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ABSTRACT

Cancer is the second leading cause of death worldwide. Despite the immense research focused in this area, one is still not able to predict disease trajectory. To overcome shortcomings in cancer disease study and monitoring, we describe an exciting research direction: cellular mechanophenotyping. Cancer cells must overcome many challenges involving external forces from neighboring cells, the extracellular matrix, and the vasculature to survive and thrive. Identifying and understanding their mechanical behavior in response to these forces would advance our understanding of cancer. Moreover, used alongside traditional methods of immunostaining and genetic analysis, mechanophenotyping could provide a comprehensive view of a heterogeneous tumor. In this perspective, we focus on new technologies that enable single-cell mechanophenotyping. Single-cell analysis is vitally important, as mechanical stimuli from the environment may obscure the inherent mechanical properties of a cell that can change over time. Moreover, bulk studies mask the heterogeneity in mechanical properties of single cells, especially those rare subpopulations that aggressively lead to cancer progression or therapeutic resistance. The technologies on which we focus include atomic force microscopy, suspended microchannel resonators, hydrodynamic and optical stretching, and mechano-node pore sensing. These technologies are poised to contribute to our understanding of disease progression as well as present clinical opportunities.

INTRODUCTION

At the bench or bedside, cancer is often viewed through a "biochemical lens." Genetic mutations, protein pathways and expression, and risk factors such as age and genetic variants[1–5] are investigated, identified, and acted upon. Yet, we still cannot predict who will develop cancer, who will respond to treatment, and who will relapse years later when the cancer was thought to be in remission. Indeed, despite the ever-growing number of molecular-targeted therapies[6–11] and immunotherapies,[12–19] cancer still remains the second leading cause of death worldwide, with approximately 10.1×10^6 cancer-related deaths projected for 2020 alone.[20] That new therapies have not fulfilled their promise may be due to the underlying heterogeneity of cancer, with bulk analyses failing to take into account the differential responses of multiple cellular phenotypes within the tumors. Consequently, new approaches to cancer, and correspondingly new tools to investigate and assess individual cancer cells within heterogeneous tumors, are greatly needed.

One exciting new approach in cancer research involves examining the intrinsic mechanical properties of cells.[21–25] There is strong biological rationale for this: cells continually experience different and varying forces in the body—from shear flow in the vasculature to compressive forces from interstitial pressure within organized tissue or the local microenvironment.[23,26] While these forces are necessary for healthy tissue to maintain homeostasis, in malignant cells, abnormal stress and defective mechanosensing can drive cancer progression.[27–29] For cancer cells that escape the primary tumor, these forces present obstacles that challenge their survival. How these cells respond to these forces could potentially serve as a biomarker for cancer, whether it is in its earliest stage or when it recurs. Already, a number of studies performed using atomic force microscopy (AFM) have shown that cancer cells

generally have a lower Young's modulus than non-malignant cells[30–33] and that the metastatic and invasive potential of cancer cells are related to their elasticity.[32,34–36] Given these studies and those that we highlight below, it is therefore an intriguing hypothesis that a "mechanical biomarker" could be used alongside traditional methods (e.g., immunostaining, genetic analysis, etc.) to analyze a tumor and its neighboring cells, thereby providing a more comprehensive view of the tumor in regard to its biology, potential responsiveness to treatment, and metastatic potential.

In this perspective, we discuss further the rationale of a mechanical biomarker for cancer. While there are a number of single-cell mechanophenotyping methods currently in development, we highlight specific examples of those that have been directly applied to clinical samples and that have led to promising pre-clinical results in support of a mechanical biomarker.

A BIOLOGICAL RATIONALE FOR CELLULAR MECHANOPHENOTYPING

Cellular anatomy that affect mechanical properties

The intrinsic mechanical properties of a cell are a function of its various subcellular components and its interactions with its surroundings. Broadly speaking, the nucleus, cytoplasm, and cell membrane all contribute to the mechanical properties of cells (Fig. 1). At approximately 10 times the stiffness of cytoplasm,[37,38] the nucleus is the largest, stiffest organelle[38] and is thought to be the primary contributor to a cell's resistance to deformation. The nucleus houses chromatin which is organized into chromosomes

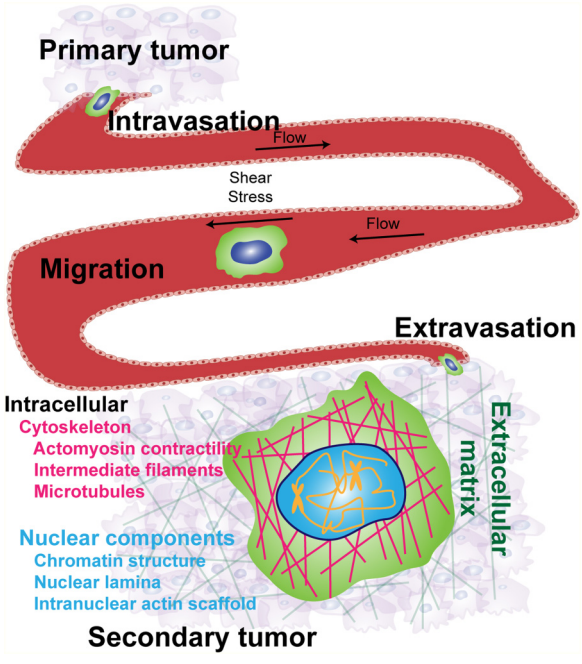

FIG. 1. Mechanical stimuli and mechanical properties in the metastatic cascade.

for most of the cell cycle, with DNA wound around histones. Chromatin organization and compaction controls the size and density of the nucleus and its deformability. Likewise, protein expression and distribution in the lamina also affect nuclear deformability.[27,29] The tethering of the nuclear lamina to the cytoskeleton allows both the movement of the nucleus via transmembrane actin-associated nuclear (TAN) lines[38] during cell polarization and migration and also during the transduction of mechanical signaling outside the cell to the nucleus.

Within the cell's cytoplasm exists the cytoskeleton. Consisting of actin, microtubules, and intermediate filaments,[23] the cytoskeleton serves multiple functions, including providing cellular structure, transporting organelles and cargo within the cell, orchestrating cell division, and enacting cell movement. The cytoskeleton can undergo changes in structure and composition based on signals it receives from the outside environment (via an adaptor protein, such as paxillin) or as a result of force-induced changes to gene expression (via environmental signaling proteins).[23] The relative composition of the actin, microtubules, and intermediate filaments within the cytoskeleton, as well as the degree of polymerization and cross-linking of actin, contribute to a cell's elasticity and viscosity. Through actin or myosin-disrupting agents, actin has been shown to contribute more to changes in cellular elasticity than microtubules.[39] The presence and activity of actin binding partners (e.g., ARP 2/3) is particularly important for cell stiffness, and the deactivation or destabilization of these associated complexes leads to less organized actin and a more compliant cell. Studies involving cell lines have shown that both actin remodeling and low actin filament junction density lead to the high compliance of more metastatic cells,[40] in agreement with the fact that metastatic tumor cells must be sufficiently deformable to navigate small openings in the extracellular matrix (ECM) and endothelium.[23] Cancer cells have also demonstrated higher actomyosin contractility, thereby applying abnormally high forces on their environment and increasing tumor stiffness in response to a stiff ECM.[23] Additionally, actomyosin contraction, membrane tension, mechanosensitive channels, and mechanotransduction ion channels—all of which are dysregulated in many cancers—regulate a cell's internal hydrostatic pressure, which is another contributor to cell stiffness.

Environmental contributions that affect the cellular mechanical phenotype in cancer

Cellular mechanical properties are also affected by environmental factors such as the ECM, and there is an extensive and dynamic interplay between the matrix's effect on a cell and that cell's ability to remodel the matrix. Importantly, the combination of a cell's own mechanical properties (e.g., contractibility), properties of the matrix (i.e., stiffness and degree of degradation), and environmental properties (e.g., interstitial flow) affects its ability to navigate the ECM.[37] Given that stiffer, more difficult to traverse, stroma is associated with worse clinical outcomes,[37] the properties of tumor tissue ECM have direct implications for disease progression.

Neighboring cells influence a cancer cell's mechanical properties. For example, in both primary and secondary locations, activated fibroblasts, i.e., cancer associated fibroblasts (CAFs),[41] remodel the ECM, which, in turn, alters cell mechanical properties.

As another example, intercellular connections can transmit intercellular tension through adherens junctions.[23] The transmission of mechanical tension and compression is commonly found in cell sheet migration, wherein the junctions and cytoskeletons of migrating cells reorganize to minimize shear stress.[42] Traveling clusters of tumor cells exhibit cell sheet migration, and during the circulation phase of metastasis, circulating tumor cell (CTC) clusters use this type of migration as a potential strategy to avoid anoikis (i.e., cell death due to lack of necessary adhesion). They are, thus, more viable than individual CTCs.[43] Direct tumor cell interaction with neighboring cells in the microenvironment and with the ECM affects their ability to migrate. Cells undergo several mechanical changes during migration: actin polymerization occurs, cell polarization may change, and cells respond to matrix-based cues as they move through the stroma.[44]

While neighboring cells and the extracellular matrix are crucial factors in disease progression, the current focus on bulk tissue measurements by many researchers obscures some of the dynamics at the cellular level.[45,46] For example, while CTCs may be stiffer than normal blood cells (i.e., red blood cells and leukocytes),[47] multiple studies have found that the cancer cells are still softer than non-malignant cells from the same tissue and that *decreased* stiffness is associated with metastatic potential. As another example, even though breast cancer *tissue* is generally stiffer than healthy tissue (a result of increased ECM deposition and ECM crosslinking), the individual cancer cells are less stiff than non-malignant cells.[48] Within the tumor, there is a distribution of cellular stiffnesses, leading to a range of metastatic potential that correspondingly poses different levels of risk to the patient. Thus, to gain higher resolution in the mechanical properties that facilitate disease progression, it is necessary to examine disease at the single cell level.

The importance of single-cell mechanical phenotyping in cancer

The many challenges associated with characterizing tumor heterogeneity and the interplay among the different cellular properties required to complete the metastatic cascade necessitate a single-cell level analysis of the tumor. While the emphasis of single-cell studies has been on RNA-seq analysis in order to understand the biochemical basis of a tumor, the physical landscape of cancer cells motivates the exploration of mechanical properties as an additional avenue for analysis. Mechanical phenotyping could help address the challenge of understanding why some cancer patients respond to specific therapies and others do not, and it may provide new insights into predicting tumor progression and recurrence. In examining mechanical properties as a potential cancer biomarker, it is crucial to utilize single-cell rather than bulk-tumor analysis because mechanical stimuli from the environment may obscure the inherent mechanical properties of the cell that can change over time. Moreover, bulk studies mask the heterogeneity in mechanical properties of single cells.

A prime example of why single-cell mechanophenotyping is necessary can be found with rare cells within the tumor, including those of epithelial–mesenchymal transition (EMT) and cancer stem cell (CSC) phenotypes. EMT is a broad spectrum and has a direct bearing on cancer, as it both enables progression and is associated with therapeutic resistance.[49] EMT causes a number of cellular mechanical changes as it acquires a more mobile phenotype.[37] Cells lose their intercellular junctions, become less adhesive and less polarized, and form additional extracellular features like filopodia.[44,47] Hallmarks of EMT include changes in adhesion molecules and intermediate filaments in the cells, in particular the downregulation of E-cadherin and upregulation of vimentin,[47] furthering changes in cellular mechanical properties.[25,50–52] Within the heterogeneous tumor, cells display many EMT-related changes to varying degrees. The cells of the intermediate phenotype that embody pro-metastatic characteristics from both the epithelial and mesenchymal phenotypes are likely best equipped to overcome the obstacles (e.g., immune response, shear stress, lack of the propensity to migrate and/or proliferate in various locations, and anoikis) with which they are presented when establishing secondary tumors. Similar to EMT cells, CSCs have also been associated with therapeutic resistance.[49] Like EMT, CSC phenotypes are associated with changes in mechanical properties, again suggesting that mechanical phenotyping may be more effective in identifying these rare cells in a population. Mechanophenotyping individual cells may provide opportunities to identify these cells given its sensitivity to functional changes that may not be apparent solely based on marker expression. This is particularly true of cells undergoing EMT, where there are a variety of intermediate phenotypes, and CSCs, where markers are incompletely determined.

Overall, transient and time- and location-dependent phenotypes, of which the EMT and CSC are just two examples, are of particular interest to biologists and clinicians, as different phenotypes support the tumor cell at different stages of disease progression (e.g., a mesenchymal phenotype is advantageous in migration but disadvantageous in the context of ultimate colonization of a distal tumor). It is thus of great importance to characterize individual cells mechanically and determine the contributions of subcellular components to the mechanical phenotype. To meet these needs, several different strategies have been employed to investigate cancer cells, some of which have been applied to clinical samples.

CLINICAL APPLICATIONS OF SINGLE-CELL MECHANOPHENOTYPING TECHNOLOGIES

The investigation of mechanical properties of single cells has tremendous potential to improve the diagnosis and monitoring of malignancies, and a number of mechanophenotyping techniques, e.g., microfluidic micropipette aspiration[53] or microconstriction channels,[54] have extensively focused on studying cells from different cancer cell lines. Here, we highlight those promising single-cell mechanophenotyping platforms that have transitioned beyond preliminary cell line study to investigating clinical or primary samples.

Atomic force microscopy (AFM) and cantilever-based analysis of clinical samples

Because of their high resolution and instrumentation maturity, AFM and other cantilever tapping technologies have been used to investigate the mechanical properties of single non-malignant and malignant cells within a population. Cross *et al.* used AFM to characterize single cells from the pleural fluids of three patients: one

with suspected lung cancer, one with breast cancer, and one with pancreatic cancer.[55] Then, they compared the Young's moduli of these cells to those obtained from the pleural fluids of four patients with other diagnoses: liver cirrhosis, hepatic failure, and anasarca peripheral edema. They found that tumor cells had significantly lower Young's moduli as compared to normal mesothelial cells found in the same pleural fluid. A comparison of the distribution of the cells' Young's modulus shows that while tumor cells have a normal distribution with small variance, normal mesothelial cells have a log-normal distribution. Intriguingly, tumor cells from the different cancer types were found to have similar stiffnesses, although further investigation is necessary to draw conclusions given the small sample size in this study (total n = 7 patients).

AFM has also been used to contrast cancer cell stiffness in patients with varying outcomes. Following isolation of leukemic blasts using density-gradient centrifugation, Lam *et al.*[56] performed AFM measurements on cells positioned within a fabricated microwell that enabled continuous media perfusion. At least 15 cells per patient were analyzed from 15 acute lymphoblastic leukemia (ALL) patients, four of whom showed symptoms of leucostasis, a life-threatening complication due to the accumulation of leukemic blasts in microvessels. Lam *et al.* found that cells from symptomatic patients had significantly higher median stiffness values. This is in direct contrast to that found in studies involving solid tumors, highlighting differences observed in blood cancers. Of particular interest, the high variance that Lam *et al.* found in symptomatic patients suggests the presence of a subpopulation within a heterogeneous population that may be of particular clinical interest in the study of leucostasis.

In another study, Pandya *et al.* utilized cantilevers to investigate the mechanical phenotype of breast cancer tissue samples.[45] Using hematoxylin and eosin staining to identify first cancerous and benign cells in the tissue samples, Pandya *et al.* measured seven (four cancerous and three benign) samples with custom-fabricated piezoresistive microcantilevers [PMC, Fig. 2(a)]. Just as Cross *et al.* observed,[55] and consistent with other AFM studies,[57–59] Pandya *et al.* found that cancer cells were less stiff than benign cells.

With its nano-scale tip, AFM is capable of analyzing the mechanical properties of single cells within distinct regions of a tissue; however, properties of these measured cells are influenced by neighboring cells and ECM. Additionally, the cellular mechanical properties an AFM measures are influenced heavily by the substrate on which the cells are immobilized.[51,60] Finally, AFM's low throughput (a few cells/h)[21] limits its widespread use as a diagnostic and monitoring tool.

Suspended microchannel resonator (SMR) analysis of clinical samples

A cell's transit through a suspended microchannel resonator (SMR) can be used to determine its passage time and buoyant mass.[61–63] The passage time of a cell in a microfabricated SMR with a constriction is related to the cell's deformability, with shorter passage time indicating higher deformability. Bagnall *et al.* used an SMR [Fig. 2(b)] to analyze the effect of EMT and platelet treatment on the mechanical properties of a murine tumor cell line.[64] They found that cancer cells undergoing EMT had shorter passage times than those of control cells, while those incubated with platelets had longer transit times than control cells. When compared with blood cells (i.e., red blood cells and leukocytes), Bagnall *et al.* found that cancer cell lines (non-small cell lung cancer H1975, breast cancer MDA-MB-231 and SKBR, prostate adenocarcinoma PC3-9) had longer passage times. Moreover, CTCs from tumors formed in mice following tail vein injection had passage times that were more similar to their parent cell lines than to murine blood cells. Finally, CTCs isolated from two metastatic prostate cancer patient samples using the CTC-iChip[65] were stained to identify CTCs and then analyzed using an SMR. There were fewer long passage time events than there were previously enumerated CTCs (as determined by staining), suggesting that some human patient CTCs mechanically behave more like blood cells than cancer cell lines. However, a limitation of the SMR system used was the inability to make a one-to-one comparison of this highly deformable CTC subpopulation to stained cells.

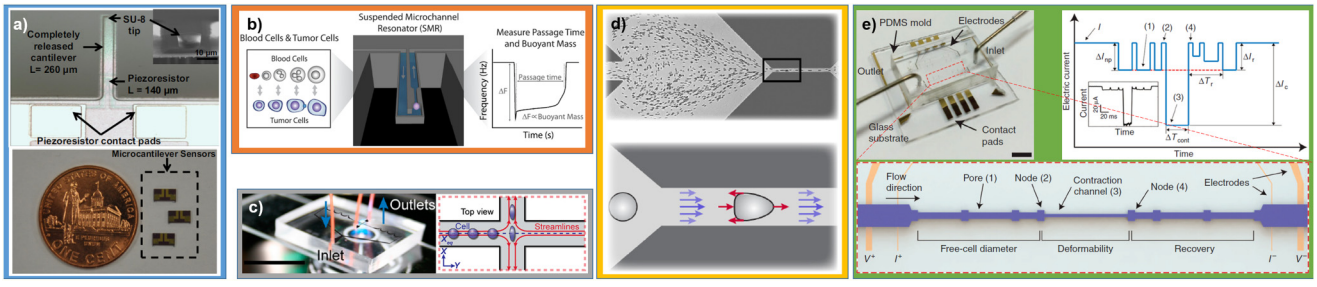

FIG. 2. Technologies that have been applied in the mechanophenotyping of clinical samples and single cells within primary tissues. (a) Piezoresistive microcantilever. Reprinted with permission from Pandya *et al.* Biosens. Bioelectron., **63**, 414–424 (2014), Copyright 2014 Elsevier. (b) Suspended microchannel resonator. Bagnall *et al.*, Sci. Rep., **5**, 18542 (2015). Copyright 2015 Author(s), licensed under a Creative Commons Attribution (CC BY 4.0) License. (c) Microfluidic hydrodynamic stretching. Reproduced with permission from Gossett *et al.* Proc. Natl. Acad. Sci. U.S.A. **109**(20), 7630–7635 (2012) Copyright 2012 Author(s). (d) Shear-induced deformability cytometry. Toepfner *et al.* eLife, **7**, e29213 (2018), Copyright 2018 Author(s), licensed under a Creative Commons Attribution (CC BY) License. (e) Mechano node-pore sensing. Kim *et al.* Microsyst. Nanoeng., **4**, 17091 (2018), Copyright 2018 Author(s), licensed under a Creative Commons Attribution (CC BY 4.0) License.

Bagnall *et al.* advanced their platform by incorporating enhanced fluid handling, thereby enabling the comparison of SMR events with immunofluorescence images.[64] CTCs isolated from the CTC-iChip[65] were first stained for EpCAM (indicative of tumor cells in the blood) and CD45 (a leukocyte surface marker) and then processed by SMR. Cells were gated based on a buoyant mass of greater than 50 pg and a transit time of greater than 10 s, and those that met that criteria were collected in a 96-well plate for imaging. Although samples from seven metastatic prostate cancer patients were processed as proof-of-concept, five samples did not have CTCs, and of the two remaining patients with confirmed CTCs, only one patient had a single CTC that fell within the gating parameters.

Overall, a combination of low throughput (45 μl/h)[64] and the rarity of CTCs make SMR more applicable to studying the underlying biology rather than disease diagnosis and monitoring of solid tumors.

Optical, hydrodynamic, and shear-based deformation of cells derived from clinical samples

Stretching of cells through optical and hydrodynamic methods has been used to determine their deformability. Microfluidic optical stretchers[66] use two axially aligned, counter propagating laser beams to apply an optical stress on a cell, causing it to stretch. Using micrographs to measure radial deformation, one can then calculate a cell's tensile creep compliance.[66] Remmerbach *et al.* used an optical stretcher to analyze cells from four healthy donors and five oral squamous cell carcinoma patients.[66] They found that cancer cells were 3.5 times more deformable than normal cells. In addition to having a higher mean compliance, cancer cells had a deformability distribution that was far broader than that of normal cells, which the authors speculated could be a result of cancer's multiclonal nature.

Hydrodynamic stretching, or deformability cytometry [DC, Fig. 2(c)], is another method to measure cellular deformability, which has shown promise in clinical mechanophenotyping because of its high throughput. Developed by Gossett *et al.*,[67] DC uses inertial focusing to align cells within a microfluidic channel, and then exposes them to an "extensional flow" region where they are deformed. High speed microscopy and image analysis are used to determine the initial dimeter of a cell as well as its deformed shape. Cell deformability is then calculated as the ratio of the long axis of deformation to the shorter perpendicular axis. DC was able to detect different mechanical phenotypes that were associated with different disease states. In 47 patient samples, pleural fluid from patients without malignancy or inflammation contained mostly had small, rigid cells, while fluid from those with chronic inflammation had both large and small cells that were more deformable. This cell population shift was consistent with activated immune phenotypes. Patients with known malignancy also presented high numbers of large diameter, highly deformable cells. Using gating criteria based on these findings, the authors achieved a sensitivity of 91% in detecting malignancy and a specificity ranging from 65% to 86%. Additionally, DC was used to determine the role of different subcellular components in stiffness. The authors found that actin polymerization had only a small effect on deformability, as did myosin contractibility, suggesting that nuclear size, contents, and structure contribute most significantly to deformability. This demonstrates how mechanophenotyping can identify subcellular differences that may have major implications in disease progression.

In another study, Tse *et al.*,[68] used DC to study 119 patient pleural effusion samples from patients with varying malignancies, including solid tumors (breast and prostate cancers), and liquid tumors (lymphoma and leukemia). Measurements from malignant samples and samples from patients with varying degrees of inflammation were used to train a classifier that used 11 features based on cell size and deformability. The classifier assigned a score to each sample ranging from 1 to 10, with 10 representing the highest probability of malignancy. This scoring method was evaluated to have an AUC of 0.86. Because DC demonstrates relatively high throughput and is fully automated, Tse *et al.* suggest that this method could be a valuable pre-screening technique to minimize processing of pathology specimens.

A variant of "deformability cytometry" is a non-contact microfluidic constriction channel that uses shear stress to deform cells [Fig. 2(d)].[69] Using a CMOS camera and LED light source to image the characteristic bullet shape of a cell flowing in the device, Otto *et al.*[69] quantified a cell's deformation (D = 1 – circularity) and cross-sectional area. Operating at 1000 cells/s and screening 1:20 diluted blood samples, Toepfner *et al.*[70] used this platform to determine the morpho-rheological (MORE) phenotype of a cell, i.e., its size, brightness, and deformation. After validating MORE measurements taken with RT-DC, they applied this technology to a number of clinical scenarios, including the analysis of blood cells taken from patients with either acute myeloid leukemia (AML, n = 7 patients) or acute lymphoblastic leukemia (ALL, n = 4 patients), and healthy donors (n = 21 patients). AML cells were similar in size to differentiated myelocytes, but were less deformable. In contrast, ALL cells did not show consistent differences in deformability from mature lymphocytes, but they were larger than mature lymphocytes. Toepfner *et al.*[70] were also able to perform serial biopsies and analyses on an ALL patient being treated with methylprednisolone, showing the potential of this application to monitor treatment.

Overall, DC approaches the throughput necessary for clinical applications, and has already been used to screen clinical samples. Urbanska *et al.*[71] compared deformability cytometry techniques in a recent study and found that changes in subcellular structures, i.e., actin polymerization, affect the deformability readouts of the various DC technologies differently. Both SMR and RT-DC showed an increase in relative deformability with the administration of the actin destabilization agent latrunculin B (LatB), and the subsequent dose response was sigmoidal. In contrast, DC via extensional flow showed little difference in response to LatB. Urbanska *et al.* suggest selecting a DC method based on desired sensitivity, throughput, and whether contact with channel walls is physiologically relevant. Overall, no matter which technology variation is used, DC does measure single cells, but its "readouts" are effectively a distribution, potentially masking the contributions of individual cells to disease progression.

Mechano-node-pore sensing (mechano-NPS) for the mechanical phenotyping of cancer cells

Mechano-NPS is a label-free technique that Kim *et al.* developed to mechanophenotype cells electronically.[24] Based on the Coulter principle,[72] mechano-NPS measures the current pulse

produced when a cell transits a channel that has been segmented by wider nodes and one segment (i.e., "the contraction" segment), in particular, is narrower than the cell diameter. By analyzing the current pulse, which is uniquely modulated, Kim *et al.* could obtain a cell's free diameter (D_{cell}), its deformed diameter (D_{deform}), its transit time through the narrow contraction segment (ΔT_{cont}), and its transverse deformation (defined as D_{deform}/D_{cell}). In contrast to other mechanophenotyping methods, Kim *et al.* could use mechano-NPS to determine a cell's time to recover from deformation ($\Delta T_{recover}$). To take into account cell size, fluid velocity, and channel dimensions, they also defined a new dimensionless physical parameter, the whole-cell deformability index (*wCDI*), which they showed is inversely related to both cortical tension and the Young's modulus.

Kim *et al.*[24] demonstrated that mechano-NPS [Fig. 2(e)] could distinguish between malignant and non-malignant cells in two different epithelial cell lines (breast and lung) based on mechanical properties alone (the malignant cells were less stiff than the non-malignant cells). Moreover, they demonstrated that mechano-NPS could distinguish between subpopulations of human mammary epithelial cells (HMECs) based on just their *wCDI*: myoepithelial cells (MEPs) were found to be more stiff than luminal epithelial cells (LEPs), which is consistent with the fact that LEPs are thought to be a target cell type for carcinogenesis.[73] Interestingly and with potentially profound impact on aging and breast cancer research, they also showed that the recovery times of HMECs of postmenopausal women were significantly slower than those of premenopausal women. Finally, Kim *et al.* showed that mechano-NPS could track the progression of HMECs as they traverse the stages of malignant progression through distinctive mechanical signatures at each stage.

Since their original work, Kim *et al.* advanced mechano-NPS such that it is also capable of performing viscoelastic studies of cells.[25] In "visco-NPS," the contraction segment is sinusoidal rather than straight, and as such, cells experience periodic deformation as they transit the channel. visco-NPS measures both the storage (elasticity) and loss (viscosity) moduli of cells, and Kim *et al.* demonstrated that the viscoelastic properties of malignant breast epithelial cells (MCF-7) were distinctly different than non-malignant cells (MCF-10A). Kim *et al.* used visco-NPS to dissect the individual contributions of different cytoskeletal components (actin and microtubules) to whole-cell mechanical properties. Moreover, they measured the mechanical transitions of cells as they entered different stages of the cell cycle or underwent EMT. Specifically, they observed that the elasticity of MCF-7 cells is highly dependent on cell-cycle phase, with cells having the stiffest mechanical response in G2-phase, i.e., when cells experience the maximum accumulation of cytoplasmic material, assembly of microtubules, and structuring of the nuclear envelope all in preparation of cell division.[25] They also observed the viscosity of MCF-7 cells showed a dramatic decrease in S-phase, when cellular DNA is actively being unwound by helicases and replicated and would be less mechanically stable than DNA packaged around histones.[25] With regard to EMT, Kim *et al.* found MCF-10A cells induced into EMT via overexpression of SLUG were more viscous than normal MCF-10A cells. This result in particular is counter to previous AFM measurements of MCF-10A cells that were EMT-induced and showed solid-like behavior.[74,75] Kim *et al.* hypothesized that the difference is due to the fact that the AFM measurements were performed on localized areas of the cells, which were adhered to substrates of defined stiffness. visco-NPS, in contrast, measures the whole cell in suspension, thereby decoupling substrate properties from cell mechanical properties.

Overall, mechano-NPS and visco-NPS both have moderate-throughput (~500 cells/min) and can be used for screening cells or to elucidate the underlying biology of cellular mechanical properties. Although these devices have significantly lower throughput than DC, they are able to measure multiple mechanical features and provide more comprehensive and valuable information including cell recovery time and viscoelastic properties.

DISCUSSION

Clinical and biological interest in the mechanical properties of tumor cells has motivated the development of several analysis modalities, each with their own advantages and drawbacks. AFM has the longest history given its prior establishment in materials testing, and it allows measurements of stiffness at single-cell and even subcellular resolution. However, AFM is typically used to measure cell within the context of tissues, and single cell measurements are influenced by the substrate on which cells are immobilized.[51,60]

In contrast, SMR analyses the mechanical properties of single cells without the influence of neighboring cells or ECM. It has been applied downstream of a CTC isolation, enabling the analysis of a cancer cell population in the midst of the metastatic cascade. Cell stretching, through either optical or hydrodynamic methods, also provides information about cells independent of tissue or ECM context. It features higher throughput than SMR and provides a single metric—deformability. mechano-NPS and visco-NPS are single-cell mechanophenotyping methods that quantify multiple mechanical properties, including deformability, transit time, recovery time, and even viscoelastic properties.

SMR, cell stretching, and NPS all have an advantage over AFM in that they can measure inherent properties of a cell without the influence of neighboring cells or substrates. However, direct comparison of a cell's mechanophenotype with other information (e.g., single-cell sequencing or immunofluorescence staining) is challenging, as it requires cell sorting after mechanical measurement. This limitation could potentially be overcome with advances in barcoding[76] or microfluidic cell trapping technologies.[77–79] Compared to AFM, the relatively high throughput of DC cell stretching, the moderate throughput of mechano-NPS, and even the lower throughput of SMR better position these technologies for screening or monitoring in a clinical setting.

High-throughput is important when considering tumor heterogeneity, and all the mechanophenotyping technologies described here do not approach the throughput of flow cytometry—a "workhorse" in biomedical research and in the clinic which can operate at throughputs of up to tens of thousands of cells per second.[80] In order to inform clinical decision making, mechanophenotyping must be able to efficiently sample a sufficient number of cells in order to characterize the different subpopulations present in a tumor. The clinical application of many mechanophenotyping technologies may be hindered by the high cost of high-speed cameras (for DC and optical stretching) or complex fabrication methods (for SMR). These techniques are also limited by the

highly specialized laboratory equipment required for operation, from specialized instrumentation to custom device fabrication. In the instance where microfluidic devices are required, partnerships with commercial fabrication facilities for the mass production could facilitate widespread adoption

Overcoming these obstacles could enable mechanophenotyping to address challenges in the monitoring of cancer and in the study of its underlying biology. Mechanophenotyping can be applied to cells obtained from multiple types of biopsies, including liquid biopsies. This capability is advantageous when serial biopsies are performed to monitor disease progression or response to treatment. Crucially, unlike biomarkers such as the prostate-specific antigen (PSA), mechanophenotyping analysis can go beyond basic detection and provide information about how a tumor changes over time.

Cancer progression features highly dynamic phenotypes, and further investigation is necessary to determine the role of EMT and CSCs in the different steps of the metastatic cascade. Given that the complex biological processes underlying these phenotypes can manifest as changes in cellular structure, mechanophenotyping can serve as a significant tool to probe the intermediate points along the EMT or CSC continua. Studies of EMT in particular have produced conflicting data about which phenotypes are required for the completion of metastasis, and mechanophenotyping could provide clues as to the physical changes involved in this process. This, in turn, could aid the development of therapeutics and strategies for assessing disease response.

Thus, a host of technologies have been developed to mechanically phenotype cells. These technologies have shown promise in identifying the presence of cells of varying structural phenotypes in cancer. Subsequent studies and technologies will need to address the logistical hurdles (e.g., throughput, accessibility) that currently preclude mechanophenotyping from routine clinical use and from clinical studies involving large numbers of patients. Correlating a cell's mechanophenotype with other properties will not only further our understanding of the underlying mechanisms of disease progression and but also increase the attractiveness of mechanophenotyping, overall. As a tool at the bedside and benchside, mechanophenotyping continues to be an exciting direction in cancer research.

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DATA AVAILABILITY

Data sharing is not applicable to this article as no new data were created or analyzed in this study.

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