Y. Tseng, I. Jiang, J. L. Katz, and D. Wirtz, “Intracellular mechanics of migrating fibroblasts,” Mol. Biol. Cell 16(1), 328–338 (2005).
3. R. G. Wells, “The role of matrix stiffness in regulating cell behavior,” Hepatology 47(4), 1394–1400 (2008).
4. E. U. Azeloglu, J. Bhattacharya, and K. D. Costa, “Atomic force microscope elastography reveals phenotypic differences in alveolar cell stiffness,” J. Appl. Physiol. 105(2), 652–661 (2008).
5. D. Fudge, D. Russell, D. Beriault, W. Moore, E. B. Lane, and A. W. Vogl, “The intermediate filament network in cultured human keratinocytes is remarkably extensible and resilient,” PLoS ONE 3(6), e2327 (2008).
6. H. Huang, A. Asimaki, D. Lo, W. McKenna, and J. Saffitz, “Disparate effects of different mutations in plakoglobin on cell mechanical behavior,” Cell Motil. Cytoskeleton 65(12), 964–978 (2008).
7. J. Lammerding, P. C. Schulze, T. Takahashi, S. Kozlov, T. Sullivan, R. D. Kamm, C. L. Stewart, and R. T. Lee, “Lamin A/C deficiency causes defective nuclear mechanics and mechanotransduction,” J. Clin. Invest. 113(3), 370–378 (2004).
8. D. Wirtz, “Particle-tracking microrheology of living cells: principles and applications,” Annu Rev Biophys 38(1), 301–326 (2009).
9. J. C. Crocker and B. D. Hoffman, “Multiple-particle tracking and two-point microrheology in cells,” Methods Cell Biol. 83, 141–178 (2007).
10. M. Jonas, H. Huang, R. D. Kamm, and P. T. So, “Fast fluorescence laser tracking microrheometry, II: quantitative studies of cytoskeletal mechanotransduction,” Biophys. J. 95(2), 895–909 (2008).
11. A. W. Lau, B. D. Hoffman, A. Davies, J. C. Crocker, and T. C. Lubensky, “Microrheology, stress fluctuations, and active behavior of living cells,” Phys. Rev. Lett. 91(19), 198101 (2003).
12. C. M. Hale, A. L. Shrestha, S. B. Khatau, P. J. Stewart-Hutchinson, L. Hernandez, C. L. Stewart, D. Hodzac, and D. Wirtz, “Dysfunctional connections between the nucleus and the actin and microtubule networks in laminopathic models,” Biophys. J. 95(11), 5462–5475 (2008).
13. J. S. Lee, P. Panorecan, C. M. Hale, S. B. Khatau, T. P. Kole, Y. Tseng, and D. Wirtz, “Ballistic intracellular nanorheology reveals ROCK-hard cytoplasmic stiffening response to fluid flow,” J. Cell Sci. 119(9), 1760–1768 (2006).
14. A. Pai, P. Sundd, and D. F. Tews, “In situ microrheological determination of neutrophil stiffening following adhesion in a model capillary,” Ann. Biomed. Eng. 36(4), 596–603 (2008).
16. P. Panorchan, J. S. Lee, B. R. Daniels, T. P. Kole, Y. Tseng, and D. Wirtz, “Probing cellular mechanical responses to stimuli using ballistic intracellular nanorheology,” Methods Cell Biol. 83, 113–140 (2007).
17. P. J. Stewart-Hutchinson, C. M. Hale, D. Wirtz, and D. Hodzic, “Structural requirements for the assembly of LINC complexes and their function in cellular mechanical stiffness,” Exp. Cell Res. 314(8), 1892–1905 (2008).
18. A. D. van der Meer, Y. Li, M. H. Duits, A. A. Poot, J. Feijen, and I. Vermeers, “Shear stress induces a transient and VEGF-2-dependent decrease in the motion of injected particles in endothelial cells,” Biomech. 47(3-4), 179–192 (2010).
19. Y. Tseng, T. P. Kole, and D. Wirtz, “Micromechanical mapping of live cells by multiple-particle-tracking microrheology,” Biophys. J. 83(6), 3162–3176 (2002).
20. K. Nishida, M. Yamato, Y. Hayashida, K. Watanabe, K. Yamamoto, E. Adachi, S. Nagai, A. Kikuchi, N. Maeda, H. Watanabe, T. Okano, and Y. Tano, “Corneal reconstruction with tissue-engineered cell sheets composed of autologous oral mucosal epithelium,” N. Engl. J. Med. 351(12), 1187–1196 (2004).
21. S. L. Ishaug-Riley, G. M. Crane-Kruger, M. J. Yaszemski, and A. G. Mikos, “Three-dimensional culture of rat calvarial osteoblasts in porous biodegradable polymers,” Biomaterials 19(15), 1405–1412 (1998).
22. S. L. Ishaug, G. M. Crane, M. J. Miller, A. W. Yasko, M. J. Yaszemski, and A. G. Mikos, “Bone formation by three-dimensional stromal osteoblast culture in biodegradable polymer scaffolds,” J. Biomed. Mater. Res. 36(1), 17–28 (1997).
23. T. Eschenhagen, C. Fink, U. Remmers, H. Scholz, J. Wattchow, J. Weil, W. Zimmermann, H. H. Dohmen, H. Schäfer, N. Bishopric, T. Wakeda, and E. L. Elson, “Three-dimensional reconstitution of embryonic cardiomyocytes in a collagen matrix: a new heart muscle model system,” FASEB J. 11(8), 683–694 (1997).
24. M. A. Neil, R. Juskaits, and T. Wilson, “Method of obtaining optical sectioning by using structured light in a conventional microscope,” Opt. Lett. 22(24), 1905–1907 (1997).
25. N. Bozinovic, C. Ventalon, T. Ford, and J. Mertz, “Fluorescence endomicroscopy with structured illumination,” Opt. Express 16(11), 8016–8025 (2008).
26. M. F. Langhorst, J. Schaffer, and B. Goetze, “Structure brings clarity: structured illumination microscopy in cell biology,” Biotechnol. J. 4(6), 858–865 (2009).
27. S. Santos, K. K. Chu, D. Lim, N. Bozinovic, T. N. Ford, C. Hourtoule, A. C. Bartoo, S. K. Singh, and J. Mertz, “Optically sectioned fluorescence endomicroscopy with hybrid-illumination imaging through a flexible fiber bundle,” J. Biomed. Opt. 14(3), 030502 (2009).
28. D. Lim, K. K. Chu, and J. Mertz, “Wide-field fluorescence sectioning with hybrid speckle and uniform-illumination microscopy,” Opt. Lett. 33(16), 1819–1821 (2008).
29. J. Mertz and J. Kim, “Scanning light-sheet microscopy in the whole mouse brain with HiLo background rejection,” J. Biomed. Opt. 15(1), 016027 (2010).
30. J. C. Crocker and D. G. Grier, “When like attract shapes: the effects of geometrical confinement on long-range colloidal interactions,” Phys. Rev. Lett. 77(9), 1897–1900 (1996).
31. T. G. Mason, “Estimating the viscoelastic moduli of complex fluids using the generalized Stokes-Einstein equation,” Rheologica Acta 39(4), 371–378 (2000).
32. B. S. Elkin, E. U. Azevoldo, K. D. Costa, and B. Morrison 3rd, “Mechanical heterogeneity of the rat hippocampus measured by atomic force microscope indentation,” J. Neurotrauma 24(5), 812–822 (2007).
33. D. C. Lin, E. K. Dimitriadis, and F. Horkay, “Robust strategies for automated AFM force curve analysis—an non-adhesive indentation of soft, inhomogeneous materials,” J. Biomech. Eng. 129(3), 430–440 (2007).
34. J. Solon, I. Levental, K. Sengupta, P. C. Georges, and P. A. Janmey, “Fibroblast adaptation and stiffness matching to soft elastic substrates,” Biophys. J. 93(12), 4453–4461 (2007).
35. X. Shi, L. Qin, X. Zhang, K. He, C. Xiong, J. Fang, X. Fang, and Y. Zhang, “Elasticity of cardiac cells on the polymer substrates with different stiffness: an atomic force microscopy study,” Phys. Chem. Chem. Phys. 13(16), 7540–7545 (2011).
36. L. Cao, A. Wu, and G. A. Truskey, “Biomechanical effects of flow and coculture on human aortic and cord blood-derived endothelial cells,” J. Biomech. 44(11), 2150–2157 (2011).
37. J. G. McNally, T. Karpova, J. Cooper, and J. A. Conchello, “Three-dimensional imaging by deconvolution microscopy,” Methods 19(3), 373–385 (1999).
38. A. Ganz, M. Lambert, A. Saez, P. Silberzan, A. Buguin, R. M. Mège, and B. Ladoux, “Traction forces exerted through N-cadherin contacts,” Biol. Cell 98(12), 721–730 (2006).
39. Y. Maruthamuthu, B. Sabass, U. S. Schwarz, and M. L. Gardel, “Cell-ECM traction force modulates endogenous tension at cell-cell contacts,” Proc. Natl. Acad. Sci. U.S.A. 108(12), 4708–4713 (2011).
40. M. C. DeSantis, S. K. Zareh, X. Li, R. E. Blankenship, and Y. M. Wang, “Single-image axial localization precision analysis for individual fluorophores,” Opt. Express 20(3), 3057–3065 (2012).
41. P. H. Wu, S. H. Arce, P. R. Burney, and Y. Tseng, “A novel approach to high accuracy of video-based micro rheology,” Biophys. J. 96(12), 5103–5111 (2009).
42. P. Prabhat, S. Ram, E. S. Ward, and R. J. Ober, “Simultaneous imaging of different focal planes in fluorescence microscopy for the study of cellular dynamics in three dimensions,” IEEE Trans. Nanobioscience 3(4), 237–242 (2004).
43. H. P. Kao and A. S. Verkman, “Tracking of single fluorescent particles in three dimensions: use of cylindrical optics to encode particle position,” Biophys. J. 67(3), 1291–1300 (1994).
44. V. Levi, Q. Ruan, and E. Gratton, “3-D particle tracking in a two-photon microscope: application to the study of molecular dynamics in cells,” Biophys. J. 88(4), 2919–2928 (2005).
1. Introduction

The viscoelastic properties of the cell are crucial to many cellular and tissue processes such as organelle translocation, wound response, and force response [1–5]. Additionally, changes to the cell’s mechanical properties have been correlated with disease states such as arrhythmogenic right ventricular cardiomyopathy (ARVC), progeria, and muscular dystrophy [6,7]. Particle tracking microrheology (PTM) is a passive spatiotemporal technique used to probe local viscoelastic properties of a wide range of cell types under a variety of conditions [8–17]. In PTM, fluorescent probes are introduced to the cell cytoplasm, which are then imaged using fluorescent wide-field microscopy. These particles’ displacements are then tracked over time, allowing for the calculation of the storage and loss modulus of the cytoplasm using mathematical models, such as the generalized Stokes-Einstein relation [18].

As researchers study more physiologically representative cell constructs, it becomes increasingly necessary to measure the mechanical properties of cells in a three-dimensional environment [19–22]. This includes stacked cell layers as well as cells embedded in scaffolds. In both cases, it is difficult to measure cell mechanical properties using conventional cell probing techniques, as they require direct contact with the cell or require imaging through multiple cell layers. PTM, being a passive readout, allows for the measurement of the mechanical properties of cells without requiring direct contact with the cell. This facilitates the measurement of mechanical properties of cells in a greater variety of plating conditions, including more sophisticated constructs [8]. However, as PTM generally requires tracking bright fluorescent probes, out-of-plane probes can have a significant effect on the measurement of the cells’ properties. The accuracy of the measurement of the cell’s mechanical properties can be improved by removing out-of-plane data. Methods such as deconvolution or confocal imaging can address this issue, but are difficult, expensive, or are limited by the need for high-speed particle tracking.

A class of depth-resolved imaging techniques based on Structured Illumination Microscopy (SIM) have been proposed to select a particular imaging plane and to reject out-of-plane background for standard wide-field microscopy [23–25]. In essence, a structured pattern (a grid) is projected onto a sample at the focal plane. The in-plane portion of the image is modulated along with the structure with the emitted fluorescence reproducing the pattern. The amplitude of out-of-plane portions of the images decrease according to the modulation transfer function, thereby blurring those portions of the pattern which are out-of-plane [23]. The optically sectioned image is extracted from the images that have been illuminated with a grid set at three different phases.

More recently, a new SIM-based optical sectioning technique has been developed, termed HiLo microscopy, which requires only one structured image and one uniform image [26]. In this technique, the high-frequency in-plane components of the image are extracted from the uniform image, while the low frequency components are extracted from the non-uniform (structured) image. To extract in-plane low frequency data, the local image contrast is multiplied by the uniform image to get a low frequency optically sectioned image. This low-resolution image is then combined with the high-resolution image to get a full-resolution optically sectioned image [26].

Here we describe a method using HiLo microscopy for removing out-of-plane data from particle tracking images of cell-embedded probes. By using a thresholding algorithm that calculates the percent drop in intensity of the probes in the HiLo image relative to the uniform image, we eliminated almost all probe data 0.5 µm or further from the image plane.

Using this technique, we were able to separate the probes from the top and bottom layer of a cell bilayer. Analyzing the data obtained from the two cell layers separately revealed an increase in the mean-squared displacement (MSD) of the bottom layer and a reduction in the MSD of the top layer. By analyzing the MSDs, we found that cells in the top layer had a significantly lower average mean-squared displacement, with a correspondingly larger storage.
and loss modulus than cells in the bottom layer. These results are supported by atomic force microscopy measurements. By removing out-of-plane probes using HiLo microscopy with PTM, we were able to more accurately measure the mechanical properties of individual cell layers. Our results indicate that there are significant differences in the mechanical behavior of both cell layers that was not clear when analyzing the aggregate, non-thresholded probe data.

2. Methods

2.1. HiLo optical setup and image processing

We first acquired structured illumination data on fluorescent beads. The standard ‘wide-field’ fluorescence image is called the uniform image. For non-uniform (structured) images, a Ronchi ruling (20 lines / mm, Edmund Optics, Barrington, NJ) was placed at the field stop between the mercury light source and the objective (60x, NA 1.3) of an Olympus IX81 inverted microscope. The grid was projected onto the focal plane of the sample during structured image acquisition. The Ronchi ruling was then removed to acquire a sequence of uniform images for PTM. All images were recorded with an ORCA R2 CCD camera (Hamamatsu, Bridgewater, NJ). The non-uniform image and the first frame of the uniform image sequence are then processed using the HiLo algorithm.

The details of the HiLo analysis algorithm have been described in detail elsewhere [26–28]. Briefly, the non-uniform $I_n(\hat{\rho})$ and uniform images $I_u(\hat{\rho})$ are the two raw images needed for acquiring the HiLo image, where $\hat{\rho} = \{x, y\}$ are the spatial coordinates in the image plane. These two images are used to define a partially demodulated image:

$$I_d(\hat{\rho}) = |I_u(\hat{\rho}) - I_n(\hat{\rho})|$$  \hspace{1cm} (1)

where $I_n(\hat{\rho}) - I_u(\hat{\rho})$ is then high-pass filtered prior to taking the absolute value to ensure the term is locally centered about zero. The low-resolution image is then obtained by applying a low-pass filter to the partially demodulated image:

$$I_{lp}(\hat{\rho}) = LP\left[I_d(\hat{\rho})\right];$$  \hspace{1cm} (2)

The cutoff frequency used for the low-pass filter is half of the spatial frequency of the grid pattern projected on the structured image. The high-resolution information is obtained by applying a high-pass filter to the uniform image:

$$I_{hp}(\hat{\rho}) = HP\left[I_u(\hat{\rho})\right]$$  \hspace{1cm} (3)

The HiLo image is obtained using

$$I_{hi\rho}(\hat{\rho}) = \eta I_{hp}(\hat{\rho}) + I_{lp}(\hat{\rho})$$  \hspace{1cm} (4)

where $\eta$ is an custom scaling factor, empirically adjusted until there is a seamless transition from high to low frequencies. HiLo image sequences were generated using the initial non-uniform image with each image in the uniform image sequence.

2.2. HiLo-based image thresholding

In order to generate depth resolved images for PTM, we used thresholding to objectively determine which probes were more than 0.5 µm from the focal plane. We calculated the percentage drop between the average intensity of each probe between the HiLo and uniform images. Trajectories of probes that experienced an average intensity drop greater than an empirically-determined threshold were removed from the MSD calculation.

To determine this threshold, a z-stack was taken of a polyacrylamide gel seeded with 200nm fluorescent probes (Invitrogen), with 100 images taken at intervals of 0.1µm. A
structured image was acquired near the center of the stack, where there were multiple in-plane and out-of-plane beads at various depths throughout the PAA gel. Because of the clarity of the gel, it was possible to determine the z-location of the embedded beads based on visual assessment of the bead sharpness. The uniform and HiLo-processed images were then compared to determine the drop in bead intensity as a function of the distance from the image plane using

$$\%Drop = 100\frac{(I_u - I_{\text{HiLo}})}{I_{\text{HiLo}}}$$  \hspace{1cm} (5)$$

These data were then used to set a threshold at which 90% probes greater than 0.5 µm from the focal plane were removed.

### 2.3. Cell culture

NIH 3T3 cells were cultured with high-glucose DMEM (Sigma, St Louis, MO), 10% Fetal Bovine Serum (Aleken, Nash, TX), 50 units / mL penicillin and 50 µg / mL streptomycin at 5% CO₂. For particle tracking bilayer experiments, confluent NIH 3T3 cells plated on a collagen-coated glass-bottomed cell culture dish (MatTek, Ashland, MA) were seeded with 400nm fluorescent beads as our PTM probes (Bangslabs, Fishers, IN) and allowed to incubate overnight. After 18 hours, another batch of 3T3 cells, which were also seeded with fluorescent probes, were plated on top of the confluent cell layer. The dish was then allowed to incubate for another 18 hours before imaging.

Cardiac fibroblast and myocyte bilayers were constructed using neonatal rat cardiac fibroblasts (CFs) and myocytes (CMs), which were isolated from 1-day old Wistar rat pups (Charles River, Wilmington, MA). Briefly, hearts were removed and placed in a dish containing Hank’s Buffered Salt Solution (HBSS, Cellgro, Manassas, VA) supplemented with 10 units / mL penicillin and 10 µg/mL streptomycin. The hearts were then finely minced into another dish of HBSS with Pen-Strep. The heart pieces were then digested through a series of four digestions with trypsin (0.14%) and pancreatin (0.22mg/mL, Sigma) in HBSS. Following the digestions, the cell solution was plated in a cell-culture treated flask, followed by an incubation at 37C at 1% CO₂ for three hours. The CFs remained adhered to the flask while the CMs were removed with the supernatant. The supernatant was then passed through a 40 µm filter to remove all remaining debris and the CMs were plated as described below. All animal protocols were approved by the Institutional Animal Care and Use Committee at Columbia University.

The CFs were plated on collagen-coated glass-bottom dishes (MatTek) and cultured at 37C and 5% CO₂ in the same media used for the NIH 3T3 cells. Once the CFs became confluent, they were stained with 20 µM Cell Tracker Green (CTG, Invitrogen, Grand Island, NY) for 30 minutes. Immediately following the staining, CMs were plated on top of the confluent CF layer. Once the myocytes adhered, the media was changed to M199, 5% FBS, 10 units / mL penicillin, 10µg / mL streptomycin, and 0.1mM 5-Bromo-2′-deoxyuridine (Sigma), and the cells were incubated at 37C and 1% CO₂. After three days, the samples were fixed with 3.7% formaldehyde for 15 minutes, permeabilized with 0.1% Triton-X for 10 minutes, and stained with Alexa-Fluor 488 phalloidin (Invitrogen) for 1hr at a 1:20 dilution. Structured and uniform images were then taken at the CF layer and the CM layer.

### 2.4. Particle tracking microrheology of a NIH 3T3 cell bilayer

Particle tracking data were acquired from the top and bottom layers of living NIH 3T3 cell bilayer. Structured images were taken of each layer, followed by acquisition of an image sequence of the probes’ movements. Six image sequences (for each of the top and bottom layers), with each sequence containing 20-60 probes, were imaged using a 60 × (NA 1.42) oil immersion objective at 16 frames per second for 1000 frames.
We used a publicly available particle tracking code adapted from code by Crocker and Grier to select suitable probes [29] on uniform images (termed Uniform data). Probes were than tracked using a custom multiple particle tracking MATLAB (The Mathworks, Natick, MA) program. As discussed in the above section, the trajectories of all probes, which experienced an average drop in intensity greater than the empirically determined threshold, were removed from the data prior to the calculation of the MSD (termed HiLo data).

The change in position of particles was tracked over all frames, correcting for whole-field shifting. By averaging over the squared displacements of all particles at various time lags, the mean squared displacement (MSD) was obtained:

$$\left\langle \Delta r^2 \right(\tau) \right\rangle = \left\{ (r(t+\tau) - r(t))^2 \right\},$$

where \(r(t)\) describes the particle’s two-dimensional trajectory, and \(\tau\) corresponds to a various time lags. The average MSD (for the bottom and top cell layer with and without HiLo-processing) was then used to calculate the frequency-dependent storage \(G'\) and loss \(G''\) moduli using an algebraic form of the generalized Stokes-Einstein relation (GSER) [30]:

$$G'(\omega) = \left[G' (\omega) \right] \cos(\pi \alpha(\omega)/2),$$

$$G''(\omega) = \left[G' (\omega) \right] \sin(\pi \alpha(\omega)/2),$$

where

$$\alpha(\omega) = \frac{\partial \ln \left\langle \Delta r^2 (\tau) \right\rangle}{\partial \ln \tau}_{\tau \rightarrow 1/\omega}$$

$$\left|G' (\omega) \right| \approx \frac{2k_B T}{3\pi a \left(\Delta r^2 (1/\omega) \right) \Gamma \left[1 + \alpha(\omega)\right]},$$

where \(k_B\) is the Boltzmann’s constant, \(a\) is the radius of the probe, \(T\) is temperature of the probe environment (in Kelvins), and

$$\Gamma \left[1 + \alpha\right] \approx 0.457(1 + \alpha)^2 - 1.36(1 + \alpha) + 1.90 .$$

The data is presented as line plots of the MSD, \(G', G''\) of both the top and bottom cell layer with and without out-of-plane bead removal (labeled as HiLo and Uniform data, respectively). Extracted values (including the standard error) are compared at three different time lags (0.2s, 1s, 5s), which were chosen to represent the data across over two orders of magnitude. The positive and negative error for \(G'\) and \(G''\) was calculated by determining \(G'\) and \(G''\) for the MSD plus and minus the MSD standard error, respectively. The MSD values at the bottom and top layer at specified time lags were compared using a student’s t-test. Statistical testing was not performed on \(G'\) and \(G''\), as these are derived parameters, and have unequal positive and negative error values.

2.5. Atomic Force Microscopy

Atomic Force Microscopy (AFM) was used to compare the stiffness of 3T3 cells plated as a monolayer on glass to the top layer of cells of a 3T3 cell bilayer. An atomic force microscope (Bruker, Santa Barbara, CA) mounted on an inverted light microscope (Olympus IX81, Center Valley, PA) was used to measure the elastic moduli of the cells. The cantilever probe used was a silicon-nitride DNP probe (Bruker) with a nominal spring constant of 0.12N/m. Each cell was indented at 1Hz in five different locations to find the average Young’s modulus for the cell.
The deflection versus displacement data was then fit to an 8th order polynomial. The contact point was then identified as the first point at which the first and second derivatives were greater than an empirically determined threshold [31]. The force is calculated as the product of the cantilever spring constant and the cantilever deflection. The following equation was then used to calculate the point-wise elastic modulus [32]:

\[ E = \frac{F(1-\nu^2)}{\pi \cdot \Phi(D)} \]  

(10)

where

\[ \Phi(D) = \frac{4}{3\pi} \left( R \cdot D^3 \right)^{1/2} \]  

(11)

when \( D < b^2 / R \) and

\[ \Phi(D) = \frac{4}{2\pi} \left( a \cdot D \right) - \left[ m \cdot \left( \frac{a^2}{\tan \phi} \right) \left( \frac{\pi}{2} - \arcsin \left( \frac{b}{a} \right) \right) \right] - \left( \frac{a^3}{3R} \right) + \left( a^2 - b^2 \right)^{1/2} \left[ m \cdot \frac{b}{\tan \phi} + \frac{a^2 - b^2}{3R} \right] \]  

(12)

when \( D \geq b^2 / R \).

For this formulation, \( D \) is the indentation depth, \( 2\phi \) is the tip angle, \( b \) is the radius at which the tapered sides transition into a spherical tip \( R \) (set equal to \( b = R \cos \phi \)), and \( m = 2^{1/2}/\pi \) for a pyramid-shaped probe. The contact radius \( a \) is found by numerically solving the following equation:

\[ D + \frac{a}{R} \left[ \left( a^2 - b^2 \right)^{1/2} - a \right] - \frac{n \cdot a}{\tan \phi} \left[ \frac{\pi}{2} - \arcsin \left( \frac{b}{a} \right) \right] = 0 \]  

(13)

where \( n = 2^{3/2}/\pi \) for a pyramid-shaped probe.

2.6. General approach summary

The experimental imaging and analysis protocol for the cell bilayer microrheology experiment is

1. Locate area of cell bilayer suitable for microrheology (has beads).
2. Adjust focal plane until bottom cell layer is in plane.
3. Place Ronchi ruling at field stop and take one structured image of bottom layer.
4. Remove Ronchi ruling and take sequence of uniform images.
5. Adjust focal plane until top cell layer is in-plane.
6. Repeat steps 3 and 4 at top layer.
7. Process the first frame of the uniform image sequences against the corresponding structured image for each layer using HiLo processing.
8. Choose trackable beads from the remaining beads (e.g., beads that are too close together are excluded).
9. Using thresholds, remove beads that are more than 0.5 \( \mu \)m out-of-plane, based on the intensity reduction from the uniform to the HiLo frame.
10. Track the remaining beads, using the uniform image sequences, and extract the MSD.
11. Extract $G'$ and $G''$ from the MSD.

These results, denoted “HiLo” were compared to similar results without removal of out-of-plane beads, using only the uniform image sequence to track all trackable beads, denoted “Uniform.” Thus the Uniform results contain beads that are out-of-plane but still considered trackable.

3. Results

3.1. Validation of HiLo algorithm for depth-resolved imaging of a multi-layer cell plating

To assess the quality of the structured illumination microscopy (HiLo) setup, cardiac myocytes (CMs) are plated atop cardiac fibroblasts (CFs), with the CF layer labeled with Cell Tracker Green, a whole-cell label, and both types of cells are labeled with phalloidin, which targets actin stress fibers. For demonstrating the filtering capabilities of HiLo imaging, we use this combination of cells because cardiac myocytes exhibit distinct sarcomeric patterns upon actin staining, allowing us to distinguish between the two cell layers more easily. Processing the image of the top CM layer with the HiLo algorithm produces a reduction in the presence of background signal from the bottom CF layer, including both the actin stain and the Cell Tracker Green (CTG) stain. Processing the bottom cell layer with the HiLo algorithm retains both the actin and cytoplasmic fluorescence (Fig. 1). These results demonstrate that the HiLo method is suitable for differentiating between the top and bottom layers of a cell-bilayer and thus can be used for depth-resolved imaging.

![Fig. 1](image_url)

Fig. 1. For the uniform (A) and HiLo-processed (C) images of the top CM layer, HiLo processing reduces the presence of the background signal from the bottom CF layer. For the uniform (B) and HiLo-processed (D) images of the bottom CF layer, HiLo processing reduces the presence of the signal from the top CM layer, while retaining actin and cytoplasmic fluorescence of the CF layer. For example, the sarcomeres that are seen in the top CM layer (white arrow) are less clear in the HiLo image of the bottom CF layer. Similarly, a circular actin structure that can clearly be seen in the top CM layer (gray arrow) cannot be seen in the HiLo image of the bottom CF layer. Images brightness/contrast adjusted for clarity. Scale bar is 30um.
3.2. HiLo-based threshold determination

As demonstrated with the CM and CF bilayer, a major advantage of HiLo processing is the reduction of out-of-plane signals. Because fluorescent beads generally exhibit high fluorescence intensities, their signals are not entirely quenched by HiLo processing even if they are out-of-plane. However, HiLo processing does attenuate their intensities, with greater reduction of intensities occurring at greater distances out-of-plane. We determined a threshold for the reduction of intensity to maintain beads within ±0.5 µm of the imaging plane so that beads out of this 1 µm band could be excluded from analysis.

To determine this threshold, we compare the intensity of polyacrylamide gel-embedded fluorescent beads in uniform and HiLo-processed images at a range of distances from the focal plane (also called the imaging plane), which is approximately ten frames above the midplane of the image stack. The reduction in average bead intensities between uniform and HiLo images increase with the beads’ distances from the focal plane (Fig. 2). Since the percent drop in bead intensity increases with distance from the focal plane, we set a threshold which we could remove a majority of beads 0.5 µm or further from the image while retaining a majority of beads 0.5 µm or closer to the image plane. For the z-stack taken in the PAA gel, the critical intensity reduction threshold is 20%. All beads 0.5 µm or closer to the focal plane have an intensity reduction less than 20%, while 88% of the beads greater than 0.5 µm from the focal plane have a reduction greater than 20%. Thus, by using this intensity reduction threshold, we are able to conserve all beads within the imaging plane and exclude all but about 10% of the out-of-plane beads. Additionally, due to the relationship between intensity drop and distance from the plane, it is likely that most of the included out-of-plane beads are close to the imaging plane.

![Fig. 2. The percentage drop in bead intensity between uniform and HiLo images (relative to HiLo) at increasing distances from the focal plane. Beads 0.5 µm or closer to the image plane have a drop in intensity less than 20%, and 88% of beads 0.5 µm or further from the image plane had a drop in intensity greater than 20%. The asymmetry in the bead distribution results from the inverted configuration of the microscope; objects below the imaging plane could be observed to a far greater out-of-plane distance than objects above the imaging plane.](image)

3.3. HiLo-based depth resolved microrheology of a cell bilayer

For the bilayer microrheology experiment, we use HiLo to separate out probes in the bottom and top layer of a living 3T3 cell bilayer. Processing uniform image sequences with the HiLo algorithm reduces the intensity of out-of-plane probes. In order to improve the depth resolution of the images, we remove bead trajectories from the uniform data, which
Fig. 3. Plot of MSD (nm$^2$) vs. tau (s) (A), $G'$ (Pa) vs. frequency (Hz) (B), and $G''$ (Pa) vs. frequency (Hz) (C) for bottom (Media 1) and top (Media 2) cell layers with and without HiLo image processing. HiLo processing allows for the removal of out-of-plane beads. Removing the out-of-plane beads from each image produces a small increase in the MSD of the bottom layer and a larger decrease in the MSD of the top layer over all time lags. These alterations in MSD produce a decrease in the storage and loss modulus in bottom layer and an increase of the storage and loss modulus in the top layer. The MSD (D, in mm$^2$), storage modulus (E, in Pa), and loss modulus (F, in Pa) are compared at three different time lags. All data are compared using a student’s t-test (*p < 0.05, **p < 0.01, ^p < 0.001). Statistical analysis at not performed for $G'$ and $G''$, as these are derived parameters. $G'$ drops to zero above 0.2Hz, so was not included in the plot (E).

experience a drop in intensity above 20%, as determined in the previous section. The remaining trajectories are used to calculate the MSD of the optically sectioned cell layer. The trajectories of the probes are used to calculate the average mean-squared displacement and then the storage and loss moduli of the cell layers over a continuous range of time lags (Fig. 3). Using the uniform image sequence (without out-of-plane bead removal), we show that the MSD is elevated for the bottom layer of cells except at low time lags. Comparisons of values at 0.2, 1 and 5 seconds (for MSD) or Hz (for $G'$ and $G''$) show somewhat mixed results when the top layer is compared to the bottom layer. For MSD, the top layer is significantly higher (p < 0.05) at 0.2s, non-significantly smaller at 1s and significantly smaller (p < 0.05) at 5s (Figs. 3(a), 3(d)). Further, $G'$ exhibits a small decrease at 5 Hz and a more moderate increase at 1 Hz (Figs. 3(b), 3(e)). The loss modulus $G''$ was mixed, showing a large decrease at 5Hz, but small increases at 1 and 0.2 Hz (Figs. 3(c), 3(f)).
Next, HiLo processing is used to exclude beads greater than 0.5 µm from the focal plane. When we remove these probes’ trajectories from the MSD calculation, significant changes result. First, the top layer MSD became significantly lower than the bottom layer at 0.2s (p < 0.05, Fig. 3(d)) and 1s (p < 0.01), and the p-value for 5s decreased (p < 0.001). Second, both G’ and G” show clear increases at all frequencies for the top layer compared to the bottom layer, and in almost all cases, the magnitude of the increase become much larger (Figs. 3(e), 3(f)). For example, in the uniform data, the G’ of the top layer at 5 Hz is 21% smaller than the bottom layer, whereas it is 119% higher for the HiLo data. These data demonstrate that depth-resolved microrheology yields much cleaner, more consistent results in heterogeneously plated samples.

3.4. Causes of changes to MSD and complex moduli

HiLo-based bead removal does not have a significant effect on the MSD (and subsequently, G’ and G”) when analyzing the probes from the bottom cell layer. The reason for this is that out of the bead trajectories removed from the bottom layer data (44 beads removed from a total of 185), it’s unlikely that many of the removed beads are from the top layer of cells due to the asymmetry in bead visibility (Fig. 2). The removal of out-of-plane beads produces a small reduction in MSD at short time lags (3% at 0.2s) and a small increase in MSD at mid-range (11% at 1s) and long time lags (15% at 5s).

However, bead removal does have a significant effect on the MSD of the top layer (108 beads removed from 268 total). This is because beads below the focal plane are more likely to appear clearly in the top layer image (Fig. 2). Therefore, the trajectories of beads in the (softer) bottom layer are more likely to be included in the calculation of the top layer MSD. It follows that the removal of all out-of-plane bead trajectories from the top layer data has a significant impact on the average MSD, G’ and G” of the top layer (Fig. 3(a)). This effect is most clearly seen at low time lags (on a log-log plot), where there is a significant drop (64%) in MSD at τ = 0.2s (Fig. 3(d)). Additionally, there is a clear change in slope of the top layer MSD at low time lags.

Because the probes in the bottom layer cells exhibit a higher average MSD compared to the probes in the top layer cells, removal of the bottom layer probes from the top images should result in a decrease in MSD, which is observed (Fig. 3(a)). However, there is also a change in slope at low time lags. The reason for the change in slope relates to the fact that bottom layer probes will be out-of-plane in the top layer images. Because these probes are out-of-plane, they will exhibit a smaller bead profile with lower, more uniform intensity magnitudes. Thus, the beads will exhibit apparently increased MSDs at all time lags due to the overrepresentation of signal fluctuations. This apparent increase to MSD makes a larger difference at smaller time lags, as the storage and loss modulus are dependent on the slope of the MSD on a log-log scale (Fig. 4).

3.5. Confirmation of cell layer stiffness comparison

Other groups have shown that many cell types can exhibit elastic moduli equal to, or lower than, the moduli of the underlying substrates [33,34]. However, certain cells are stiffer when plated on a confluent cell layer due to an increase in cortical stress fiber density [35]. Because of this ambiguity, we validate the microrheological results using AFM. We confirm that the upper layer of a 3T3 cell bilayer exhibits a significantly higher elastic modulus than 3T3 cells plated on glass (p < 0.05; Fig. 5).

Since conventional AFM requires direct contact with a cell to measure its mechanical properties, we are unable to measure the bottom layer of the cell bilayer. However, it is possible that the presence of the top layer of cells also changes the properties of the bottom layer cells. To address this, we compare the mechanical properties of the bottom layer of a bilayer with the properties of a monolayer plated on glass using PTM. We found that cells in
the monolayer exhibited similar MSD to cells in the bottom layer of a cell bilayer, indicating that the addition of cells to the top of a monolayer does not substantially alter the monolayer’s properties (Fig. 6).

Our results thus indicate that the correction of out-of-plane beads using HiLo-based thresholding and processing is both accurate and vital for more precise characterization of cell properties.
Fig. 5. AFM testing shows that 3T3 cells plated on a 3T3 monolayer have a significantly higher elastic modulus (kPa) than 3T3 cells plated on glass (n = 11 for both cases; *p < 0.05 as measured by a student’s t-test).

Fig. 6. MSD (nm²) vs. Tau (s) plot comparing properties of 3T3 cell monolayer with the top and bottom layers of a 3T3 cell bilayer. The monolayer is shown here as having properties similar to the bottom layer of the bilayer, showing that the presence of the top layer of cells does not have a significant effect on the mechanical properties of the bottom layer.

4. Discussion

We developed a technique using HiLo microscopy and post-processing to measure the viscoelastic properties of optically sectioned specimens, permitting quantitative characterization of cells cultured in 3D constructs. Our method removes out-of-plane probes, permitting depth-resolved images that contain mostly probes within a 1 µm layer. While this method can be used for removing probes both above and below the image plane, the data above the image plane is less likely to be visible in the image for an inverted imaging configuration. This is because spherical aberrations become significant for objects above the image plane of an inverted microscope [36]. Thus, the probes above the focal plane appear...
severely warped, whereas objects below the focal plane will appear out-of-focus but will retain approximately the same shape and much of their fluorescence intensity.

To demonstrate the effectiveness of this technique we measured the viscoelastic properties of the top and bottom layers of a cell bilayer. While raw data showed some trends, use of HiLo processing and thresholding removed the majority of out-of-plane beads and resulted in much cleaner results with lower p-values and more clearly accentuated and consistent differences, showing that the upper layer of cells are both stiffer and more viscous compared to the lower layer cells across over 2 orders of magnitude. These results were confirmed by AFM. The difference in cell layer properties has many potential interpretations. One possible interpretation is that the top layer of cells becomes stiffer in order to protect the bottom layer from normal and shear forces. It is also possible that the top layer cell must create excess extra-cellular matrix components in order to adhere and spread onto the cell surface, as cell-cell forces have generally been found to be weaker than cell-matrix adhesion forces [37,38]. It is not currently known whether 3T3 cells will become stiffer on all softer substrates, as these experiments have not been systematically performed.

We demonstrated that out-of-plane probes affect PTM analysis in two ways: first, by shifting the MSDs when the out-of-plane probes exhibit different average MSDs from the in-plane probes, and second, by contributing frequency-dependent noise resulting from diminished probe intensity profiles. The latter is most prominent on a log-log scale at low time-lags, or high frequencies.

Other techniques may offer similar benefits to the method used in this study. Confocal or two-photon microscopy can be used to the same effect by eliminating out-of-plane noise. However, these systems are expensive, and certain modes (such as raster-scanning) are not always compatible with PTM. In general, scanning techniques cannot be used for PTM because the motions of the entire probe would no longer be simultaneously acquired. Our method can be cheaply and readily adapted using conventional wide-field fluorescence microscopes. Use of point-spread function analysis, raw probe intensity thresholding, or image aberration-based techniques can also serve to attenuate out-of-plane beads. The addition of a precision axial-localization method may be required for the tracking of smaller probes than we used here [39]. However, these methods may not be readily adaptable in more turbid situations where fainter signals are attenuated and variability in intensities are high. Since our HiLo method depends on the change in signal intensity rather than the raw magnitude, it is likely more adaptable to a wider range of environments. Deconvolution may achieve the same effect, but at the expense of taking image stacks or performing computationally intensive blind deconvolution.

One limitation of our technique stems from using a single image to determine which beads are within the image plane. As a result, our method does not allow for very long-term particle tracking (hours), as bead displacements in the axis perpendicular to the imaging plane may become large, thereby rendering the original bead choices largely non-relevant. However, short of beads being transferred from one cell to another, this is unlikely to affect the cell-level resolution of our method. Similarly, particle tracking in very compliant and low-viscosity samples is limited by large bead displacements in shorter times. This limitation is unlikely to be a major issue in cell characterization since most cells are stiff and viscous enough for tracking for a moderate period of time, as we demonstrated in this study. Longer-term tracking is further confounded by photobleaching, phototoxicity and active cell processes.

The thresholding process is imperfect. As a result, one has to be very careful in using this method to characterize viscoelastic properties. All beads within the imaging plane were likely preserved; however, ~10% of the beads out-of-plane were included. In this study, imaging the bottom layer included 24% out-of-plane beads. Thus, out of every hundred beads imaged, 2-3 out-of-plane beads are included, after HiLo-based thresholding. Approximately 40% of the imaged beads were out-of-plane in the top layer images. Thus, out of every hundred beads
imaged, 4-5 beads from out-of-plane regions will remain after HiLo-based thresholding. Since this represents less than 8% of all beads analyzed in each layer, the contribution of these out-of-plane beads are not likely to be significant, especially since they are unlikely far from the imaging plane. However, if one were to perform a study where there were high numbers of out-of-plane beads compared to in-plane beads, the contribution of out-of-plane beads may render the analysis incomplete or inaccurate. It should be noted that the threshold determined from the polyacrylamide gel may actually remove a higher percentage of out-of-plane beads in the cell bilayer than in the gel, as the out-of-plane bead intensity may drop more in the cell bilayer compared to the diminished scattering conditions of the gel. We additionally note that the HiLo and thresholding parameters used in this study were tailored specifically for our system, and that comparisons with different systems must be performed carefully. Future work may include parameter validation based on other optical sectioning methods such as three-phase SIM or spinning disc confocal (at a low frequency range) and numerical simulation methods to assess centroid determination accuracy in the presence of out-of-plane object motions. The technique developed in this study will allow for quantitative measurements of the viscoelastic properties of cells, and perhaps tissues, in more physiologic cell constructs. Additionally, this technique can also be used to enhance conventional in vitro 2D PTM experiments, by allowing for the study of subcellular planar sections, to assess apical versus basal properties, for example. However, it should be noted that there is a limit to the information we can gain from HiLo processing due to the presence of image distortion. This is especially true of very thick or opaque samples.

Future improvements include developing a technique for alternating structured and uniform images, allowing for analysis of a greater variety of samples. An additional possibility is to combine our technique with the Monte Carlo simulation method developed by Wu et al. (2009), in order further increase the accuracy of our PTM measurements [40]. We also expect that as PTM becomes used in a wider variety of cells, other optical sectioning and three-dimensional imaging techniques such as deconvolution microscopy, aberration based axial position determining methods, two-photon orbital scanning, and simultaneous focal plane imaging will be employed towards improving the accuracy of cell mechanics measurements in a variety of three-dimensional culture scenarios [36,41–43].

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