A new approach for high-content traction force microscopy to characterize large cell ensembles

Condensed running title: *A new approach for high-content TFM*

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1.1 Abstract

Accurate measurements of cellular forces are important for understanding a wide range of biological processes where traction plays a major role. The characterization of mechanical properties is needed to unravel complex phenomena like migration, morphogenesis, mechanotransduction, or shape regulation, but accurate data on large numbers of single cells remain scarce and challenging. The capacity to measure forces in populations of cells and to identify subsets within heterogeneous ensembles would enable to reveal and manipulate their intrinsic complexity. Traction Force Microscopy (TFM) is a technology that can quantify the contractile forces exerted by cells via measuring the displacement of fluorescent beads embedded on the surface of a soft substrate with precisely defined mechanical properties. However, conventional numerical approaches for measuring cellular forces using TFM are labor-intensive and often yield significant artifacts, making them ill-suited for high-throughput analysis. In this work, we propose using the Demons algorithm instead, leading to significant improvements in both computational efficiency and accuracy. Based computer simulations, we show that this methodology outperforms conventional approaches in terms of speed, it is less sensitive to the blur induced by out-of-focus images and improves the accuracy of force field reconstructions. Additionally, we conducted experiments using various cell lines and gels of distinct stiffness to demonstrate that the Demons algorithm is capable of real-time and effective at clustering cells according to their mechanotype.

1.2 Statement of significance

Large cellular ensembles encompass a wide variety of traits that require detailed but high-throughput technologies for characterisation. Granular descriptions of cell populations enable to describe their heterogeneity are essential to understand biological phenomena in the search of effective treatments. Most advances in the field of Traction Force Microscopy (TFM) are oriented towards high resolution force measurements at sub-micron adhesion scale that reveal the intricacies of molecular interactions. Here, we propose a strategy to exploit TFM for characterizing the mechanotypes of large cell ensembles. We benchmark and show the capacities of using the Demons algorithm to measure bead displacement fields, the improvement in force reconstructions obtained, and experimentally demonstrate that single cell analysis of cell dynamics can be used to cluster and describe different cell types.

1.3 Introduction

Measurement of traction is required to study the ability of cells to sense, generate and transmit forces to their microenvironment and neighboring cells. Cells initiate intracellular signaling cascades in response to external mechanical stimuli that impact their fate and functions, such as adhesion dynamics (1–4) , cell shape (5), proliferation, differentiation and metabolism (6, 7). On a macroscopic scale, coordinated changes in cell shape lead to force propagation throughout tissues, which is essential to morphogenesis; these forces can also serve as cues for immune response and wound healing (8–10). On a microscopic scale, migrating single cells contract, stretch and apply forces as they maneuver through complex environments. These shape transitions are influenced by expression of cytoskeletal and adhesion proteins, and the mechanical properties of the substrate. In the case of disease, migrating metastatic cells represent an important determinant of progression: they navigate through densely packed microenvironments, exerting pressure to penetrate the extracellular matrix, distort, and relocate to invade distant organs (11). Taken together, the thorough characterization of cellular traction is important to understand the role of mechanical forces on biological phenomena that determine both organismal homeostasis and the outbreak of maladies.

Most biological ensembles display highly heterogeneous traits, thereby requiring single-cell characterization of many cells for a complete description. High-content force measurement techniques are needed to describe the wide variety of mechanical phenotypes that are found experimentally. Notably, even cells from a genetically homogeneous background display epigenetic and expression variance that yield substantial changes in force phenotypes; cancer cells are even more heterogenous (12), due to elevated mutation rates and chromosomic alterations (13, 14). High-content techniques must characterize not only the details of single adhesions or individual cells but assess the variability within cell populations and the capacity to probe critical but rare events.

The study of the signaling pathways, molecular properties and factors that determine the mechanotype has been an active research field for decades (15–17), highlighting key molecular players involved in cellular force generation. Recently, researchers have been trying to dissect the mechanical heterogeneity found in metastatic cell populations by isolating cells based on various properties like their adhesiveness (18), speed (19), or migration ability through a transwell (20).

Traction Force Microscopy (TFM), the most widespread technique to measure cellular contractility, uses commonly available reagents and tools to assess force fields generated by cells. In TFM, fluorescent beads are dispersed on the surface of a soft hydrogel before cells are seeded. After adhesion, the forces that cells exert on the substrate, as they contract and extend, are translated to movement of the beads. Thus, using the mechanical properties of hydrogels, it is possible to calculate traction forces from bead displacements. TFM experiments are mostly used to study a low number of cells with high accuracy, as the imaging modality is often based on laser scanning microscopy and the computation of bead displacement field is slow. Furthermore, current advances on TFM focus on high-resolution, sub-adhesion scale traction measurement, exploiting nanoscopy techniques (21).

It is widely recognized that errors in the bead displacements can propagate and lead to important artifacts in the force reconstruction (22), thus, a common numerical approach to minimize errors is to perform a parametrized Thikonov regularization in the force calculation. However, inaccuracies cannot be fully corrected with regularization and the precise assessment of the bead displacement field is still of prime importance. Computationally intensive cross-correlation techniques are typically used to measure the bead displacements but are slow to calculate and may result in significant error rates in force reconstruction, often underestimating traction by approximately 50% (23). Recently, optical flow techniques, which are faster to compute, have been introduced to effectively measure small displacements, and demonstrated superior performance compared to cross-correlation methods for this specific application (24). The Demons algorithm, which we analyze here, is novel in TFM, but has been extensively utilized in the domain of medical image processing (25, 26). Demons iteratively traces spatio-temporal gradients of intensity to converge on a displacement field between two consecutive images and uses regularization by applying a Gaussian filter at each iteration, leading to a diffusion-like behavior. Here, we benchmark the Demons algorithm and demonstrate that it can be used to increase the throughput of TFM and minimize bead displacement errors (25, 26). We show, using simulated data, that this approach outperforms both the gold standard cross-correlation techniques and optical flow. Finally, we implement high content TFM to highlight the capacity to characterize the mechanoptype of three different cancer cell lines.

1.4 Methods

1.4.1 Computing displacement fields

The present study compares three techniques to measure bead displacements and the subsequent precision of the force fields reconstructions. While cross-correlation techniques are time consuming, Optical-Flow and the Demons algorithm are faster to compute and allow high throughput live measurements of displacement fields. Figure S1 illustrates the calculation time per evaluation point.

1.4.2 Cross-correlation

Cross-correlation methods have been widely employed in TFM, with numerous variations developed over time. Cross-correlation methods seek to localize regions of similarity between a moving image and a reference fixed image within a specified neighborhood; they are effectively used to determine the optimal displacement compatible with the deformation observed when beads are pulled. A reference template window $\boldsymbol{W}_{ref,ci}(m_1,m_2)$ is extracted from the reference fixed image at coordinates $[m_1,m_2]$. A second window, $\boldsymbol{W}_{ci}(m_1+b_1,\ m_2+b_2)$, is then extracted from the moving image, where coordinates are translated from $[m_1,m_2]$ by $[b_1,b_2]$.

The cross-correlation matrix $\boldsymbol{cc}(b_1,\ b_2)$ is computed by assessing the correlation between $\boldsymbol{W}_{ref,ci}(m_1,m_2)$ and $\boldsymbol{W}_{ci}(m_1+b_1,m_2+b_2)$ across the defined neighborhood. This is mathematically represented as:

$$\boldsymbol{cc}(b_1,b_2) = \frac{1}{2}\sum_{i=1,2} \boldsymbol{W}_{ci}(m_1+b_1,m_2+b_2)\boldsymbol{W}_{ref,ci}(m_1,m_2) \tag{6}$$

Sub-pixel bead displacement (b_1^*,b_2^*) is calculated at the maximum of a gaussian fit on the cross-correlation matrix $\boldsymbol{cc}(b_1,b_2)$ (27, 28). In this work, we utilized a MATLAB package for particle image velocimetry (28). For all experiments, we used a window size of 11 pixels, and this value was obtained after numerical testing.

1.4.3 Optical Flow

Optical flow refers to the distribution of velocities associated with the movement of brightness patterns within a scene as perceived by an observer (29). The method assumes that a moving object maintains a constant brightness and that displacement is small. Optical flow constraint is expressed as (30) :

$$\nabla \boldsymbol{I}(\boldsymbol{x},t)\cdot \boldsymbol{v} + \frac{\partial \boldsymbol{I}}{\partial t}(\boldsymbol{x},t) = 0 \tag{7}$$

where $\nabla \boldsymbol{I}$ represents the spatial intensity gradient of the image, $\boldsymbol{v}$ is the velocity of the optical flow, and $\boldsymbol{I}_t$ is the partial derivative of the intensity with respect to time. Consequently, the component of the optical flow in the direction of the brightness gradient ($\nabla \boldsymbol{I}$) can be formulated as:

$$\boldsymbol{v} = -\frac{\partial \boldsymbol{I}/\partial t}{|\nabla \boldsymbol{I}|} \tag{8}$$

Numerous techniques to measure movement are based on the concept of optical flow(31). Here, we used the version proposed by Lucas and Kanade(24, 32) implemented in the Vision toolbox of Matlab, where the displacement $\boldsymbol{v}$ is determined by minimizing the following expression within a small spatial neighborhood Ω :

$$\sum_{\boldsymbol{x}\in\Omega} \boldsymbol{W}^2(\boldsymbol{x})[\nabla \boldsymbol{I}\,(\boldsymbol{x},t)\cdot \boldsymbol{v} + \boldsymbol{I}_t(\boldsymbol{x},t)]^2 \tag{9}$$

where $\boldsymbol{W(x)}$ denotes a square function that regularizes v.

1.4.4 The Demons algorithm

Demons is a non-rigid image registration technique inspired by Maxwell's demon thought experiment (25). To determine the pixel displacements that registers a moving image to a reference image, this approach iteratively calculates and applies a series of small transformations. In each iteration a Gaussian smoothing is applied to the transformation, mimicking a diffusion process (25, 26). While the technique performs the best in measuring displacements of freely diffusing particles, it also applies to beads embedded in an elastic medium (33). The small transformation, caused by "demonic" forces, makes the pixels move along the temporal and spatial gradients of the images, following the equation:

$$\boldsymbol{u}(p) = -\frac{\boldsymbol{F}(p) - \boldsymbol{M}\circ s(p)}{\left|\left|J^p\right|\right|^2 + \frac{\sigma_i^2(p)}{\sigma_x^2}}\, J^{p^T} \tag{10}$$

where $\boldsymbol{u}$ represents the iterative small displacement, $\boldsymbol{F}$ is the reference fixed image, $\boldsymbol{M}$ is the moving image, and s denotes the cumulative transformation up to the current iteration. The term J^p refers to the Jacobian, while $\sigma_i(p)=|\boldsymbol{F}(p)-\boldsymbol{M}\circ s(p)|$ quantifies the noise in the image. Additionally, the constraint $||\boldsymbol{u}(p)||\leq \sigma_x$ accounts for the spatial uncertainty associated with the transformation. The smoothing parameter, which represents the standard deviation of the Gaussian, can be tuned. In this work, we used the MATLAB function imregDemons with only one pyramid level, a total gaussian smoothing of 0.75 pixels and 100 iterations. These values were obtained after numerical testing and optimization.

1.5 Reconstruction of traction forces

The bead displacements between the reference image (without cells) and the moving image (gel under contractile activity of cells) enable the reconstruction of the force field. We solved the inverse problem of the Boussinesq approximation to the elastostatic equation in the Fourier domain, which allows fast computation (22, 34). This approach limits the calculations to a 2-dimensional plane but greatly accelerates the computation of the traction forces.

Briefly, the formulation of the stress reconstruction works as follows: the strain tensor inside an infinite linearly elastic material can be written in the form of a 3x3 matrix of elements $\epsilon_{i,j}$ that is a function of the local displacement $\boldsymbol{u} = [u_1, u_2, u_3]$ and spatial coordinates $\boldsymbol{x} = [x_1, x_2, x_3]$:

$$\epsilon_{i,j} = \frac{1}{2}\left(\frac{\partial u_j}{\partial x_i} + \frac{\partial u_i}{\partial x_i}\right) \tag{11}$$

where *i* and *j* are the indices of the 3 dimensions.

For linearly elastic materials, the stress σ_{ij} is proportional to the strain ε_{ij}.

$$\sigma_{ij} = \frac{E}{s+1}\left(\epsilon_{ij} + \frac{s}{1-2s}\delta_{ij}\sum_{k=1}^{3}\epsilon_{kk}\right) \tag{12}$$

where *E* is the Young's modulus, *s* is the Poisson ratio and δ_{ij} the Kronecker delta.

The Boussinesq approximation to the elastostatic equation (34) assumes a semi-infinite medium with stresses applied at the surface, which simplifies the problem, as forces and displacements in 2D only can cab be expressed as:

$$u_i(\boldsymbol{x}) = \int \sum_{j=1}^{2} G_{ij}(\boldsymbol{x}-\boldsymbol{x}')f_j(\boldsymbol{x}')d\boldsymbol{x}' \tag{13}$$

$$G_{ij}(\vec{x}-\vec{x'}) = \frac{1+s}{\pi E r^3}\begin{pmatrix}(1-s)r^2 + sx^2 & sxy \\ sxy & (1-s)r^2 + sy^2\end{pmatrix} \tag{14}$$

where $r = |\boldsymbol{x}|$, *s* is the Poisson ratio, *G* the Green's tensor for the Boussinesq approximation.

To obtain the force field, the inverse problem must be solved. Importantly, this is an ill-posed problem, and even minor discrepancies in displacement data can significantly alter the resulting force estimation. Regularization approaches are used in the force field calculation to obtain smooth solutions despite the noise in the displacement field measurement (22, 35, 36). Thikonov regularization, where force magnitude is penalized, is typically used by requiring the tuning of a regularization parameter λ, where we minimize:

$$argmin\{|G\boldsymbol{f} - \boldsymbol{u}|^2 + \lambda^2|\boldsymbol{f}|^2\} \tag{15}$$

where *G* is the Green's tensor for the Boussinesq approximation, $\boldsymbol{f}$ is the calculated force field, $\boldsymbol{u}$ the measured displacement filed and λ, the regularization parameter.

For automatic selection of the parameter, a Bayesian approach has been developed which requires a statistical measurement of displacement noise performed outside the cell, where the displacements are assumed to be null (35). For faster computation, the inverse problem can be solved in the Fourier domain (23, 37).

1.6 Cellular traction simulations and error definitions

To compare the various methods used to measure bead displacements, we simulated bead images in two different scenarios: one where all beads are homogeneously displaced in the same direction, and one where we simulated a realistic displacement field generated by a distribution of focal cell adhesions.

1.6.1 Homogeneous displacement field

Beads where displaced homogeneously from 0 to 20 pixels with a step of 0.25 pixels. To mimic well-resolved vs. blurred beads, we adjusted the standard deviation of a Gaussian point spread function (PSF) to 0.33 pixels, 1.81 pixels and 10 pixels. We also varied the bead density, ranging from sparse 0.01 px^{-2}, to 0.5 px^{-2}, to a dense 1 px^{-2}. For all the 84 displacement values, 3 PSF sizes and 3 bead densities, we generated 100 images by randomly shuffling the bead location. For each method, we fine-tuned and optimized parameters, i.e. the widow size for cross-correlation and optical flow, and the total smoothing for Demons, and the and kept them constant throughout the experiments, independently from the size of the displacements, the PSF or the bead density. One displacement error measure was extracted per image, comparing the simulated ground truth to the measured displacement field. The error of the measurement was expressed using the root mean square error (RMSE). Detailed mathematical description of the error follows in subsection 4.3.3.

1.6.2 Cell-like traction field

To evaluate the impact of the accuracy of bead displacement measurements with each technique on the force reconstruction, we simulated the displacement field that is typically observed for a distribution of focal adhesions (FA) of a polarized cell. We segmented focal adhesions (FA) from a microscopic image of migrating breast cancer cells, specifically MDA-MB-231 cells (Figure 2a), and simulated equal forces for every adhesion pointing towards the center of mass of the cell (Figure 2b). We used the Boussinesq approximation to determine the motion of each bead (Figure 2c) and varied the traction, ranging it between 200 to 2000 Pascal, which resulted in average particle displacements under the FAs of 1-10 pixels, respectively. Similarly to the previous experiment, we replicated the experiment for 4 PSF standard deviation to values (0.33, 1, 2 and 5 pixels), and 4 bead densities (0.01, 0.1, 0.5 and 1 $pixels^{-2}$). For each traction value, PSF size and bead density, we generated 200 images.

We then measured bead displacement errors and force reconstruction errors for each image by comparing the simulated to the reconstructed traction fields, and the simulated and measured bead displacement fields.

1.6.3 Error measurements

To quantify the bead displacement errors, we measured a root mean square error (RMSE) per image

$$RMSE = \frac{1}{n}\sqrt{\sum_{i=1}(\boldsymbol{d}_i - \widehat{\boldsymbol{d}}_i)^2} \tag{16}$$

Where $\boldsymbol{d}_i = (d_i^x, d_i^y)$ and $\widehat{\boldsymbol{d}}_i = (\hat{d}_i^x, \hat{d}_i^y)$ represent the simulated and measured displacements for the ith pixel and *n* represents the total number of pixels considered.

To quantify the errors on force reconstructions, we measured the deviation from magnitude (DTM), and the deviation from angle (DTA), comparing the simulated force field and the reconstructed force field.

$$DTM = \frac{1}{n}\sum_{i=1}\frac{|\widehat{\boldsymbol{f}}_i| - |\boldsymbol{f}_i|}{|\boldsymbol{f}_i|} \tag{17}$$

$$DTA = \frac{1}{n}\sum_{i=1}\operatorname{acos}\left(\frac{\widehat{f_i^x} f_i^x + \widehat{f_i^y} f_i^y}{|\boldsymbol{f}_i||\widehat{\boldsymbol{f}}_i|}\right) \tag{18}$$

Where $\boldsymbol{f}_i = (f_i^x, f_i^y)$ and $\widehat{\boldsymbol{f}}_i = (\hat{f}_i^x, \hat{f}_i^y)$ represent the simulated and reconstructed tractions for the ith pixel and *n* represents the total number of pixels considered.

1.7 Traction force experiments

For this study, we detached the cells at the end of the timelapses and then captured the reference frame. Cells were allowed to adhere for 12 hours on TFM hydrogels mounted on 35mm glass-bottom imaging dishes (IBIDI, #**81218-200)**, followed by a 4-hour time-lapse acquisitions at the microscope. We used a temperature- and gas-controlled stage-top incubator (IBIDI, #**10720)** for imaging. We added 2 mL of 2% triton-X-100 (Millipore sigma, #**9036-19-5)** in ddH2O at 37 degrees Celsius to the 1 mL of culture medium to detach cells and capture the reference frame.

1.8 Gel fabrication

We fabricated polyacrylamide hydrogels following established protocols (27, 38, 39). We added a 4 µL drop of a solution containing 40% acrylamide (Bio-Rad, #161-0140) and 2% bis-acrylamide (Bio-Rad, #161-0142) to glass-bottom 35 mm dishes (IBIDI, #81218-200). Dishes had previously been activated with (3-Aminopropyl) trimethoxysilane (APTMS, Sigma-Aldrich, #281778) and a 25% glutaraldehyde solution (Electron Microscopy Sciences, #16200). To manipulate the desired rigidity, polyacrylamide mixing ratios can be found in previously published resources (27). We placed a 12 mm cover glass coated in poly-l-lysine (Sigma #25988-63-0) and dark red fluorescent micro beads (ThermoFisher, #F8807) on the drop before gels polymerized. We used tweezers to remove the coverslip, creating a TFM gel with fluorescent beads located on its surface. To prepare for cell seeding, we activated the gels using sulfo-SANPAH (ThermoFisher, #22589) and coated them with fibronectin (Millipore Sigma #FC010).

1.9 Cell culture

We used three cell lines in this study: MDA-MB-231 (ATCC, HTB-26), Lewis lung carcinoma (ATCC, CRL-1642) and U2OS (ATCC, HTB-96). All cell lines were cultured in Dulbecco's modification eagle's medium (Wicent, #319-005 CL) with added 10% Fetal bovine serum (Wisent, #090-150- FBS), 5% Penicillin-Streptomycin-Neomycin (Fisher scientific, #15640055), 5% Glutamax (Fischer scientific, #35050061).

1.10 Imaging

We used a Nikon Eclipse Ti2 equipped with a 20X 0.75 NA objective and a 1.5X magnification lens. The resulting pixel size on the image is 0.37 microns. Images of cells were obtained in bright field mode and those of beads with epifluorescence. We acquired images every 90 seconds for 70-80 stage positions in parallel.

1.11 Image analysis

We used Cellpose to segment cells from the brightfield images(40). We trained a model for each cell type by retraining 2-3 times the "cyto" model from Cellpose V2 with 3-10 images containing 10-20 cells. We then used the centroids of their masks to follow individual cells using a nearest neighbor algorithm (41). Tracks were filtered according to a minimum trajectory length, average speed, and d_{max}, to avoid considering dead cells or debris.

Before computing the displacement field, all bead images were registered to the reference frame using rigid registration algorithms. The registration transformations obtained from beads images were applied to cell images as well.

To calculate the mechanical work for a single cell at a specific timepoint, we only considered the force and displacement vectors located under a force mask. The force mask included traction vectors with magnitude above an Otsu threshold on the traction magnitude. Only the overlapping objects of the force mask and the cell mask were considered.

1.12 Statistical analysis

1.12.1 Effect size

We calculated the effect size as follows:

$$ES = \frac{med(X) - med(Y)}{\sqrt{\frac{var(X) + var(Y)}{2}}}$$

where *X* and *Y* are the two compared observations, *var* is the variance and *med* the median. We can interpret the values 0.2, 0.5, 0.8 and 1.3 as small, medium, large and very large effect sizes respectively.

1.13 Results

1.13.1 Homogeneous displacement field

We first tested the case of homogeneous displacement fields and observed that the Demons algorithm consistently outperformed all other methods in terms of displacement accuracy for a wide range of PSF sizes and bead densities. More specifically, both the error of displacement measurement (RMSE) and its dispersion are almost negligible for small displacement (Figure 1, Figure S2). Interestingly, for unresolved beads that mimic out-of-focus images with a PSF size of 10 pixels or smaller, the Demons algorithm is the only approach that allows measuring displacements over 20 pixels. This suggests that larger PSF can help to track greater displacements, and that the Demons is particularly well suited for measuring beads displacements from unresolved images. For all the techniques, an undulating pattern can be observed in the error graphs for sub-pixel bead displacements: valleys occur at full-pixel offsets, leading to a boost in correlation, and error increases for fractional displacements.

Increasing the number of beads does not significantly affect the precision of Demons estimates for small movements. The small error increases abruptly after a threshold that scales with the size of the PSF according to a complex relationship (Figure 1). This likely stem from the algorithm's built-in smoothing mechanism, which minimizes fluctuations between adjacent vector components. Moreover, despite having no effect on the error in magnitude, raising bead densities improves angle accuracy. The mistakes of Demons are negligible for a large movement span, irrespective of the PSF's dimensions or the number of beads, it provides consistent outcomes and remains resilient under varying imaging circumstances such as gel expansion, and nonhomogeneous bead density.

Optical flow is often described as reliable in the case of small displacements (42); in our hands it excels at detecting sub-pixel shifts with mid-sized PFS of 1.82 pixels. However, the error and variability increase significantly when measuring displacements larger than 1 pixel. Regardless of PSF size, the error distribution of optical flow is worse with the increase of bead concentration, as shown in Figure S2, but a large fraction of the relative RMSE can be attributed to angular deviations, rather than the magnitude, as illustrated in Figure S3-S4. Using a well sampled 10-pixel PSF, optical flow struggles to maintain precise measurements, even at low bead densities, leading to an average relative RMSE between 0.2 and 0.4.

While cross-correlation tends to lag behind the other two methods, it performs the best for smaller PSF. In fact, its relative RMSE increases with PSF width, while it plateaus for increasing bead density. Across all displacement ranges, cross-correlation underestimates the values, as the magnitude error remains below 0 (Figure S3). Despite applying Wiener filtering, cross-correlation leads to incoherent bead displacement fields, where neighboring displacement vectors have limited correspondence in their orientation and magnitude. In these conditions, cross-correlation is the slowest and least accurate technique.

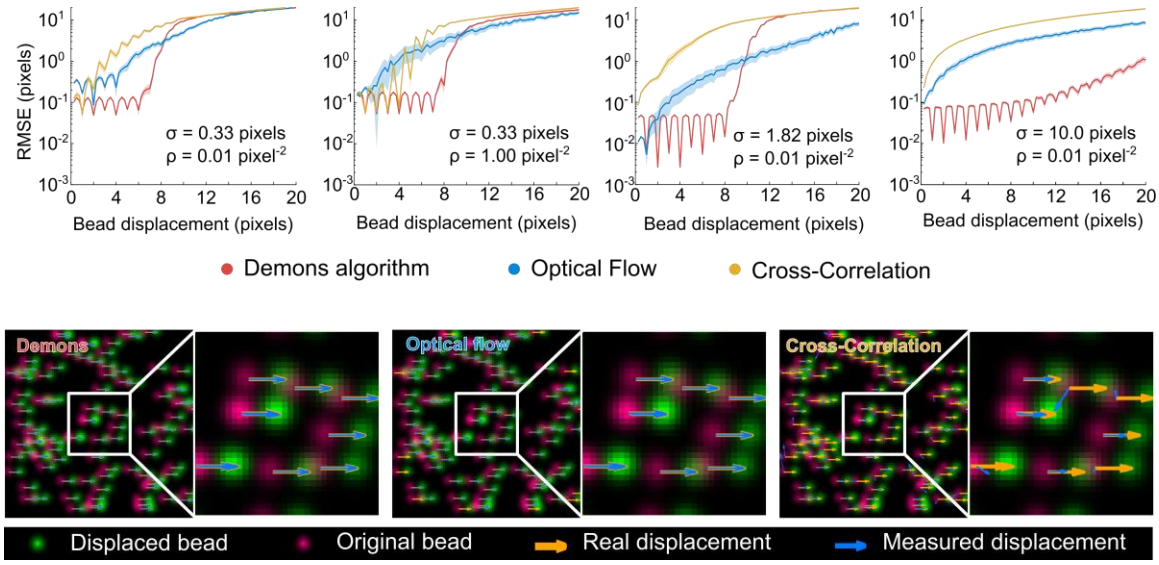

Figure 1: Bead displacement measurement error for homogeneous fields. (a) RMSE for bead displacements. Every data point represents the RMSE of an image. All conditions at a specific displacement were replicated 100 times. The shadow line thickness represents the standard error over the mean. Only four representative conditions are displayed: Gaussian PSF standard deviation (STD) of 0.33 pixels at a density of 0.01 pixels^{-2}, STD of 0.33 pixels at density 1 pixels^{-2}, STD of 1.8 pixels at density of 0.01 pixels^{-2}, STD of 10 pixels at density 0.01 pixels^{-2}. (b) Representative images of beads highlighting displacement errors for the three methods for PSF SDT 1.82 pixels, density 0.1 pixels^{-2} and displacement of 6.5 pixels.

1.14 Reconstruction of cell-like force field

We evaluated the impact of displacement field errors in the force reconstruction of cellular traction by simulating the distribution of focal adhesions found in migrating cells. We simulated the bead displacements using the Boussinesq approximation.

We first compared the errors of displacement fields produced by the Demons, cross-correlation and optical flow as bead density varies across the surface of a TFM hydrogel. All techniques exhibited worsening performance for increasing densities (Figure 2g-j, Figure S5). Demons displayed less variability and cross-correlation performed the worst across all bead densities. Interestingly, the Demons and optical flow showed improved performance when paired with the mid-sized PSF with a standard deviation of 1 pixel, and cross-correlation achieved its highest accuracy with the smallest PSF at 0.33 pixels. Errors nearly doubled when dealing with PSFs featuring 2-pixel standard deviations, emphasizing the crucial impact of PSF size on accuracy.

We assessed the precision of the reconstructed force field by measuring the deviation to traction magnitude (DTM) and angle (DTA). We evaluated the error for various regularization parameters (L) ranging from 1 to 25 pixels/pascal. Enhancing the regularization parameter led to an increase in DTM error, as it underestimated the force's magnitude (Figure S6). Interestingly, DTA showed a significant reduction when the regularization parameter increased, reaching over 20° difference between L=1 and L=25 (Figure S6). The relative RMSE errors reflect this trend, with the smallest error occurring at a mid-sized PSF of 1 pixel's standard deviation. Moreover, the Demons algorithm exhibited remarkable consistency, maintaining stable DTA values for densities between 0.01 and 0.1 beads per pixel.

The characterization of heterogeneity within large sets requires not only high throughput, but also appropriate ranking of the cells. To investigate the force ranking ability of the three techniques, we calculated the Spearman's rank correlation between the simulated ground truth and the reconstructed force fields of simulated cells. Interestingly, while increasing the regularization parameter led to lower absolute force measurements, it didn't affect how accurately we could rank cells. The ranking remained consistent, with correlation values staying close to 1 (Figure 2k). The bead density and the PSF size had more influence on ranking accuracy (Figure 2l,m). However, increased regularization improved the linear relationship between simulated and reconstructed total force as shown by better Pearson's correlation values (Figure S6, Figure S7). Demons and cross-correlation showed excellent linear relationships with correlation values above .95, while the optical flow method was less reliable at measuring proportional differences in cell forces.

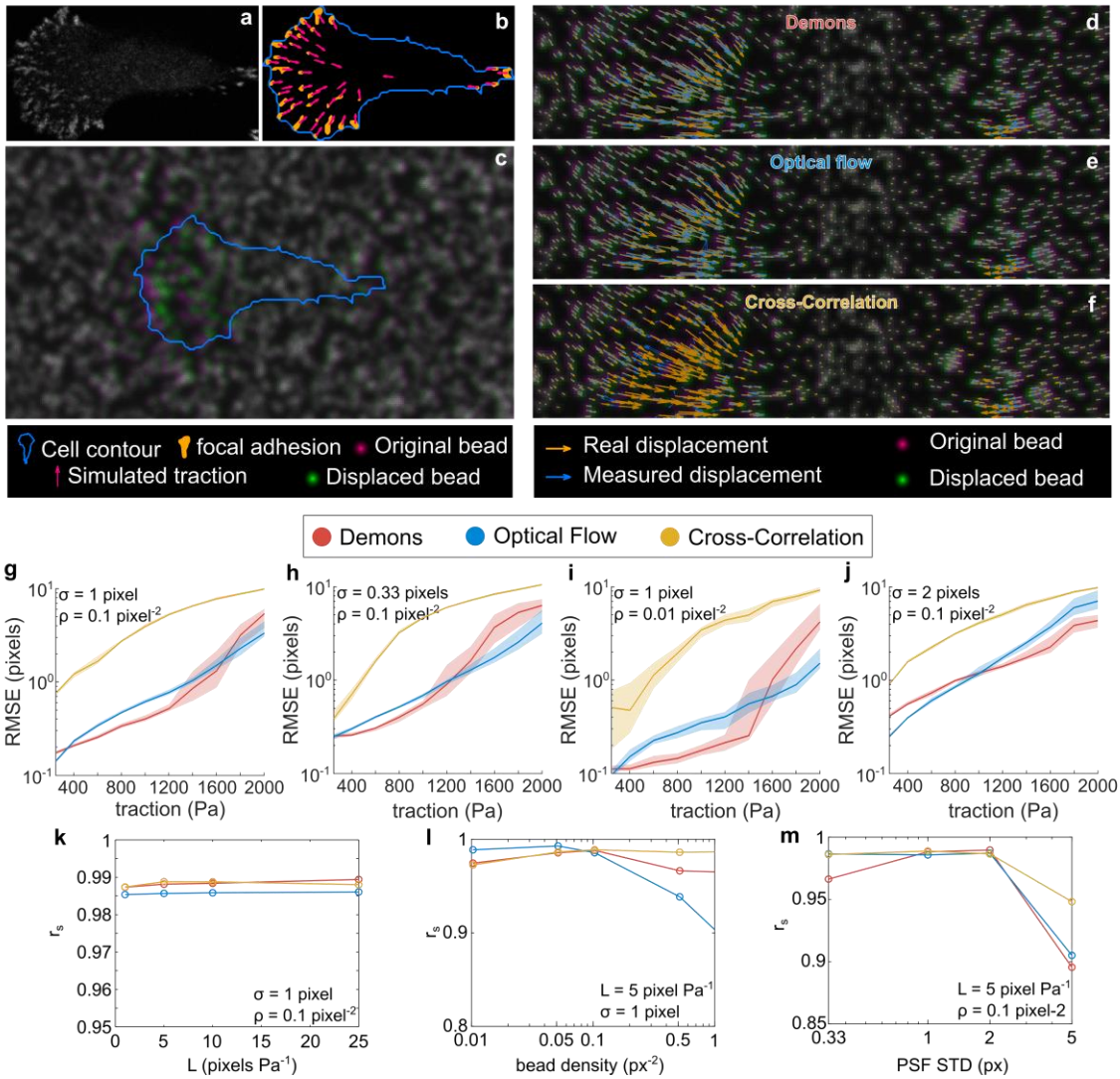

Figure 2: Traction force reconstruction error for a realistic cell-like bead displacement pattern. a) Confocal image highlighting focal adhesions of a migrating breast cancer cell (MDA-MB-231 expressing paxillin-mCherry). b) Segmentation of cellular membrane and focal adhesions and simulated of traction field. Cell body contour (blue), focal adhesion segmentation (orange) and simulated traction (magenta). A homogeneous traction is applied on the whole FA surface, oriented toward the centroid of the cell body. c) Simulated bead images before (magenta) and after (green) traction was applied. (d-f) Representative images of beads highlighting displacement errors for the three methods. Comparison of ground truth and measured bead displacement for (d) Demons algorithm, (e) Optical Flow, and (f) Cross-Correlation. (g-j) Bead displacement measurement errors (RMSE) as a function of simulated traction. Thickness of curves represent percentiles 25 and 75, and the line represents the median of 200 images. 4 conditions are depicted: (h) 1-pixel PSF STD and bead density of 0.1 pixel^{-2}, (i) PSF STD of 0.33 pixel and density 0.1 pixel^{-2}, (j) 1 pixel PSF STD and density 0.01 pixel^{-2}, and (k) PSF STD 2 pixels and density 0.1 pixel^{-2}. (k-m) Spearman's rank correlation between simulated and reconstructed total force as a function of the regularization parameter (k), bead density (l), and PSF size (m).

1.14.1 High-content TFM on cell populations

To evaluate the use of these approaches on cell populations experimentally, we acquired 4-hour TFM movies, for three cancer cell lines: two of them characterized by their highly migratory phenotypes. We analyzed the three populations cultured on gels of two distinct Young's modulus: 2.3 and 55 kilopascals.

Cell segmentation was achieved using custom Cellpose models(40), followed by automatic tracking using a Nearest Neighbor approach(41). Bead displacement fields were computed using cross-correlation, optical flow, and Demons, and we reconstructed force fields using Fourier transform traction Cytometry (35). We extracted the temporal evolution of several metrics: mechanical work, cell area, mean traction, mean velocity, and maximum distance traveled (d_{max}) (Figure 3 a-e).

We quantified the ability of the three approaches to distinguish between cell populations and substrates. Specifically, an ANOVA analysis of the mechanical output generated by these approaches reveals a F-value that is approximately twice as large for the Demons algorithm (9836,8) compared to cross-correlation (5197,7), and around three times larger for optical flow (3245,0). Furthermore, Demons leads to narrower distributions of mechanical work and better population separation. The median effect size for Demons, cross-correlation and optical flow are 4.43, 3.78 and 2.85, respectively (Figure S8).

We categorized cells based on a multivariate mechanotype consisting of five variables. Paired variables (Figure 3f) show relationships that lead to unclear segregation of cell line mechanotypes. Furthermore, we did not observe a consistent relationship between speed and mechanical work within each group (Table S1). However, dimensional reduction projections of 5-dimensional data, such as t-SNE, display differences between cell lines on different substrates (Figure 3g). As a graphical projection of multi-dimensional data cannot fully evaluate the clusterability of datasets, we used the Dips Test, a statistical analysis that evaluates unimodality, applied to the pairwise Euclidean distances between cells using the normalized mechanical properties as coordinates (43, 44). The test refuted the assumption of unimodality for all the methods, thus we can cluster cell groups.

Finally, we tested the ability to discriminate the datasets with statistical classification. We trained four classifiers (quadratic discriminant analysis, binary decision tree, k-nearest neighbors, and support vector machine) with 90% of the data and tested them on the remaining 10%. We repeated the procedure 10 times, randomly shuffling the data partitioning, and calculated the average misclassification rate across the replicates. For all classifiers, the Demons algorithm achieves the lowest error rates (Figure S9). For example, in the case of the quadratic discriminant analysis classifier, Demons achieves 12% misclassification rate, while cross correlation and optical flow are at 15% and 16%, respectively.

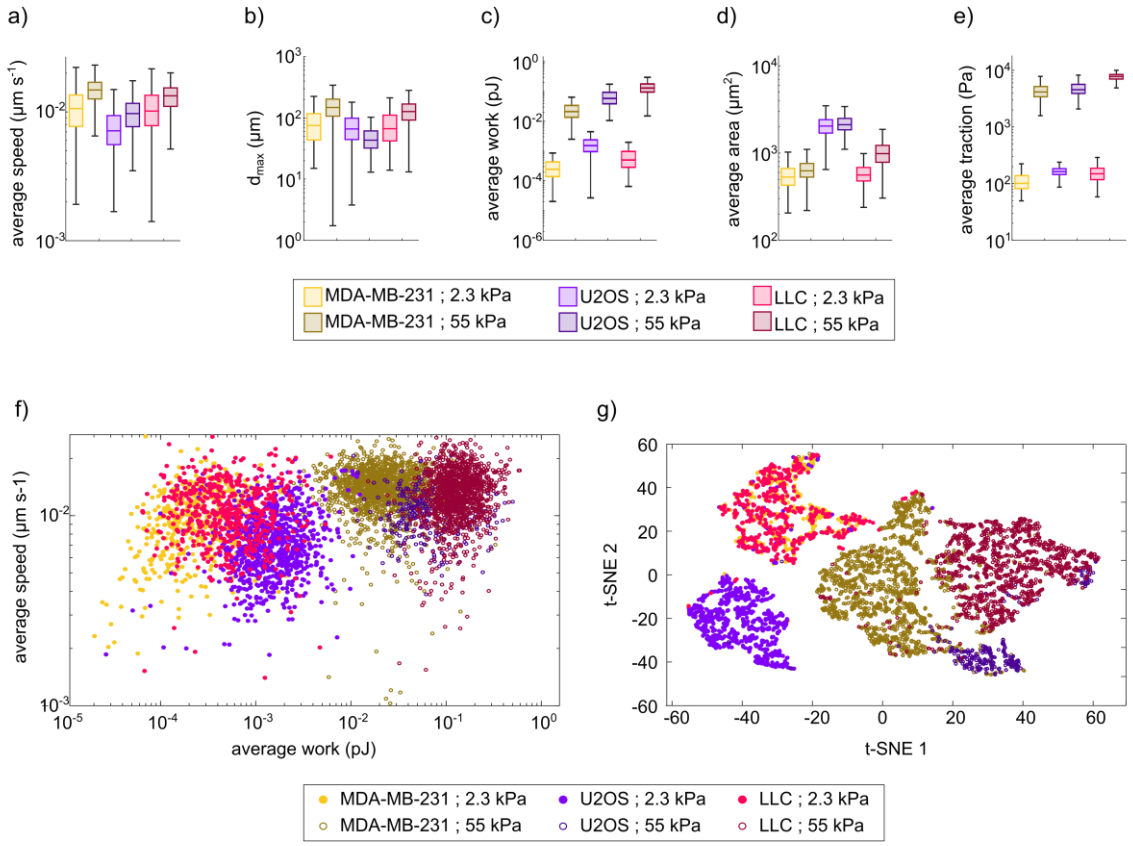

Figure 3: (a-e) Mechanical phenotype description using 5 features extracted from cell trajectory analysis, TFM and morphology analysis. Boxplots display distributions of single cells with median and 2 quartiles. (f) Cellular average migration speed vs. average contractile work. (g) t-SNE projection of the 5 cellular mechanical features.

1.15 Discussion

Experimental conditions and the specific scientific question determine a trade-off between computational time, complexity, cost, and accuracy to properly chose analytical tools; this work provides a thorough examination to guide such choices. Fine grained descriptions of the dynamics of single focal adhesions are often not needed to characterize large populations of cells, while large throughput data may not be informative about the details of protein interactions in migratory phenomena. The vast majority of the cell migration literature is dedicated to obtaining mechanistic molecular insight, tested on low numbers of individual cells, and only few studies aim at characterizing mechanical properties of cell types. Our work sought to analyze and compare a novel approach to obtain metrics that can be used to describe and mechanically sort populations of cells, in a cost-effective way.

Our simulations show that the bead displacement measurement accuracy on the surface of a TFM hydrogel has a significant impact on the reconstruction of cellular forces. We propose the use of Demons as an alternative to the most widely used image cross-correlation algorithms, as this method is significantly faster. We added optical flow to the comparative analysis, which produced great outcomes for small stresses (24). Numerical simulations demonstrated that Demons outperforms the other two alternatives and can be reliable even for displacements significantly larger than the PSF of the microscope. Demons proved to be resilient to a range of experimental conditions, particularly for displacements below 8 pixels. Optical flow excelled only in sub-pixel displacements of beads around the pixel size, outperforming other techniques, but was the least accurate for a wide distribution of measurement errors. Cross-correlation performed optimally for PSF smaller than a pixel.

We analyzed the precision of force reconstructions mimicking the traction originating from a cell. While all three techniques showed to be sensitive to changes in bead density, Demons was the most resilient. Cross-correlation performed the best with highly resolved beads, and the other two techniques worked optimally for more realistic PSFs, larger than one pixel size. Although all techniques ranked cells well according to their traction force, optical flow performed the worst at measuring proportional relashionship between simulated and reconstructed total force.

Demons showed the best capacity to distinguish machanotypes based TFM. We examined how distinct phenotypes clustered by modifying only the mechanical work in a multivariate mechanical description of the cells, which includes cell speed, greatest distance traveled, mechanical work and spreading area. Demons showed the lowest misclassification rate using a quadratic discriminant classifier.

Thikonov's method, which was used throughout this work to regularize the reconstruction of the force field, imposes a penalty on excessive norms of calculated stresses that results from inconsistencies between neighboring displacement vectors. This kind of noise is typical of cross-correlation and optical flow, but not Demons. Instead of using the norm of the force to regularize the reconstruction process, other operators, such as the curl or the divergence of the force field can be minimized (45). Otherwise, machine learning approaches can be employed to reconstruct the force from displacement field, as these tools are adaptable to various noise type (46, 47).

The Demons algorithm integrates regularization; it is a local iterative method that is prone to getting stuck in local minima which constrains the method to relatively small displacements (48). Additionally, its regularization process resembles a diffusion process, whereas we aim to monitor the transformations of an elastic substance. These limitations could potentially be addressed by using variations of the algorithm that are better suited for elastic materials or that better capture larger displacements (48, 49).

Current efforts are mostly aimed at enhancing 2D TFM by achieving high precision measurements of individual focal adhesions. Our primary interest, however, lies in characterizing forces across populations, thus enabling to pinpoint uncommon cells that exhibit distinct mechanical properties (reviewed in (21)). Our findings reveal that a wide-spread algorithm, that is not typically used for TFM, provides the right compromise between time and accuracy for high-content measurements of mechanical work. It resilient to experimental constrains, such as out-of-focus beads and uneven bead distribution on a large hydrogel substrates. Additionally, Demons enables the use of lower magnification objectives for increased throughput, accurately categorizes cells based on their strength, and enhances clustering capacity.

2 Author contributions

Nicolas Desjardins-Lecavalier designed research, performed research, analysed data, and wrote the manuscript. Santiago Costantino designed research and wrote the manuscript.

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4 Declaration of interest

There are no relevant financial or non-financial competing interests to report.

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