Quantitative measures to reveal coordinated cytoskeleton-nucleus reorganization during in vitro invasion of cancer cells

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Abstract
Metastasis formation is a major cause of mortality in cancer patients and includes tumor cell relocation to distant organs. A metastatic cell invades through other cells and extracellular matrix by biochemical attachment and mechanical force application. Force is used to move on or through a 2- or 3-dimensional (3D) environment, respectively, or to penetrate a 2D substrate. We have previously shown that even when a gel substrate is impenetrable, metastatic breast cancer cells can still indent it by applying force. Cells typically apply force through the acto-myosin network, which is mechanically connected to the nucleus. We develop a 3D image-analysis to reveal relative locations of the cell elements, and show that as cells apply force to the gel, a coordinated process occurs that involves cytoskeletal remodeling and repositioning of the nucleus. Our approach shows that the actin and microtubules reorganize in the cell, bringing the actin to the leading edge of the cell. In parallel, the nucleus is transported behind the actin, likely by the cytoskeleton, into the indentation dimple formed in the gel. The nucleus volume below the gel surface correlates with indentation depth, when metastatic breast cancer cells indent gels deeply. However, the nucleus always remains above the gel in benign cells, even when small indentations are observed. Determining mechanical processes during metastatic cell invasion can reveal how cells disseminate in the body and can uncover targets for diagnosis and treatment.

1. Introduction
Cell invasion through tissue is a critical step in the formation of metastases, the typically lethal spread of cancer in the body. During invasion, a single cancer cell can move through the extracellular matrix (ECM) and between cells in tissue or lining blood vessels. Metastatic cancer cells change their morphology and stiffness [1–4] and also apply forces to their surroundings [5–7]. The forces that a cell can apply result from a dynamic interplay between the cell type and capability and the mechanical features of the microenvironments, i.e., the stiffness, topology, and geometry of the ECM [5, 8–15]. Force is typically applied by cells through actin polymerization or the acto-myosin network [14, 16]; a marked elevation in acto-myosin generated forces has been correlated with increased invasiveness in metastatic cells [17]. Actin-containing membrane protrusions transmit extracellular forces across the membrane and to the mechanically connected nucleus [18, 19] and the cytoskeleton can transport the nucleus within the cell [20, 21]. The coordinated processes performed by the cytoskeleton and the nucleus of invading metastatic cells are important to identify and characterize as they can reveal force application mechanisms and potential targets for treatment.

Cell invasion through 3D bio-gel microenvironments is defined either as ameboid or mesenchymal [22]. Those modes of motion require, respectively, large-enough pores in the gel matrix or proteolitic degradability of the matrix for the cells to create a pathway; cancer cells can switch between the modes according to the environmental conditions [22, 23]. During ameboid and mesenchymal invasion within a 3D environment the cells change morphology and apply forces. While cell motion within a 3D environment has been studied, the
initiation of invasion—entering the 3D gel, or penetration through the 2D surface and into the 3D ECM—has yet to be studied.

While gel pores or degradability are the typical requirements for successful cell penetration [24], we have recently shown that metastatic breast-cancer cells will attempt to invade even a non-degradable, impenetrable gel when its stiffness is suitable [7]. Specifically, cells indent a 2D gel, pushing down vertically, if the gel is soft enough for the cells to push, yet stiff enough for them to grip and develop force on [7]; living cells were observed to indent the gel repeatedly. The gels that we observed as inducing the cancer cells to indent were 1.5–2.3 kPa stiffness. ECM stiffness has been shown to provide cues that trigger actomyosin contractility and increase the invasiveness of tumor cells [25] as well as induce change in nucleus stiffness [26]. The observed force-induced transition from 2D to pseudo-3D state may reveal force application mechanisms and stages in metastatic invasion not previously observed.

Here, we introduce a specialized 3D image processing tool for automated, quantitative analysis, and demonstrate that concurrently with impenetrable-gel indentation by single metastatic cells, the cytoskeleton relocalizes and rearranges in the cell and the cell and nucleus morphologies change; this likely indicates a role for them in force applications. We have imaged cells interacting with a collagen-coated, impenetrable, initially flat polyacrylamide gel. Using fluorescent microbeads embedded at the gel surface we are able to observe cell-induced indentations, while simultaneously determining locations of the cytoskeleton and nucleus; fluorescent stains are used for the cell elements. While our gel system is not a physiological model of the ECM, it provides an important way to effectively isolate mechanical cell-substrate interactions, following biochemical attachment; cells are unable to cross the gel. Using our custom MATLAB-based image analysis and 3D rendering module, we are able to automatically and quantitatively analyze confocal images. We show and quantify the dynamic changes in cell morphology and reorganization of the cytoskeleton at specific locations in the cell, highlighting differences between indenting and non-indenting cells. In indenting cells, the entire cell body is moved and the nucleus is transported into the indentation dimple formed in the gel, near the leading edge of the cell. Our work also demonstrates that cell indentation depth and the % volume of the nucleus transported below the gel surface both increase with the metastatic potential (MP) of the breast cancer cells.

2. Methods

2.1. Cell culture

We have used three human, epithelial, breast cell lines: highly-metastatic MDA-MB-231 cancer cells (HTB-26, ATCC Manassas, VA), low MP MDA-MB-468 cancer cells (HTB-132, ATCC), and as control, benign MCF-10A cells (CRL-10317, ATCC). The MCF-10A cells were kindly provided by Professor Israel Vlodavsky at the Faculty of Medicine, Technion-Israel Institute of Technology. For simplicity the cells are referred to as high metastatic potential (MP), low MP, and benign.

Cells were cultured and maintained in a humidified incubator at 37 °C, 5% CO2 and were used at passages 10–30 from stock. The high and low MP cell lines were routinely cultured in Dulbecco’s modified Eagle’s medium (DMEM, Gibco, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS, HyClone Thermo Fisher Scientific, Waltham, MA) and 1 vol% each of L-glutamine, sodium pyruvate, and penicillin-streptomycin (Biological Industries, Kibbutz Beit Haemek, Israel). The benign, MCF-10A cells were cultured in 1:1 DMEM and F-12 media (Biological Industries, Kibbutz Beit Haemek, Israel), 5% horse serum (HyClone Thermo Fisher Scientific, Waltham, MA), 1 vol% of L-glutamine and penicillin/streptomycin, 10 μg ml−1 insulin, 10 ng ml−1 epidermal growth factor, 0.5 μg ml−1 hydrocortisone, and 100 ng ml−1 cholera toxin (Sigma-Aldrich, Rehovot, Israel).

2.2. Gel preparation

Cells were seeded on polyacrylamide gels with Young’s modulus of 2300 Pa. The gels were prepared according to an established protocol [10, 27], as previously described [7]. In short, glass coverslips #5 thickness and 30 mm in diameter (Menzel, Germany), were hydroxylized using 0.1 M NaOH (Sigma, St Louis, MO), activated with 3-aminopropyltrimethoxysilane (Sigma, St Louis, MO), and glutaraldehyde fixed. Gels were prepared on ice and solutions were kept at 4 °C. The gels were composed of 40 μl of 40 vol% acryl and 3.5 μl of 2 vol% BIS acrylamide (both from Bio-rad, Israel), producing 2300 Pa gels. Gelation was induced with 1:200 vol. APS as initiator and 1:500 vol. TEMED as a catalyst (both from Sigma, St Louis, MO). Gels were prepared on the glass within plastic frames (Gene Frame, 25 μl, 10 × 10 mm, ABgene Thermo-Scientific, Waltham, MA). Fluorescent, carboxyl-coated polystyrene particles, 200 nm in diameter (Molecular Probes, Invitrogen Life Technologies, Carlsbad, CA) were added to gels and localized to the gel surface by performing gelation at 2 °C during centrifugation for 30 min at 300 g [27, 28]. Finally, the surface of the gel was activated with Sulfo-SANPAH (Pierce, Thermo
performed to reveal the gel and cell elements. A 3D model of the gel surface is obtained through detection of the rendering of the gel and the cell elements is obtained (see 3D shapes of elements in the cell: the nucleus, the cell membrane, the actin, and the microtubules. After a 3D image segmentation is performed to reveal locations and measures of the gel and then specific binding was achieved with 3% FBS (Hyclone, Thermo Fisher Scientific, Waltham, MA). Microtubule staining was performed with primary α-tubulin antibody (Sigma, St. Louis, MO) and secondary antibody Cy3 goat anti mouse IgG or alexa 647 goat anti mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). Actin staining was done with the antibody Phalloidin Tetramethylrhodamine B isothiocyanate (TRITC, Sigma, St. Louis, MO). Membrane staining was done with the primary antibody epidermal growth factor receptor (EGFR, thermo scientific, Fermont, CA) and secondary antibody Cy5 donkey anti rabbit IgG (Jackson ImmunoResearch Laboratories, West Grove, PA). In parallel with the secondary antibody, cell nuclei were labeled with 4′,6-diamidino-2-phenylindole (DAPI, Sigma, St. Louis, MO).

2.4. Confocal imaging

Imaging was performed with spectral-imaging Zeiss LSM 510 META confocal system mounted on an upright motorized Axio Observer Z1 microscope (Carl Zeiss Microscopy GmbH, Jena, Germany), using a 63×/1.0 NA water objective. Spatial resolution in x–y plane was 0.12 μm or 0.24 μm. Confocal slices were 0.73 μm thick.

2.5. Quantitative analysis of confocal images

Basic image analysis was performed using the LSM image browser software (Carl Zeiss Microscopy GmbH, Munich, Germany) and with Imaris 7.6 software (Bitplane AG, Zurich Switzerland), whereas quantitative information was automatically obtained using a custom program written in MATLAB 2012b (Mathworks, Natick, MA). The advantages of the MATLAB program are that it does not require manual measurement of lengths and provides sub-pixel and sub-slice resolution. For comparison and definition of any systematic errors, we have manually measured the depth of indentation of the cancer cells using the Zen software and compared that to the depths obtained from our MATLAB program. In both (indenting) cancer cell types the error was on average 1 μm, where measured indentation depths are consistently smaller in the custom MATLAB program. Hence, the comparison between the different cell types remains consistent.

The analysis program always requires particles to mark the gel, nucleus staining, and at least one other cell element to be stained. The custom MATLAB-based program applies image processing procedures (see figure 1) on the raw multi-channel images (confocal stack data) to generate a 3D structural model of the (indenting) cells (see figure 2). The presented results are collected from 63 high MP cells, 77 low MP cells, and 28 benign cells. Out of those cells, in about 30–40% of the cases, cells were too close physically to accurately determine cytoskeleton location and cell edge, yet indentation depth and nucleus depth were still available; this affects only number of cells in the statistics presented in figure 3 where all other figures include data from all of the cells.

Figure 1 details the steps of the algorithm, developed to analyze the confocal images and to provide an automated measure of element morphologies and distributions. In short, the stack data is pre-processed, basic image segmentation is performed to reveal locations and measures of the gel and then specific analyses are performed to reveal the gel and cell elements. A 3D model of the gel surface is obtained through detection of the fluorescent particles embedded within it. From the fluorescent staining images, we can in parallel determine the 3D shapes of elements in the cell: the nucleus, the cell membrane, the actin, and the microtubules. After a 3D rendering of the gel and the cell elements is obtained (see figure 2), relative distances and distributions in the cell and relative to the gel are automatically obtained.

Following pre-processing, the analysis process begins with determination by fitting the optimal gel plane. The optimal height (z-coordinate) for each particle in the gel is determined by calculating the intensity center of mass for each particle at the respective x–y coordinates. The gel surface is then defined by fitting a 1st order (linear), 2-dimensional polynomial (e.g., \(ax + by + c = 0\)) to the gel 3D surface point array; the polynomial was fit with the linear least squares method. The other various channels (stained elements) were then segmented, where initially a Gaussian blur filter was applied to avoid local intensity discretization and false edge detection due to potentially diluted and sparse coloring of the different channels.

For each cell we detect the location and structure of the nucleus, and the actin, microtubules and membrane, depending on what was stained in the image. The nucleus shape was determined by thresholding the 3D image array and removing outliers by size; large, faint blobs outside the main structure were considered noise. The threshold was a fractional percent of the maximum intensity, which was empirically determined as 1–2% for
most cases; nucleus staining was very bright and was insensitive to threshold value selection. Similarly, locations of the stained microtubules, actin, and membrane were determined. Using the membrane or the actin stains, the cell surface (edge) was determined, as both are good indicators for the cell edge. The cell edges were identified by sending out hundreds of rays from the nucleus center, interpolating the channel intensity signature along each radial ray, and estimating the cell surface edge point of each ray vector (local, maximum negative derivative criterion). Once the 3D images of the nucleus and cell elements are obtained, the analysis program can provide many parameters. Following 3D rendering of the cell elements, we quantitatively determine, for the nucleus: volume, indentation depth, and the % vol. under the gel; for the cell (membrane/actin): cell edges in 3D, including indentation depth and height above the gel; for the cytoskeleton (actin/microtubules): distribution of the cytoskeleton elements relative to the nucleus, i.e. above, below, or centered.
3. Results and discussion

We used a custom written program in MATLAB 2012b (MathWorks, Natick, MA) to provide 3D-rendering and automated analysis of confocal images to obtain quantitative measures of each cell, and the locations of its cytoskeleton and nucleus relative to the gel surface. We show differences in localization of the cytoskeleton and nucleus between indenting metastatic cells and non-indenting benign cells. Single cells were seeded on a soft gel (Young’s modulus 2300 Pa) with fluorescent particles marking its surface, and were allowed to adhere for 2 h. About 25% of seeded metastatic breast-cancer cells indent the gel by applying forces, while <10% of benign cells exhibit (small) indentations (see figure 3 and figure S1 and figure in [7]). After 2 h, cells were fixed, fluorescently stained to show actin, microtubules, membrane, and nucleus, and imaged in 3D using a confocal microscope.

Figure 3 shows the rounded morphology and condensed cytoskeleton of cells cultured on the soft gel; while in the work presented here we show fixed cells, we have previously observed similar structure in live stained cells as well [7]. On a soft gel, a cell is unable to spread [12] and thus it remains rounded and its cytoskeleton is not distinctly fibrous [30, 31]; on glass, the cells spread out and exhibit (not shown) a typically structured cytoskeleton network including actin stress fibers, and an oval shaped nucleus [1, 12, 30–34]. On the gels, we observe that in the benign cells the actin and the microtubules are nearly uniformly distributed at the cell
periphery and in the cytoplasm, respectively (figures 3(d)–(e)). In the cancer cells, however, actin accumulates at
grip handles on the gel and at the indenting cell’s leading edge, at the bottom of the indentation dimple, mostly at
the nucleus height or under it (figure 3(d)). Concurrently, microtubules may localize to above or to below the
nucleus, or to both locations (figure 3(e)). The observed differences in structural localization of the cytoskeleton
between the metastatic and benign cells, likely relates to force application during mobility of the cells, as actin is
typically at the leading edge of a moving cell and within membrane protrusions [18, 19].

Figure 4 shows that the higher MP (more invasive) cells indent the gels more deeply [7] and their nuclei are
also found deeper below the gel surface. Both metastatic cell lines exhibited a wide range of indentation depths,
where 60–70% of the evaluated indenting cells indented the gels more than 2.67 μm (dashed line in figure 4(a)). Concurrently, microtubules may localize to above or to below the
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where 60–70% of the evaluated indenting cells indented the gels more than 2.67 μm (dashed line in figure 4(a)). We consider the indentation depth of 2.67 μm a threshold value, as it is the maximal indentation exhibited by the benign cells; 40% of those cells did not indent the gels at all. Negative apparent indentations obtained in the
benign cells, likely relate to the cells situating in micron-scale grooves in the gel (figure 2(a)) and may also result
from buckling of the soft gels under strong lateral forces applied by the cells; those were not removed from the
data to show all the statistics. The small indentations generated by the benign cells may occur in response to
mainly lateral traction forces that are applied by the cells. Similar small indentations of 0.5–1 μm have been
observed in other non-cancerous endothelial and ameboid cells [35, 36], as the cells pull the substrate upward
along the contact line with the gel and downwards under the cell center. It is important to note that the
indentation depth and cell response depends on the gel stiffness and the specific cells [37]. For example,
endothelial cells have been shown to induce indentations of 0.5 μm on a 3.78 kPa [36], which may correlate to
the indentations of up to 2.67 μm of our benign cells on the 2.3 kPa gels used here. Hence, we suggest a definition
of invasively indenting cells in our system, as (the metastatic) cells exhibiting indentation depths that are larger
than 2.67 μm, the maximal indentation generated by the benign cells. The intersection between the high and low

![Figure 4](image-url)
MP cells, in figure 4(b), likely indicates different structure and repositioning of the nuclei and cell body of indenting cells.

We observe that deeper cell indentations involve repositioning of the cell body and of the nucleus into the formed gel-dimple. Figure S3 shows that increased cell indentation depth increases nucleus depth, while cell height above the gel decreases. Interestingly, the correlation between nucleus depth and cell height relative to the gel level is, however, the same in all the evaluated cells, regardless of their malignancy and indentation depth (figure S4). This could suggest that the entire cell body is moved together during the indentation process, perhaps in contrast to typical 2D and 3D cell migration models in penetrable gels with a clear leading cell edge; cell invasion and migration is typically described as cell sent leading, membrane-protrusions, followed by the cell body, with the nucleus at its back [24]. Nucleus repositioning likely occurs as a result of cytoskeleton reorganization and morphological changes in the cells, yet the average cell indentation depth most likely relates to the cells’ invasive capabilities.

Figure 5 shows that the percent volume of nucleus below the gel surface-level notably increases as large indentations occur in the metastatic cells (>2.67 μm). In the benign cells, where indentations are small the
nucleus is always above the gel; i.e., zero %vol. of the nucleus is below the gel surface. Interestingly, in the cancer cells, two populations can be identified with indentation depths smaller or larger than the 2.67 μm threshold (dashed line in figure 5). Below the 2.67 μm threshold defined, the nucleus of all the metastatic cells is always above the gel, as in the benign cells. However, as cell indentation depths increase above 2.67 μm, a rapid increase in the percent of the nucleus volume below the gel surface is observed in contrast with the uniform depth changes in figures 3 and 4. In some deeply indenting cells we note that the nucleus is still at or above the gel surface. We suggest that raising of the nucleus may result from fixation at times of transient release of applied stress as observed in living, indenting cells [7]; staining images used here are of fixed cells.

4. Conclusions

In summary, our quantitative image-processing approach reveals differences in the structure and internal organization between indenting, metastatic cells and benign cells. Using our custom tool, we show that in the indenting cells actin is at the leading edge of the cell, microtubules may appear at one side of the cell or both, and the nucleus is repositioned between them, typically below the gel surface and close to the leading edge of the cell. This does not contradict the current concept of the nucleus dragged behind the main cell body [24], as in our experimental setup the nucleus location and cell structure may also be affected by the impenetrability of the encountered substrate as observed in changes in the entire cell morphology. In addition, we demonstrate that large indentations observed in the high- and low- MP cells correlate with the flattening of the cell body and its motion into the indentation dimple, as well as an increase in percent volume of the nucleus below the gel surface. In contrast, the benign cells do not significantly indent the gels and their nucleus is always above gel level and away from the cell edge. Hence, in the current work we present the basic assay and analysis approach using sample cell lines, where the quantitative measures provided here could potentially allow us to distinguish between different types of invasive and non-invasive cells.

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