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## Tumor cell migration in complex microenvironments

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### Abstract

Tumor cell migration is essential for invasion and dissemination from primary solid tumors and for the establishment of lethal secondary metastases at distant organs. *In vivo* and *in vitro* models enabled identification of different factors in the tumor microenvironment that regulate tumor progression and metastasis. However, the mechanisms by which tumor cells integrate these chemical and mechanical signals from multiple sources to navigate the complex microenvironment remain poorly understood. In this review, we discuss the factors that influence tumor cell migration with a focus on the migration of transformed carcinoma cells. We provide an overview of the experimental and computational methods that allow the investigation of tumor cell migration, and we highlight the benefits and shortcomings of the various assays. We emphasize that the chemical and mechanical stimulus paradigms are not independent and that crosstalk between them motivates the development of new assays capable of applying multiple, simultaneous stimuli and imaging the cellular migratory response in real-time. These next-generation assays will more closely mimic the *in vivo* microenvironment to provide new insights into tumor progression, inform techniques to control tumor cell migration, and render cancer more treatable.

### Keywords

cell migration; mechanotransduction; chemotaxis; interstitial flow; tumor microenvironment; microfluidics

### Introduction

The metastatic cascade is a complex, multistage process involving modulation of cell phenotype, cell migration, and dynamic homeotypic and heterotypic cell-cell interactions [1]. There are various proposed models for carcinoma progression and the formation of metastases (reviewed: [2]), and tumors likely employ multiple mechanisms *in vivo*. Metastatic carcinoma progression follows the general pattern of primary tumor growth, tumor cell invasion, intravasation, circulation, extravasation, and growth of the secondary tumor. However, the heterogeneous tumor microenvironment [3], plasticity of invasion [4], and hypoxia-induced genomic instability [5] are among many factors that contribute to tumor heterogeneity, and there is debate as to the source and onset of the metastatic

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phenotype [6], [7]. Most cancer-related deaths result from the formation of metastases [8], which are difficult to detect and can remain dormant for years after treatment of the primary tumor [9, 10]. The formation of these metastases by disseminated tumor cells is preceded by and requires tumor cell invasion at the primary tumor site, a ubiquitous step in early tumor progression that represents a viable target for therapy [11].

Disseminating carcinoma cells navigate through the tumor microenvironment, across the basement membrane and into the surrounding stroma. Migration is a highly orchestrated process in which cells are guided both by internal and external signals. Mechanical signals, sensed by integrins [12] and other adhesion receptors [13], and chemical signals, sensed by chemokine and growth factor receptors [14], influence the migration of tumor cells. Hence, understanding the mechanisms that guide cell migration in response to various stimuli in the tumor and stromal microenvironments is key to developing therapies that prevent tumor cell migration and render cancer more treatable.

Because it is difficult to isolate the effects of an individual stimulus on cell migration *in vivo*, *in vitro* models have emerged as powerful tools for investigating tumor cell migration. These reductionist *in vitro* assays isolate a subset of stimuli that can be examined in detail to enhance our overall understanding of the important chemical and mechanical signals that guide tumor cell migration. A key assumption in many reductionist experiments is that chemical and mechanical stimuli act in parallel. However, the migrating cell acts as a signal integrator, sensing simultaneous stimuli, activating intracellular pathways, and responding through organized processes that culminate in the extension of protrusions and subsequent migration. Furthermore, tumor cells invade stromal tissue through a variety of mechanisms, and the process of migration is dynamic and a function of tissue substrate [4]. As we look to develop the next generation of assays for tumor cell migration, it is important to consider the crosstalk between chemical and mechanical stimuli, and the role it plays in guiding the migration of tumor cells.

We start by summarizing the experimental and computational approaches that have been developed to study tumor cell migration, and highlight their benefits and shortcomings. We then discuss the results of these studies and introduce the various stimuli that guide tumor cell migration. We focus on single migrating tumor cells and we broadly divide the stimuli into mechanical and chemical cues. We conclude by highlighting recent data demonstrating that chemical and mechanical stimuli are not independent and the crosstalk among them strongly influences cell migration. In conjunction with computational models, assays that allow the application of several, simultaneous stimuli will provide insight into tumor cell migration *in vivo* and help in the development of new methods to control and limit cell migration, improving the efficacy of cancer therapy.

## Experimental Methods to Study Tumor Cell Migration

The tumor microenvironment is comprised of a dynamic network of extracellular matrix (ECM) proteins [15] bathed in interstitial fluid and a host of associated cells including fibroblasts, bone marrow-derived cells, endothelial cells, and infiltrating immune cells (reviewed: [16]). These stromal cells remodel the ECM and provide mechanical and chemical signals to the tumor cells. The many components and the dynamic nature of the tumor microenvironment (Figure 1) contribute to its complexity, but investigation of the effect of individual stimuli on migration requires an environment in which the mechanical and chemical properties can be tuned precisely with reproducibility. The requirement for such control has led to the development of *in vitro* assays that mimic aspects of the *in vivo* tissue. *In vitro* studies are well suited for dissecting the signaling pathways that govern cell migration in response to a particular factor of interest, while *in vivo* studies can be utilized

to investigate the relevance of these signaling pathways in the intrinsic tumor microenvironment during different steps of the metastatic cascade. Different experimental methods to assay tumor cell migration *in vitro* are presented schematically in Figure 2 and summarized in Table 1 with their key advantages/limitations, parameters that can be manipulated, and practical information for implementation.

### Traditional *in vitro* assays

Single cell migration in response to soluble biochemical factors has been traditionally assayed using Boyden chambers [17], or modifications of the original design (e.g. Zigmond [18] and Dunn [19] chambers). Boyden chambers, also known as transwell systems, incorporate a stiff, porous membrane between two cell culture chambers. Soluble factor gradients can be established across the membrane, and the number of tumor cells that migrate through the membrane, from one chamber to the other, is used as a metric for migration (Figure 2). To improve upon the traditional transwell assay design, a hydrogel, often Matrigel or collagen type I, can be placed on top of the membrane to study invasion through a 3D matrix [17]. An alternative method to investigate cell migration in response to localized chemokine gradients is the micropipette assay [20, 21], where a micromanipulator-controlled micropipette is used to dispense predefined chemokine solutions in close proximity to cells on a 2D substrate. However, chemokine gradients generated by micropipette are transient and difficult to quantify.

Collective cell migration can be assayed by using a cell scraper [22] or a micropipette [23] to generate a “wound,” or an area without cells in a cellular monolayer. Cell motility is assayed by monitoring the time required for the “wound” area to be covered by the collectively migrating cells. Alternatively, cell damage can be avoided by positioning a barrier to migration, allowing cells to migrate in the area covered by the barrier upon its removal [24]. These assays are known as “wound healing assays” and are typically limited to two-dimensional substrates with uniform stimulation conditions, although stimulus gradients can be created through modified assays involving microfabrication [25]. An important advantage of these assays is the ability to perform live cell imaging and conduct multiple parallel assays, enabling high throughput data generation [26].

### Microfluidic *in vitro* assays

The limitations of conventional assays to precisely control microenvironmental stimuli have led to the development of novel migration assays using microfabrication technology and 3D ECM matrices. In particular, the ability to accurately position fluid streams in microfluidic channels has enabled the development of microfluidic wound-healing assays [27], and multi-channel systems that allow the seeding of multiple cell types in 2D or 3D [28]. Other approaches utilize microcontact printing methods to create patterned substrate-bound gradients [29]. These novel assays enable the creation of user-defined microenvironments, where the magnitude, direction, and temporal characteristics of biochemical and biophysical stimuli can be precisely controlled.

Recent advances in microfluidic technology have made it possible to create novel assays that allow precise control of the cellular microenvironment [30]. Microfluidic assays can also be readily adapted for live cell imaging to reveal tumor cell migration dynamics, in response to critical factors in the tumor microenvironment, such as chemokine gradients [31], interstitial flow [32], electrical fields [33] and paracrine and juxtacrine interactions with other cell types [34, 35]. Cell seeding in microfluidic platforms is performed by loading cells suspended in fluid or hydrogel solution, and cell motility is monitored after establishment of chemokine gradient or flow conditions. Microfluidic platforms enable the accurate control of these microenvironmental factors, allowing the establishment of well-defined

combinatorial conditions to dissect the interplay of mechanical and chemical signals. Small sample volumes and low cell numbers in microfluidic assays pose challenges for traditional biochemical assays, and often data analysis in microfluidic migration assays is performed using live cell imaging and immunofluorescence staining for proteins of interest.

### Macroscale models

Mesenchymal cells in their intrinsic environment interact with a 3D ECM, characterized by physical parameters including pore size and stiffness [36] and chemical parameters including adhesion site density and bound ligand concentration. The development of novel biomaterials has enabled the creation of 3D environments that provide control of both the chemical and physical parameters of the ECM to study the effects of these factors on cell migration [37]. Hydrogel matrices, such as collagen type I, Matrigel and synthetic matrices have been used to investigate how ECM physical properties (e.g stiffness [38]), and tumor cell proteolytic activity [39] influences tumor cell invasion. In these assays, tumor cells are uniformly seeded inside a homogeneous 3D ECM and their migration characteristics are monitored in real-time or after a given time period [39]. These are termed “macroscale migration assays” because of the inability to control biochemical and biophysical factors at the single cell level compared to microfluidic- or micropatterned-based assays. Elegant methods have also been developed to investigate the effects of stiffness gradients (durotaxis) on cell migration [40].

### Micropatterned models

Integration of microfabrication technology with ECM patterning has enabled the creation of 1D, 2D or 3D micropatterned substrates to selectively define the topography for culturing single or multiple cell types under well-defined cell positioning [41]. Micropatterned substrates can be fabricated using different techniques, such as microstencil stamping [42] or optical patterning [43]. Cell seeding in micropatterned assays is achieved in a similar way as microfluidic platforms, where cells are positioned in different areas of the device by pipetting cell suspensions. Interestingly, advanced micropatterning methods have also been developed that allow for active control of the substrates and impose tunable constraints on cell migration [44]. Doyle *et al.* developed an assay to investigate tumor cell migration along 1-D paths [43] and Irimia *et al.* studied the effect of physical geometrical constraints on tumor cell migration persistence [45]. These technologies can recreate a more physiologically relevant tumor microenvironment, including multiple cell types [46], mimicking tumor cell migration along fibers in the tumor stroma and invasion in 3D [47]. Although these micropatterned assays offer scalability and the capability to culture large cell numbers, they do not typically include fluid flow, and hence the application of chemical gradients and localized mechanical (e.g. fluid shear stress) stimuli to cell subpopulations is generally not possible.

### Tumor cell migration assays in vivo

Intravital imaging of live animals at sufficient resolution to identify individual tumor cells has allowed monitoring tumor cell migration in the native tumor microenvironment *in vivo* [48, 49]. In these studies, human tumor cells are transplanted into different model organisms such as mouse [50], zebrafish [51] and chicken embryo [52], or alternatively genetically engineered mouse tumor models incorporating constitutively expressed fluorophores [53, 54] are employed. Intravital studies using multiphoton microscopy enable deep imaging (~mm) into the tissue and also allow for second harmonic imaging to visualize ECM fibers [53]. Wyckoff *et al.* have developed an *in vivo* chemotaxis migration assay to be used in combination with monitoring tumor cell motility [55], which allows for the collection of the tumor cells as they migrate inside a cytokine-filled needle.

## Computational Modeling of Tumors

Computational methods have been developed to augment experiments and gain deeper insight into tumor development. Cancer progression has been modeled with a variety of approaches (Figure 3), ranging in length scale from microscopic and discrete models that consider individual cells, to macroscopic models that treat the tumor mass as a continuum (reviewed: [56]). Such computational models provide insight into biological processes and guide experimental development. For example, Baxter and Jain demonstrated that interstitial flow velocity varies significantly for constant interstitial fluid pressure when the ratio of transvascular to interstitial resistance is varied [57]. Interstitial fluid pressure can be measured experimentally, while flow velocity is more difficult to assay. However, flow is a strong regulator of drug delivery, so these computational models provide insight into the barriers to drug delivery and inform more effective cancer treatment [58]. Although these models have provided valuable insight into solid tumor progression, the treatment of a tumor as a continuum does not allow explicit consideration of single cell behavior or the effectors that regulate the behavior of individual tumor cells.

To improve on continuum models, Jeon *et al.* developed a hybrid discrete-continuum model that implemented a continuum sub-model for chemical transport within the tumor and a discrete sub-model for individual cell behavior such as cell adhesion strength (Figure 3D) [59]. Because the hybrid model explicitly considers cell-cell adhesion sites, the model produced realistic tumor morphologies that could not be generated by previous continuum models. Several studies have also implemented a force-based, microscale approach to effectively model the biphasic haptotactic and haptokinetic migratory response for tumor cells in 3D matrices, and these models have provided insight into the parameter space of adhesion and ECM stiffness that governs 3D migration speed [60], [61]. Although these models establish a framework for studying cell migration through 3D matrices, they are limited by certain assumptions such as fixed values for matrix density and cell adhesivity to the matrix. Furthermore, the model described by Zaman *et al.* lacked consideration of soluble ligand fields and thus could not account for chemotaxis. Anderson *et al.* developed an agent-based model that predicts clonal evolution of a tumor as a function of the microenvironment properties [62]. Such agent-based models have the advantages of simulating multiple interacting cells, but require the input of numerous parameters or assumptions, not all of which can be precisely or independently determined.

Subcellular models provide insight into mechanical and cellular processes that are difficult to measure experimentally. The subcellular element model successfully captures cell-level mechanical properties of cells by modeling the cell as a subset of nodes whose interaction is governed by mechanical potentials between the nodes (Figure 3B) [63]; however, this model lacks chemical reaction kinetics and cannot capture signal transduction. On the other hand, chemical reaction models provide insight into signal transduction and have long been used to predict the effect of a drug on cell function (Figure 3A) [64], but these models lack the mechanical consideration to adequately capture cell migration.

Cell migration is inherently a multi-scale process, involving both mechanical and chemical signal integration, and coarse-graining provides a technique for modeling multi-scale phenomena. Contact guidance in migrating cell populations has been effectively modeled by treating the cells as a continuum, where the continuum properties are derived from single cell migration models [65]. To further improve this multi-scale approach, Ramis-Conde *et al.* developed a hybrid discrete-continuum model and explicitly considered cell signaling in the discretely modeled cells (Figure 3C). The signaling model allowed dynamic modulation of cell-cell adhesion properties, which allowed simulation of intravasation [66]. However, by considering signaling on a whole-cell level, the authors could not capture localized

signaling events or simulate the effects of signaling gradients, which are critical in carcinogenesis and metastasis formation. Parameter estimation and assumption validation for computational models remains an issue for maintaining the physiologic relevance of the models (a list of parameters relevant to tumor cell migration is provided in Table 2), but advanced experimental platforms such as microfluidics provide resolution over parameters that are difficult to estimate with traditional assays. For example, Irimia *et al.* recently coupled their computational model for neutrophil chemotaxis to experiments performed in a microfluidic device to aid in parameter estimation and model verification [67]. Thus, while the potential for using computational models to gain insight into the complex and interactive mechanisms that regulate tumor cell migration, there remains room for new approaches that simultaneously encompass both chemical and mechanical stimuli, and are capable of bridging scales from molecular to cellular to tissue.

## Mesenchymal Migration

The experimental platforms and computational methods discussed above have provided much insight into the mechanisms by which tumor cells migrate in the surrounding stroma. In epithelial tissues, cells are tightly connected to their neighbors and form polarized cell sheets with high levels of E-cadherin expression. During the formation of metastatic lesions in carcinomas, epithelial cells undergo genetic and epigenetic changes that induce a phenotypic transition, in which cells lose E-cadherin expression [68], reducing homophilic cell-cell connections, and express vimentin [69], N-cadherin [70], and other fibroblastic markers (Reviewed: [71]). This phenotypic switch is known as the epithelial to mesenchymal transition (EMT), and results in cells of epithelial origin acquiring a phenotype more amenable to migration and invasion into the surrounding tissue. The migratory phenotype is varied and dynamic, with invading cells migrating individually or collectively and modulating the mechanism of migration with changes in the stromal properties (review of migration mechanisms: [4]).

Mesenchymal cell migration is an orchestrated process, requiring ECM remodeling, extension of protrusions through actin polymerization, formation of new adhesions, molecular motor-mediated cell contraction, and the release of adhesions at the trailing edge (Reviewed: [72], [73]). Migration through 3D matrices is termed invasion, and tumor cell invasion is mediated by invadopodia, specialized Src-kinase-dependent protrusions that are selectively observed in metastatic cells and extend into the ECM to probe the proximal chemical and mechanical microenvironment [74]. In general, cell migration is a stochastic process [75], and in the absence of an external gradient or directional cue, cells migrate randomly [76]. However, when presented with a directional cue, such as a chemokine gradient, the internal signaling machinery becomes polarized and cells migrate with directional bias [77]. Several metrics are commonly used to quantify aspects of cell migration beyond speed and direction. Detailed persistence metrics such as root-mean-squared speed, directional persistence time, and a random motility coefficient can be determined by fitting experimental mean-squared displacement data with a theoretical expression that models cell migration as a biased random walk [78]. Directionality, defined as the ratio of net migration distance to the total distance traveled for a given period of time, is a commonly used metric that is more straightforward to compute [79].

The stochasticity of cell migration and the fact that direction and speed can be modulated independently complicates the use of end-point migration assays. For example, migration with high speed and low persistence cannot be distinguished from low speed, highly persistent migration. This issue is compounded in transwell assays, where only the number of cells reaching and crossing the membrane is measured, and increased migration speed with random direction could be confused with directionally biased migration. Furthermore,

there is often significant cell-cell heterogeneity in cell migration characteristics, and migration statistics are often averaged over a cell population. These bulk, or population-averaged metrics can obscure aspects of the migratory response that may be important for understanding the response of tumor cells to various stimuli. These disadvantages can be overcome by the use of live-cell imaging in microfluidic systems. For example Haessler *et al.* used population histograms and scatter plots to identify subpopulations of cells that respond uniquely to interstitial flow, with downstream migrating cells moving with high directional persistence and upstream migrating cells moving faster but with less directional persistence, supporting the hypothesis that flow imparts simultaneous and competing stimuli to tumor cells [80] [32].

## Mechanical Signals

### ECM stiffness and density

Traditionally, extracellular stimuli to migration are divided into mechanical and chemical signals, and the ECM imparts a variety of mechanical signals to migrating cells. The relationship between the density and organization of stromal tissue and tumor progression is bilateral and dynamic, with tumor cell phenotype dictated by the stromal properties and tumor cells modifying the chemical and mechanical profile of the stroma [81]. In particular, the density and stiffness of the stromal ECM has been shown to influence the invasion of tumor cells. Mammographically dense breast tissue has long been known to be a risk factor for breast cancer [82], but recently, it was demonstrated that increased matrix density can promote invasion of tumor cells even in the absence of stromal cells [83]. Collagen crosslinking [84] and lysyl oxidase (LOX) over-expression [85] contribute to increased ECM stiffness in the tumor stroma [38], and stiffness of the ECM is a critical regulator of mammary gland function and morphology [86]. The surrounding stromal tissue in a normal mammary gland is characterized by an elastic modulus of ~200 Pa, while the elastic modulus of mammary tumor-associated stroma is ~4kPa (Table 2) [12].

The stiffness and density of the substrate are sensed by the cell through adhesion molecules, primarily integrins [87], a class of tension-sensitive transmembrane molecules that can initiate the formation of a continuous mechanical link between the ECM fibers and cellular cytoskeleton [88]. Integrins are both substrate and tension dependent [89], and activation and clustering of integrins initiates a mechanosensitive signaling cascade [90]. The mechanical strength of cell-ECM adhesions is dependent on integrin engagement [91]. Vinculin recruitment, a measure of matrix adhesion maturation and an integrin signaling component, is dependent both on externally applied force [92], and internally generated myosin-mediated force [93]. Integrins are overexpressed in cancer cells, and a drug that blocks  $\alpha_v\beta_3$ -integrins has been shown to increase apoptosis and the efficacy of radiation treatment of mice with mammary adenocarcinoma [94].

Advances in techniques to engineer substrate stiffness *in vitro* have allowed investigation of the effect of ECM stiffness on tumor cells. Similar to the mechanosensing by fibroblasts and other cell types [95], prostate tumor cells are sensitive to substrate stiffness, increasing their elastic modulus with an increase in the substrate stiffness [96]. Interestingly, breast cancer cells that overexpress the receptor tyrosine kinase ErbB2 are more sensitive to substrate stiffness [97]. Furthermore, ECM stiffening contributes to altered integrin and ERK signaling, resulting in high Rho-dependent cellular contractile stress, disrupting the breast epithelial phenotype and promoting the malignant phenotype [12].

Rho-mediated contractile stress and integrin signaling are key regulators of cell migration [98], and integrin signaling is altered in tumors (Review of integrins in cancer: [99]). Recently, a technique involving crosslinking collagen *in vitro* enabled Levental *et al.* to



demonstrate that the increased stiffness of tumor-associated stroma increases levels of  $\beta 1$  integrin and FAK<sup>Y397</sup> colocalization, and that these signals contribute to increased breast tumor cell invasion through enhanced PI3K signaling [38]. The rigidity of the substrate alters invadopodia function of breast cancer cells, with enhanced matrix degradation and more functional invadopodia per cell on stiffer substrates [100].

Cellular contractile stress generation is required for rigidity sensing [13], and interfering with myosin motor activity attenuates the effects of stiffness on migration. Myosin light chain kinase inhibitor ML-7 impedes invadopodia maturation and decreases FAK<sup>Y397</sup> and Cas<sup>Y165</sup> localization within breast carcinoma invadopodia [100], and inhibition of myosin II or Rho-associated kinase (ROCK) attenuates the ability of glioma cells to sense substrate rigidity [101]. Stiffness sensing is also cell-type specific, and the stiffness-dependent invasion of breast cancer cells into 3D matrices is dictated by the specific genetic profile of the metastatic cells. Single cell subpopulations of MDA-MB-231 have been shown to demonstrate tissue-specific metastatic lesions, tissue tropisms [102], and these subpopulations have shown varying stiffness-dependence when invading 3D tissues. For example, breast cancer cells that metastasize to bone do not show stiffness dependent invasiveness; however, cells that metastasize to the lung invade less with increasing stiffness [103].

Although most experiments to date investigate cell migration as a function of stiffness on substrates or within matrices of uniform stiffness, stiffness gradients can guide cell migration on 2D substrates [40], and in 3D [104], [105]. Furthermore, patterning stiffness on substrates has been shown to lead to self-aggregation of fibroblasts [106]. The tumor microenvironment is heterogeneous, characterized by abrupt changes in the mechanical properties of the tissue [107], and the role this mechanical heterogeneity plays in guiding cell migration has yet to be determined. However, improved resolution in measuring the mechanical properties of tumors *in vivo*, multi-scale models that address substrate and cell mechanics, and improved techniques for creating artificial ECMs with customized mechanical properties will provide insight into the relationship between stiffness gradients and cell migration.

### MMPs and matrix synthesis

As mentioned above, stiffness and stiffness gradients have been shown to influence cell migration on 2D substrates, and recently, there has been much interest in determining the role of stiffness of 3D matrices on cell invasion. In 2D, mechanical properties can be easily quantified using a variety of rheometric techniques, and these bulk mechanical properties agree well with microscale mechanical properties measured with techniques such as atomic force microscopy (Review of 2D vs. 3D for cancer cell invasion: [36]). However, 3D matrices that mimic *in vivo* ECM are fibrous and the material is structured on cellular length scales, so assessing the mechanical properties relevant to cell migration is challenging. For example, the storage modulus of a 0.5 mg/ml collagen hydrogel is less than 10 Pa [108], while the extensional modulus of a fiber is on the order of GPa [109]. The coupling of matrix ligand density, pore size, and matrix stiffness in 3D migration assays employing collagen type I hydrogels further complicates investigation of the role of mechanical properties on 3D tumor cell invasion. For example, increasing hydrogel matrix protein density increases stiffness but also decreases pore size and increases ligand density. Tumor cell invasion is regulated both by pore size and stiffness [110] and crosslinking techniques provide orthogonal control of matrix ligand density and stiffness, though pore size and stiffness remain inversely coupled [108].

Cells in matrices with subcellular pore sizes implement a combination of force and proteolytic matrix degradation to overcome the structural barrier to migration imposed by

the matrix fibers. Transformed breast carcinoma and fibrosarcoma cells apply force to collagen fibers to reorganize and reorient local matrix, while MMPs focally degrade matrix to reduce steric hindrance [111]. MMPs are key regulators of carcinogenesis, and their function extends beyond degrading matrix proteins [112]. As tumor cells secrete MMPs to degrade the interstitial matrix, this provides microtracks of weakened and digested matrix enabling migration of subsequent cells [113]. MMPs diffuse from tumor or stromal cells into the surrounding interstitium [112], and the expression, secretion, and action of MMPs couples the tumor cell chemical and mechanical microenvironment. For example, MT1-MMP expression is required for carcinoma cells to digest matrix proteins and invade into 3D matrix [114], and MT1-MMP activity is also required to activate surface-bound TGF- $\beta$  in carcinoma cells, which affects cell growth and matrix secretion [115]. Furthermore, MMP cleavage of matrix materials can release soluble cytokines that are bound to the ECM in inactive, precursor form [116], and the transport environment within the tissue can induce gradients in these soluble signals, providing directional cytokine cues to cells [117].

### Intercellular mechanical signals

Fibroblasts are stromal cells that remodel the ECM through secretion of type I, type II, and type V collagen and fibronectin and contribute to basement membrane formation through secretion of collagen type IV and laminin (Review of fibroblasts in ECM remodeling: [118]). In tumors, stromal fibroblasts acquire an activated phenotype similar to fibroblast activation in wound healing [119], with 80% of stromal fibroblasts achieving the activated phenotype in breast carcinomas [120]. Along with myofibroblasts [121], these cancer associated fibroblasts (CAFs) are characterized by increased deposition of matrix, known as desmoplasia, which contributes to the increased stiffness of tumor-associated ECM (Review of CAFs: [122]), and CAFs demonstrate altered gene expression from normal stromal fibroblasts including upregulation of genes governing ECM deposition [123]. CAFs dynamically interact with tumor cells, sensing changes to the matrix through integrin-dependent adhesions, and further regulating the ECM by modulating matrix and MMP secretion. The dynamic evolution of the tumor stroma is known as stromagenesis (Review of stromagenesis: [124]), and altered stroma is a key feature of carcinogenesis [125].

CAFs alter the mechanical landscape of the tumor stroma and contribute to increased invasiveness of carcinoma cells [126]. In addition to the desmoplasia-associated matrix stiffening, fibroblasts generate high levels of contractile stress and can thus apply tensile stresses to the surrounding ECM. Fibroblasts seeded in 3D collagen gels increase the mechanical stiffness and resistance to fracture of tissue constructs [127], and TGF $\beta$ -activated fibroblasts seeded in 3D collagen gels can induce Rho-dependent contraction of these constructs [128]. These CAF-mediated alterations to the mechanical properties of the tumor stroma promote the invasive phenotype, and fibroblasts can directly promote the invasion of carcinoma cells. Migrating fibroblasts locally degrade matrix and secrete fibronectin and tenascin-C when seeded in collagen type-I and Matrigel hybrid tissue constructs. The matrix degradation is dependent on integrin engagement and Rho- and ROCK-mediated regulation of myosin light chain activity, suggesting that cellular tension generated by the fibroblasts is necessary to degrade the matrix. These microtracks, created through matrix degradation, are sufficient to promote carcinoma cell migration, even when Rho and ROCK are blocked in the migrating tumor cells; however, the tumor cell migration requires Cdc42 and MRCK to modulate myosin light chain accumulation and activity [129]. The effects of the microtracks on tumor cell migration suggest fibroblast-mediated local mechanical and matrix architectural stimuli guide tumor cell migration, and advances in micropatterning technology and computational models for investigating pericellular matrix organization [130] will provide further insight into the mechanisms by which fibroblasts influence migration of local tumor cells.

## ECM Topography

Bulk reorganization of the tumor ECM during cancer progression promotes cell invasion, and mammary tumor explants cultured *ex vivo* within collagen hydrogels reorganize the peripheral collagen fibers [131]. In mouse models, mammary tumor stroma is characterized by structured, dense collagen type I. The collagen fibers are strained around the tumor and oriented normal to the tumor surface at the tumor margin. The aligned collagen fibers correlate with local cell invasion, and both collective and single cell invasion are observed along the radially oriented collagen fibers [131], consistent with the observation that metastatic carcinoma cells migrate in the direction of collagen fibers *in vivo* [132]. Questions remain, however, regarding the mechanism by which the ECM is altered in tumor development. For example, it remains to be seen whether dense collagen regions promote carcinogenesis and precede tumor growth or whether collagen alignment is induced by increased matrix contraction by the epithelial tumor cells and altered matrix deposition by activated fibroblasts during tumor growth. Furthermore, questions remain as to whether the radial collagen fibers are formed by invading cells at the tumor periphery or if the aligned fibers precede and promote cell invasion.

Just as fibroblasts can reorganize the stromal matrix, tumor cells can induce topographical stimuli that guide migration. ROCK-mediated epithelial cell contractility can locally deform the ECM, even in the absence of protease activity and stromal fibroblasts [133], and *in vitro*, Rho/ROCK and myosin-dependent contractility of carcinoma cells can lead to alignment of collagen fibers normal to the tumor periphery [134]. Alignment of collagen fibers has long been known to influence fibroblast migration through increased migration velocity along the fibers and suppressed migration normal to the fiber alignment direction, a mechanism known as contact guidance [135], and fibroblasts migrate rapidly along micropatterned lines of matrix [43]. Interestingly, fiber alignment promotes carcinoma cell invasion in the direction of the fibers even when Rho and ROCK activity is blocked in the migrating cells, suggesting that tension generated in the stroma could promote invasion through collagen fiber alignment [134]. These results, in conjunction with the data mentioned above from Gaggioli *et al.* on fibroblast-generated microtracks, suggest that restriction of cell-ECM adhesions, either by fiber alignment or cell confinement, provides a strong directional stimulus for tumor cell migration.

Recently, advances in experimental techniques have provided insight into the role of mechanical confinement on cell migration. Irimia and Toner developed a microfluidic platform to confine cells in channels of varying cross-sectional area, and they found that in channels with cross-sectional dimensions comparable to a cell diameter, tumor cells, including breast, lung, prostate, and colorectal adenocarcinoma, migrated rapidly with high directional persistence [45]. These channels, however, were rigid and nonporous. Recently, Ilina *et al.* implemented a two-photon laser ablation technique to generate microtracks within collagen matrices and found that not only do the microtracks guide the migration of breast cancer cells even in the absence of MMP activity, but the cells use an MMP-independent pushing mechanism to expand the track diameter during migration [136]. One limitation of these studies is that the channels were synthesized from uniform hydrogels, whereas *in vivo* fibroblasts create microtracks and secrete matrix proteins, producing spatial heterogeneity in the chemical and mechanical signals within the tissue. Advances in micropatterning techniques could provide insight into how the altered chemical, mechanical, and steric properties of the microtrack influence cell migration *in vivo*.

## Solid stress

Growth of a tumor within a confined tissue compresses the surrounding matrix and generates compressive stresses, which in combination with elevated interstitial fluid pressure

[137], contributes to high levels of solid stress within the tumor [138]. To investigate the role of the compressive stresses on tumor progression, Helmlinger *et al.* developed a technique for growing tumor spheroids within agarose gels and demonstrated that solid stress generated by the growing spheroid limited tumor growth [139]. Computational models accurately captured the experimentally observed growth curves and verified that increasing agarose density further limits growth by increasing the radial and circumferential stresses generated by growing spheroids [140]. Subsequent work has demonstrated that compressive stresses limit the growth of tumor spheroids by increasing apoptosis while decreasing proliferation, with the spatial regions of highest compressive stress corresponding to the highest rates of apoptosis [141]. High compressive stress within tumors, which can collapse intratumoral blood vessels [142], can also influence the invasive properties of cells within the tumor by creating hypoxia and high interstitial pressure. Compressive stress increased the motility, measured by the rate of migration in a wound-healing assay, of transformed and partially transformed breast carcinoma cells, while it suppressed the migration of non-malignant breast epithelial cells. The increased level of invasion was dependent on the development of leader cells [143], characterized by high directional persistence and directionality in their migration, and myosin light chain kinase inhibitor ML-7 blocked the migration of leader cells, suggesting a role for intracellular tension in the migration response to compressive stress [144].

## Chemical Signals

### Chemotaxis

Traditionally, chemical migratory stimuli, mediated primarily by soluble chemical signals, have been considered independently of mechanical stimuli. Gradients in soluble molecules can guide the migration of tumor and stromal cells and modulate cancer progression [145]. Tumor cells and stromal cells sense the local concentration of chemokines and growth factors through surface receptors, and a recent review highlights the key factors and pathways involved in chemotaxis in cancer progression [14]. Chemokine receptors are often up-regulated in cancer cells *in vivo* [146], and both tumor cells and stromal cells migrate in response to chemokine gradients. Chemokines can be secreted autologously, released from the matrix, or secreted by stromal cells, and cells respond to the magnitude of the transcellular chemokine gradient as well as the mean chemokine concentration [147].

Despite the growing body of work on the molecular mechanisms behind directional sensing and migration in cancer cells, little is known about the chemo-mechanical dynamics of chemotaxis in 3D environments. Furthermore, tumor cells secrete chemokines, and autocrine chemokine gradients can guide cell migration in the absence of an externally applied chemokine gradient [148]. Tumor cells *in vivo* are likely exposed to multiple, competing chemotactic stimuli, and it is unknown how tumor cells respond to simultaneous growth factor gradients. As discussed below, there is crosstalk and cooperation between growth factor receptors and integrins, and the mechanical environment must be considered when evaluating tumor cell chemotaxis. The advent of new, microfluidic platforms for investigating chemotaxis [149] will help to elucidate the mechanisms by which cells integrate mechanical and chemical signals *in vivo*.

### Heterotypic tumor-stromal cell paracrine signaling

Chemical signal gradients that regulate tumor cell chemotaxis can be established through secretion of soluble chemokines by non-cancerous stromal cells and diffusion through the ECM in the tumor microenvironment. This cell-cell communication through paracrine factors is critical during tumor progression and invasion [150] and involves a variety of different cell types in the tumor microenvironment, such as fibroblasts, immune and

endothelial cells [151]. Recent progress in characterization of these cells showed that stromal cells isolated from mammary tumors have altered gene expression patterns [152] compared to their normal counterparts. These stromal cells have been characterized as “cancer-associated stromal cells,” such as cancer-associated fibroblasts [122] and tumor-associated macrophages [153]. The study of these interactions *in vivo* is difficult because of the challenges with precisely dissecting the underlying molecular pathways. Traditionally transwell assays have been employed to study the migration or invasion of tumor cells in response to unidirectional conditioned medium or bi-directional signaling with stromal cells, while in recent years a large number of investigators have also developed new microsystems-based assays [28, 35, 46, 154]. Stromal-tumor cell interactions may also include juxtacrine signaling involving direct physical contact [150] and through secretion and binding of ECM molecules. Our focus here is on how tumor cell migration is regulated through stromal cell secreted factors; however, an important direction for further investigation is the mechanisms by which tumor cells modulate stromal cell phenotypes and how these changes can subsequently result in feedback loops further promoting tumor cell invasion.

### Tumor-macrophage and tumor-fibroblast interactions

*In vivo* studies utilizing a microneedle chemotaxis invasion assay and multiphoton imaging showed that carcinoma cells and macrophages interact via a paracrine loop involving colony stimulator factor 1 (CSF-1) secreted by the tumor cells to signal EGF secretion by the macrophages leading to tumor cell chemotaxis. Involvement of this paracrine loop in promoting tumor cell invasion has also been demonstrated *in vitro* through studies of macrophage-tumor cell signaling [155]. De Wever *et al.* demonstrated that myofibroblasts isolated from cancer stroma, or TGF- $\beta$  transformed fibroblasts induce proinvasive activity in colon cancer cells through production of the extracellular matrix glycoprotein tenascin-C and secretion of scatter factor/hepatocyte growth factor (HGF) [156]. In their study, however, they did not address the question of whether physical contact between the two cell types was required to establish the proinvasive effects on cancer cells. A microfluidic model incorporating 3D co-culture of carcinoma cells and fibroblasts was utilized to demonstrate that cancer cell invasion was upregulated by cancer-associated fibroblasts compared to their normal counterparts [157]. Sung *et al.* developed a microfluidic 3D compartmentalized system of co-cultured epithelial cells and fibroblasts to study the transition from a localized carcinoma into an invasive carcinoma [154]. By modulating the intercellular distance between the two cell types, the authors demonstrated that morphology of the mammary clusters changes with direct contact between the two cell types, leading to a more invasive phenotype with long protrusions.

### Tumor-endothelial cell interactions

Using a transwell-based co-culture model of human glioblastoma and microvascular endothelial cells Kenig *et al.* demonstrated that endothelial cells increased the invasiveness of cancer cells. Enhanced tumor cell invasion was driven by endothelial cell SDF-1 expression resulting in increased expression of MMP-9 by the tumor cells [17]. Kaji *et al.* used a micropatterned system to co-culture HeLa and HUVEC cells, which included fluid flow control to direct transport of soluble factors from the tumor to the endothelial cells or vice-versa. Interestingly, tumor conditioned medium resulted in retraction of the endothelial monolayer, while endothelial conditioned medium did not influence the migration of HeLa cells [46]. Recently, Franses *et al.* investigated the role of endothelial cells in tumor cell proliferation and invasion, and the authors showed that dysfunctional endothelial cells, which expressed lower levels of perlecan, secreted higher levels of interleukin-6 (IL-6), resulting in increased invasion of human breast and lung carcinoma cell lines [158]. Apart from blood vessel endothelial cells, lymphatic endothelial cells have also been implicated in

cancer metastasis. Using a co-culture transwell assay, Issa *et al.* [159] demonstrated that lymphatic endothelial cells promoted melanoma cell proteolytic activity and motility via a paracrine loop involving tumor cell secreted VEGF-C and lymphatic endothelial cell secreted CCL21.

### Interactions of tumor cells with multiple stromal cell types

Although most studies have investigated the interaction between tumor cells and a single stromal cell type, recent experiments employed three different cell types to mimic more closely the tumor microenvironment. Interestingly different stromal cell types may also communicate directly via paracrine signals and secrete factors that inhibit or promote tumor cell migration factors. Hsu *et al.* employed a microfluidic model with three chambers to demonstrate that macrophage-conditioned medium reduced the ability of myofibroblasts to promote lung adenocarcinoma cell migration [34]. Walter-Yohrling *et al.* showed that tumor cells of different origin, including breast, ovarian, prostate, and lung carcinoma, have positive or negative effects on the induction of endothelial/myofibroblast invasion in a tri-culture organotypic assay [160]. Clearly, multiple cell type interactions are implicated in tumor cell invasion, and further studies are needed that incorporate a greater variety of tissue-specific cells to better mimic the *in vivo* microenvironment.

## Chemo-mechanical crosstalk

### Interstitial flow

Interstitial fluid provides a transport medium for nutrients and signaling molecules [161, 162]. Jain and Baxter identified that elevated interstitial fluid pressure (IFP) within the tumor interstitium inhibits drug delivery to tumor tissue [163], and subsequent work established a mathematical framework for determining the critical parameters that govern transport of soluble species from the vasculature into neoplastic tissue [57]. High blood vessel permeability, elevated oncotic pressure [164], and abnormal lymphatic vessels contribute to high IFP within the tumor, and high intratumoral pressure has been correlated with poor prognosis *in vivo* [165]. IFP within tumors can be nearly as high as the microvascular pressure (up to 30 mmHg [137]), while IFP in normal tissue is close to zero [166], and this difference in pressure between normal and neoplastic tissue leads to high IFP pressure gradients at the tumor margin. IFP gradients drive convection of interstitial fluid, and Chary and Jain determined typical interstitial flow (IF) speeds to be on the order of 0.1–2.0  $\mu\text{m/s}$  [167]. The magnitude of IF velocity depends on the magnitude of the IFP gradient and the hydraulic conductivity of the tumor (Table 2), and subsequent work has indicated that the velocity can reach up to 4.0  $\mu\text{m/s}$  [168]. Computational models demonstrate that IF velocity is a function of the ratio of interstitial to vascular flow resistance [57], and when the resistance to IF transport is much greater than resistance to vascular transport, IF velocity at the tumor periphery can reach as high as 10.0  $\mu\text{m/s}$  [166].

The convection of soluble signals by interstitial flow provides a host of migration signals to invading tumor cells by transporting both autocrine and paracrine chemokines (Review of IF and effects on tumor cell invasion: [169]). In recent work by Shields *et al.*, IF was found to increase the metastatic potential of breast cancer and melanoma tumor cells through binding of self-secreted chemoattractant via the CCR7 receptor [170]. This autocrine signaling, termed “autologous chemotaxis,” arises when IF transports secreted ligand downstream, and the balance between convection and diffusion establishes a Peclet number-dependent pericellular gradient, which provides a chemotactic signal in the downstream direction [117]. IF also influences fibroblast function, promoting matrix reorganization, differentiation into myofibroblast phenotype [171], and fibroblast migration [172], and these

mechanisms prime the stromal environment through MMP secretion and Rho-dependent matrix contraction to promote tumor cell invasion [173].

Computational models have helped elucidate the effects of IF on the mechanical microenvironment, and the simulations demonstrate that IF applies stress to matrix fibers, which can be sensed by the cell through matrix adhesions [174], and IF imparts shear stress on the cell membrane, the magnitude of which is determined by the arrangement of matrix fibers [175]. Recent work demonstrates that when the CCR7 receptors are blocked, breast cancer cells migrate in the upstream direction, and the data suggest that the upstream stimulus is mechanically-mediated. This flow mechanotaxis arises primarily from pressure gradients across the cell resulting in a net force on the cell in the flow direction, which is balanced by a net force on matrix adhesions in the upstream direction (Figure 4) [32]. Originally, flow mechanotaxis was thought to be mediated by tension in integrins, supported by the increase FAK<sup>Y397</sup> in cells exposed to flow, but recently a potential role for the glycocalyx has been identified in the cellular response to interstitial flow. Interstitial flow increases the migration of vascular smooth muscle cells through increased MMP13 expression, mediated by heparan sulfate proteoglycans [176]. Although this work was performed with vascular smooth muscle cells, syndecan expression in breast cancer has been demonstrated [177], though it remains to be seen whether proteoglycans play a role in the tumor cell response to interstitial flow.

Although the details of the mechanism guiding cells to migrate upstream has yet to be verified, recent evidence supports the hypothesis that IF imparts competing stimuli on migrating breast cancer cells [80]. The flow-induced expression of MMPs in fibroblasts [172], further complicates investigation of IF as a migration cue as the MMPs degrade the matrix, altering the flow field and pressure distribution around the cell. In glioma cells, fluid stress activates MMP1 [178], and secreted soluble MMPs could be convected downstream, degrading matrix on the downstream side of the cell, further activating adhesion molecules on the upstream side, though that remains to be demonstrated.

### Focal adhesion kinase and Src kinase as mediators for crosstalk

IF applies chemical and mechanical stimuli to cells, but the signals are further coupled intracellularly through shared signaling pathways from chemical and mechanical receptors (Figure 4). Focal adhesion kinase (FAK) is an important regulator of tumor progression, with elevated FAK expression found in invasive human cancers [179], and FAK has been identified as a potential target for cancer therapeutics [180]. Autophosphorylation of FAK at Y397 requires the cell to be bound to a rigid substrate, while clustering of integrins can induce phosphorylation at other sites in FAK [181], and autophosphorylation at Y397 increases linearly with stiffness of the substrate [91]. FAK<sup>pY397</sup> increases the affinity of FAK for the Src homology 2 domain of many proteins, including Src kinase [182], which can further phosphorylate FAK at other tyrosines, resulting in full activation of FAK enzymatic activity [183].

FAK further regulates downstream signals crucial to cell migration, and FAK<sup>pY397</sup> is required for cell motility [184], [185]; however, the role of FAK in 2D cell migration and 3D invasion seems to be different. Expression of v-Src restores 2D migration defects in FAK<sup>-/-</sup> fibroblasts, but FAK expression and the formation of a FAK-Src signaling complex was required for 3D cell invasion [186]. The discrepancy between 2D and 3D is presumably due to the fact that the FAK-Src-p130<sup>cas</sup> signaling construct leads to activation of MMP-2 and MMP-9 and matrix degradation for invasion [187]. Src further contributes to mechanotransduction through selective modulation of integrin-dependent traction forces [188].

FAK and Src are also key regulators of chemokine-induced migration. FAK can be transphosphorylated by activated EGFR, and FAK function is required for EGF-stimulated chemotaxis and motility [184]. Src can also be directly phosphorylated by EGFR [187], and as FAK is recruited to sites of integrin clustering and is required for growth factor-stimulated motility, Src is likely involved in the cooperativity or crosstalk between growth factor and integrin signaling [184]. The intracellular coupling of chemical and mechanical stimuli through FAK/Src complicates cell migration assays because testing for phosphorylation of FAK, Src, or downstream components does not distinguish between chemical and mechanical activation. Thus, assays that allow independent modulation of chemical and mechanical stimuli, or assays for further upstream signals, such as vinculin, are required to test the mechanism of activation within the cell.

### Growth factor and integrin crosstalk

Integrins regulate the activity and downstream signaling of receptor protein tyrosine kinases (RPTKs) such as EGFR, coupling adhesion and growth factor signaling [189]. Matrix adhesion induces the formation of integrin-EGFR macromolecular signaling complexes [190], and integrin clustering can induce phosphorylation and activation of EGFR and downstream signals (Review of integrin and growth factor cooperation: [191]). The association of integrins and growth factor receptors introduces crosstalk between adhesion and growth factor pathways, and this crosstalk necessitates consideration of the ECM when investigating chemokines and vice versa (Figure 4). For example, when breast cells are cultured in 3D, blocking of  $\beta_1$  integrin or EGFR reduces expression of both receptors, restoring malignant breast cancer cells to the normal epithelial phenotype, an effect not seen when the cells are cultured on 2D substrates [192]. Integrins can also act to amplify growth factor signals influencing chemokine-induced invasion. In carcinoma cells  $\alpha_6\beta_4$  integrin associates with and is phosphorylated by the HGF specific receptor tyrosine kinase c-Met, and functional  $\alpha_6\beta_4$  integrin is required for HGF-dependent invasion [193]. The crosstalk between integrins and growth factor receptors results in coupling between the chemical and mechanical microenvironments, and recently it has been shown that stiffening of the ECM can increase the sensitivity of epithelial cells to EGF [194].

This relationship between integrins and growth factor receptors is bi-directional, and growth factor activation can influence integrin function. In prostate carcinoma cells, binding of the chemokine CXCL12 to its receptor CXCR4 increases adhesion to the ECM through  $\alpha_5$  and  $\beta_3$  integrin activation [195]. Recent work demonstrated that  $\alpha_5\beta_1$  integrin binds to and activates c-Met, which initiates Src/FAK signaling and promotes migration in ovarian cancer cells. Blocking  $\alpha_5\beta_1$  integrin or c-Met decreases Src/FAK activation and cell invasion *in vitro* and inhibits metastasis formation *in vivo*; however, activation of c-Met by soluble HGF can restore the ovarian cancer invasive potential even when  $\alpha_5\beta_1$  integrin is blocked. Importantly, the integrin-mediated activation of c-Met was substrate-specific, and c-Met phosphorylation by integrin was not observed on collagen substrates [196].

### Integrin and cadherin crosstalk

ECM adhesion-growth factor crosstalk is further complicated by crosstalk between cell-ECM and cell-cell adhesion molecules. A variety of mechanisms contribute to matrix and intercellular adhesion crosstalk (Review of adhesion crosstalk: [197]), including feedback in which integrin activation alters cadherin expression and vice versa. The Src/FAK signaling complex is at the heart of this crosstalk [198], and c-Src suppression in breast cancer cells results in E-cadherin upregulation, reversion of the mesenchymal phenotype to an epithelial phenotype, and reduced cell migration [199]. Using a dual-micropipette technique, Martinez-Rico and colleagues demonstrated that the force required to separate cell doublets attached by E-cadherin mediated adhesion was dependent on specific integrin engagement,



and that cells, including carcinoma cells, attached to fibronectin-coated beads required a larger force to separate cell doublets than cells attached to vitronectin- or polylysine-coated beads [200]. Patterning of ECM ligands further allows the regulation of cell-cell junction morphology and force distribution [201]. Recently, Borghi *et al.* implemented a reductionist micropatterning technique to investigate crosstalk of cadherin- and integrin-mediated adhesions in governing epithelial cell motility. The micropatterning technique allowed cells to simultaneously adhere to collagen IV and the extracellular domain of E-cadherin. Micropatterning directed traction force and migration parallel to the major axis of areas printed with collagen IV. Notably, the presence of E-cadherin stripes decreased lamellipodia activity but did not affect migration rate. Depletion of  $\alpha$ E-catenin within the epithelial cells increased lamellipodia activity, migration rate, and decreased migration coordination among epithelial cell sheets [202]. These results suggest E-cadherin engagement influences the direction of migration but not migration rate, consistent with the results of Abercrombie and Haynes who originally postulated contact inhibition in fibroblasts [203]. Interestingly, contact inhibition is lost in tumor cells [204], and recently, it has been shown that matrix stiffening results in the loss of contact inhibition and acquisition of a malignant phenotype [194].

## Conclusions and Future directions

In this review, we provide an overview of recent experimental studies that investigate the effects of different factors present in the tumor microenvironment on tumor cell migration. These experimental approaches have provided great insight into the factors that guide cell migration *in vivo*, but the experimental platforms often trade precision for physiologic relevance. Computational models have been developed to supplement experimental data, but cell migration is inherently a multi-scale process, and models developed thus far lack the capability of simulating cancer progression and cell migration at various length scales. Mechanical stimuli such as ECM topography, ECM stiffness, pore size, solid stress, and matrix contraction, and chemical stimuli from autocrine and paracrine sources influence the direction and speed of cell migration. These various signals signal simultaneously to the tumor cells, and tumor cell migration is an integrated response to multiple signals. Despite significant progress in unraveling the underlying molecular mechanisms and signaling pathways for transducing individual stimuli, the crosstalk between these different factors in the tumor microenvironment at the extracellular and intracellular levels remains largely unknown.

Integrating computational and experimental approaches will allow for a more detailed understanding of these mechanisms and their interplay, and this strategy has proven to be successful regarding the treatment of pancreatic ductal adenocarcinoma. Computational modeling has demonstrated that IFP acts as a barrier for drug delivery to tumors [57], and these models suggest that altering vascular and interstitial permeability could increase the efficacy of anti-tumor therapy [166]. *In vitro* experiments, coupled with *in vivo* data, have further demonstrated that IFP and IF alter the migration properties of migrating tumor cells and suggest that IF might increase the formation of metastases [170], [32]. Recently, Provenzano *et al.* demonstrated that by enzymatically degrading matrix elements in the tumor stroma, IFP is significantly reduced within the tumor allowing increased transport of small molecule therapeutic drug, which resulted in increased mouse survival and decreased formation of metastases [205]. Furthermore, a growing body of work, motivated in-part by computational models, suggests that targeting the tumor microenvironment in combination with anti-angiogenic therapy, increases drug transport and improves cancer therapy [58].

To this end, development of the next generation functional migration assays that enable integration of spatiotemporal control of mechanical and chemical signals with high-

resolution, real-time imaging and the ability to conduct biochemical assays is crucial. The investigation of tumor cell migration at the single cell level will allow investigation of the diverse migration strategies employed by individual tumor cells inside the complex and heterogeneous tumor microenvironment. Progress in this direction will help to develop more accurate diagnostic techniques to quantify and predict cell migration in patients, thus informing methods for constraining tumor cell dissemination and halting metastasis formation.

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## Abbreviations

<b>ECM</b>	extracellular matrix
<b>EMT</b>	epithelial to mesenchymal transition
<b>EGF</b>	epidermal growth factor
<b>LOX</b>	lysyl oxidase
<b>FAK</b>	focal adhesion kinase
<b>ROCK</b>	Rho-associated kinase
<b>MMP</b>	matrix metalloproteinase
<b>CAF</b>	cancer associated fibroblast
<b>LEGI</b>	local excitation, global inhibition
<b>LIMK1</b>	LIM domain kinase 1
<b>RPTK</b>	receptor protein kinase
<b>IF</b>	interstitial flow
<b>IFP</b>	interstitial fluid pressure

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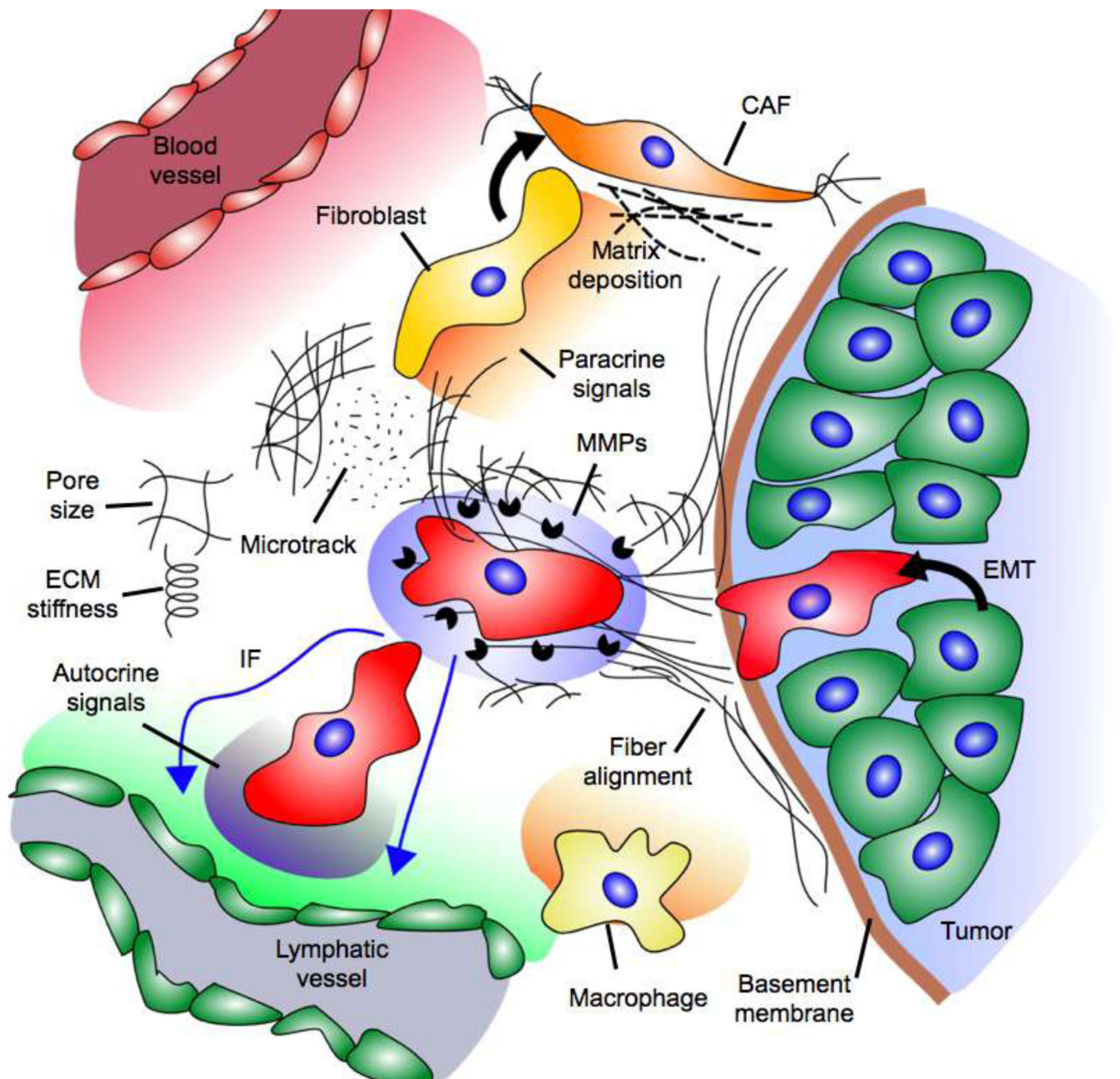


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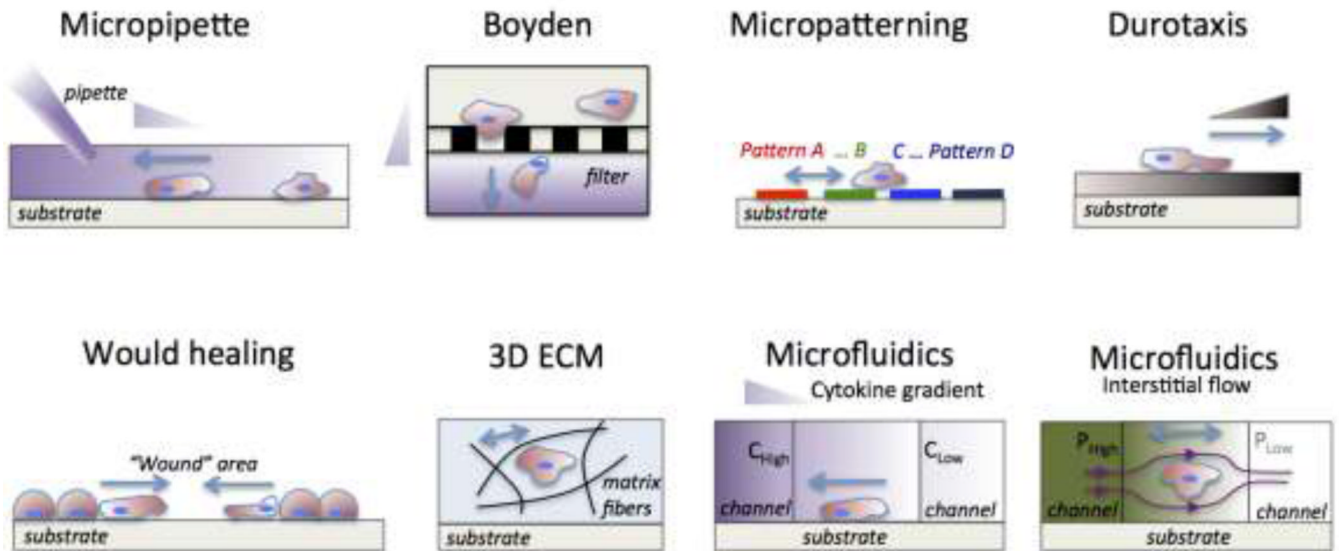
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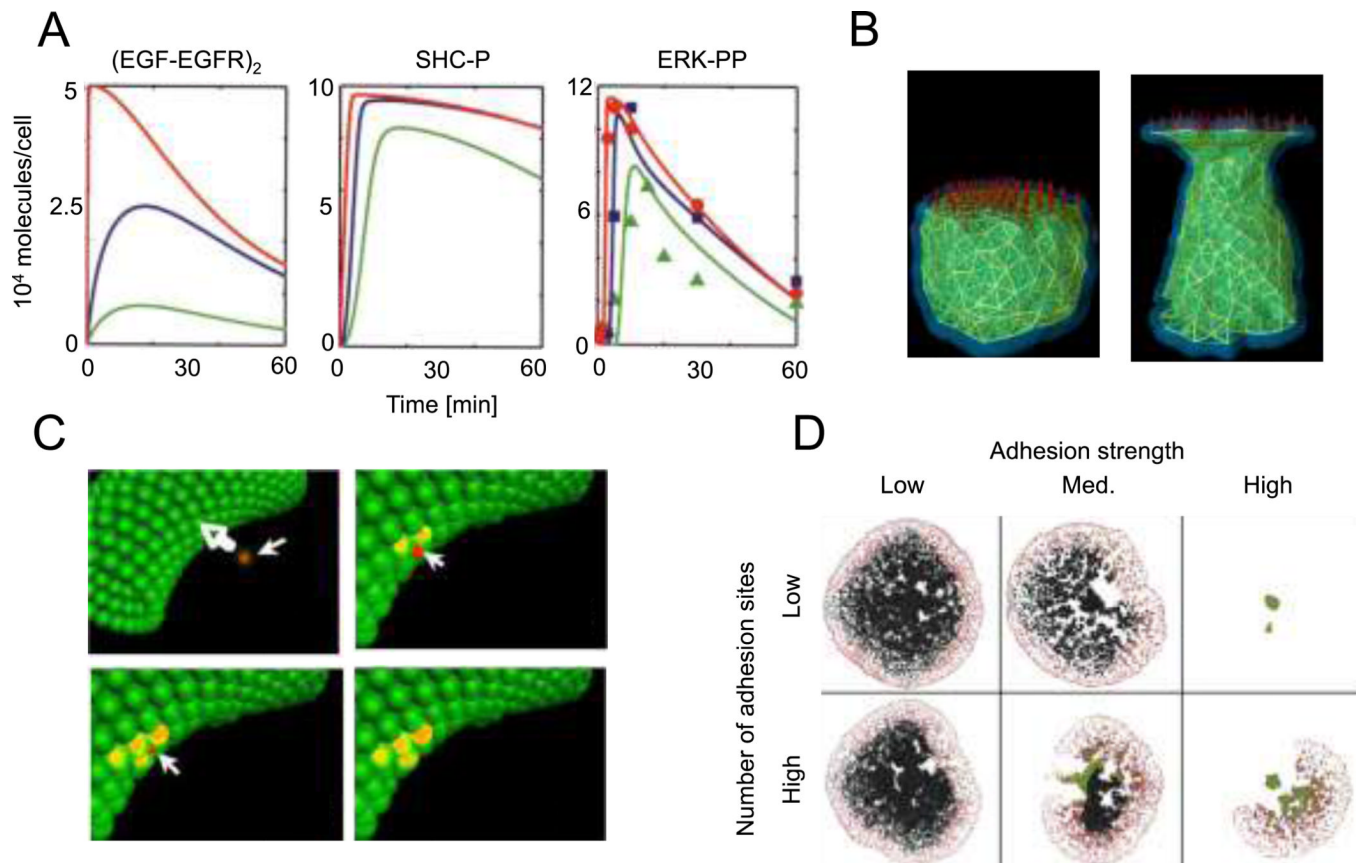
**Figure 1.**

A host of biochemical and biophysical factors influence the migration of tumor cells. Mechanical signals include stiffness of the ECM, the pore size of the ECM, solid stress, fiber alignment, and fibroblast generated matrix tension and microtracks. Fibroblasts are activated to assume the cancer associated fibroblast (CAF) phenotype, and these cells secrete altered matrix components and generate tension. Chemical signals include autocrine gradients, MMPs, oxygen tension, and paracrine signals from the vasculature, lymphatics, and stromal cells.

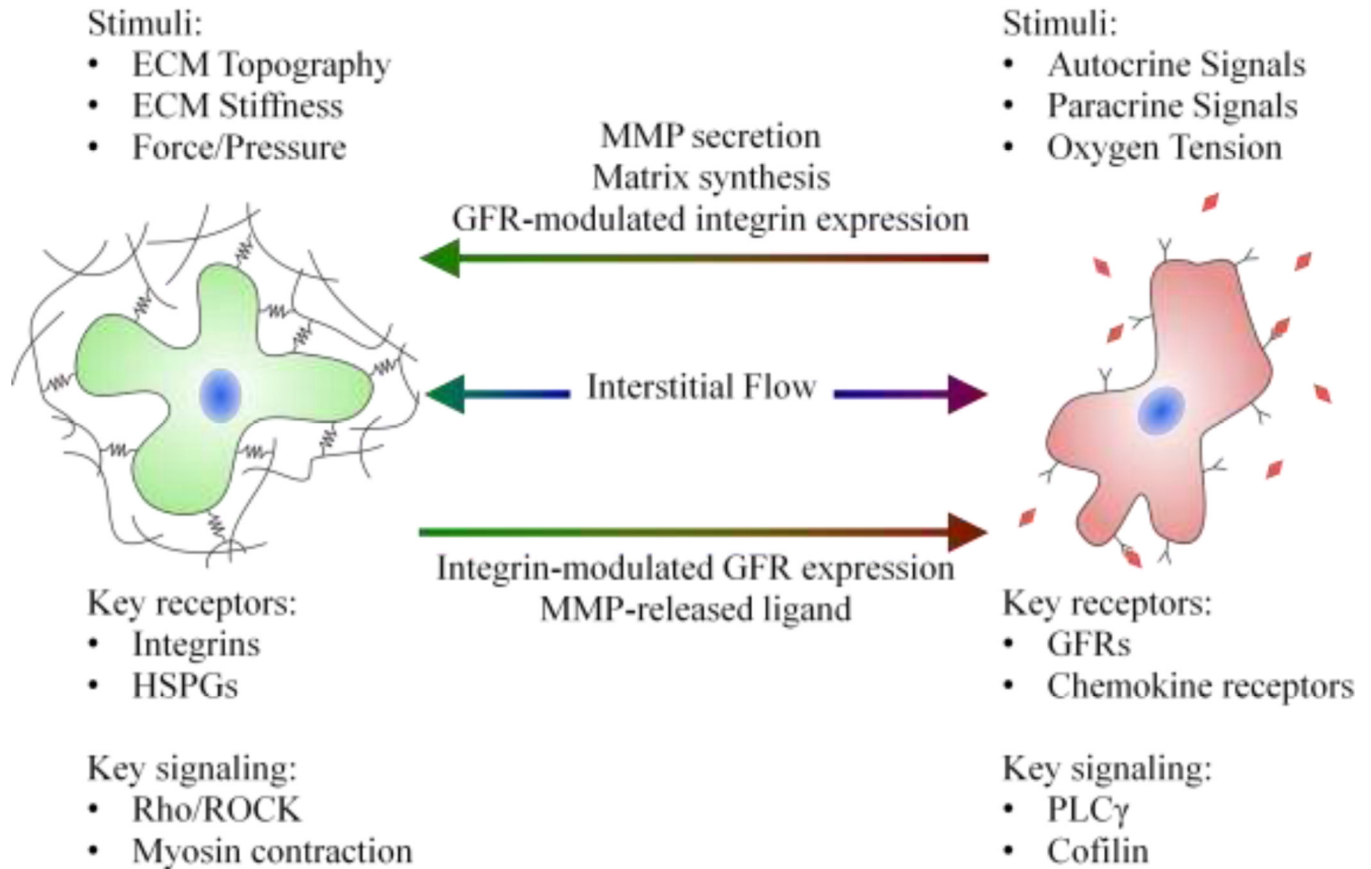


**Figure 2.**

Experimental methods for investigating factors that influence tumor cell migration *In vitro* tumor cell migration assays (reviewed in Table 1). **Micropipette assay** [21]: a pipette is placed in the vicinity of the cell and a chemoattractant solution is injected into the culture medium establishing a growth-factor gradient. **Boyden** [17] (or **transwell**) **chamber**: Cells are seeded in suspension in the top chamber and migrate through the porous filter (black rectangles) in response to a chemokine gradient, which is established by the different culture medium concentrations in the top and bottom chambers. **Micropatterning** [29]: Cells are seeded on patterns of different geometry, size and surface coatings and their migration characteristics are monitored. **Durotaxis** [40]: Cells are seeded on a substrate of variable stiffness and respond by changing traction forces, cell spread area and migration direction. **Wound healing** [26]: A “wound” is formed on a confluent tumor monolayer, and the wound closure dynamics are monitored. **3D ECM** [39]: Cells are seeded inside the 3D ECM and migrate depending on the ECM architecture (stiffness, pore size and ligand concentration); ECM fibers are outlined with black curved lines. **Microfluidics** [28, 32, 147, 206]: Cytokine gradients can be established in a 3D matrix by flowing different chemokine concentration ( $C_{HIGH} - C_{LOW}$ ) solutions in the left and right microchannels; Interstitial flow can be established by adjusting the hydrostatic pressure ( $P_{HIGH} - P_{LOW}$ ) in the left and right microchannels; streamlines are indicated with dark magenta lines. Micropipette, Boyden chamber and microfluidics assays enable control of biochemical gradients. Durotaxis, 3D ECM and microfluidics assays enable control of biophysical forces (ECM stiffness and interstitial flow). Wound healing and micropatterning assay enable control of intercellular distances, whereas only micropatterning assays enable control of substrate topography. Growth-factor gradients are indicated by the purple triangles. ECM stiffness gradients are indicated by the dark brown triangle. Blue arrows indicate direction of tumor cell migration.



**Figure 3.** Computational methods provide insight into tumor progression. A) Cell signaling models capture the dynamics of protein activation and are useful in identifying key signaling molecules and events, such as the role of EGFR concentration on ERK-1/2 phosphorylation. Colored lines represent different concentrations of EGF (red, blue, and green are 50 ng/ml, 0.5 ng/ml, and 0.125 ng/ml EGF, respectively). Symbols in panel 3 represent experimental data, demonstrating that the model well-captures the dynamics of EGF-induced ERK-1/2 phosphorylation (adapted from [64]) B) The subcellular element model recapitulates cell-level phenomena by discretizing the cell into a series of nodes and defining mechanical potentials that govern the interaction of the nodes. The panels demonstrate elongation of a cell under 1 nN of tensile force; the filaments indicate interactions between nodes, which lie at the intersection of the filaments (adapted from [63]) C) A multi-scale agent-based model captures the dynamics of tumor intravasation. The subset of models that contributed to the overall multi-scale model was comprised of a simplified set of differential equations for intracellular adhesion signaling, a modified Hertz model for intercellular adhesion mechanics, and the Langevin equation for multi-cellular interactions. The panels demonstrate a tumor cell (red) approaching the endothelium (green), forming nascent N-cadherin bonds (yellow), disrupting endothelial cell VE-cadherin bonds, and traversing the endothelium (adapted from [66]) D) An off-lattice hybrid discrete-continuum approach modeled the growth of a whole tumor and invasion at the tumor periphery. The model was comprised of a continuum model for nutrient transport, and a discrete model for single cell behavior such as motility, adhesion, and proliferation. The model demonstrated that single cell adhesion plays a role in determining the fate and morphology of the tumor mass (adapted from [59]).

MECHANICALCHEMICAL**Figure 4.**

Chemical factors influence the cellular response to mechanical factors and vice versa. MMP and matrix secretion modulates the stiffness and pore size of the surrounding matrix, while GFR activation influences integrin expression and activation. MMP-mediated matrix degradation releases chemical signals, while integrin activation and clustering can alter GFR expression and activation. GFRs and integrins can form macromolecular complexes, and elements of the intracellular signaling pathway are shared. Interstitial flow induces mechanical signals through fluid shear and pressure stresses, while simultaneously inducing chemical signals through convection of autocrine and paracrine signaling factors.



**Table 1**

Comparison of *in vitro* experimental approaches to study tumor cell migration

Assay	Key advantages / disadvantages	Applications	Important parameters that can be controlled	Implementation guidelines	References
Transwell system or Boyden chamber	<ul style="list-style-type: none"> <li>+ High throughput</li> <li>+ Easy to use</li> <li>- Live imaging</li> <li>- Gradient control</li> <li>- Cell population</li> </ul>	<ul style="list-style-type: none"> <li>• Single and collective cell migration</li> <li>• Co-culture of 2 cell types</li> </ul>	<ul style="list-style-type: none"> <li>• Pore filter and protein coating characteristics,</li> <li>• Chemoattractant to stimulate chemotaxis</li> </ul>	<ul style="list-style-type: none"> <li>• No special equipment (commercially available, e.g. Corning, BD Biosciences)</li> </ul>	[17]
Wound healing	<ul style="list-style-type: none"> <li>+ High throughput</li> <li>+ Live imaging</li> <li>- 2D substrate</li> <li>- gradient control requires modifications</li> </ul>	<ul style="list-style-type: none"> <li>• Collective cell migration</li> </ul>	<ul style="list-style-type: none"> <li>• Wound area</li> <li>• Substrate coating</li> </ul>	<ul style="list-style-type: none"> <li>• No special equipment (commercially available, e.g. Essen BioScience)</li> </ul>	[26]
Durotaxis assay	<ul style="list-style-type: none"> <li>+ Live imaging</li> <li>- 2D substrate</li> </ul>	<ul style="list-style-type: none"> <li>• ECM control</li> </ul>	<ul style="list-style-type: none"> <li>• ECM coating</li> <li>• ECM stiffness</li> </ul>	<ul style="list-style-type: none"> <li>• Polyacrylamide substrates (Bio-Rad)</li> </ul>	[40]
Micropipette	<ul style="list-style-type: none"> <li>+ Local stimulation</li> <li>+ Live imaging</li> <li>- 2D substrate</li> <li>- low throughput</li> <li>- temporal gradient decay</li> </ul>	<ul style="list-style-type: none"> <li>• Chemotaxis</li> <li>• Axon guidance</li> </ul>	<ul style="list-style-type: none"> <li>• Injection parameters</li> <li>• Substrate coating</li> </ul>	<ul style="list-style-type: none"> <li>• Special equipment (micromanipulator, e.g. Narishige)</li> </ul>	[21]
3D ECM	<ul style="list-style-type: none"> <li>+ Cell-ECM signaling</li> <li>- Confocal imaging</li> <li>- gradient control requires modifications</li> </ul>	<ul style="list-style-type: none"> <li>• EMT</li> <li>• Tumor spheroid</li> <li>• Proteolytic migration</li> </ul>	<ul style="list-style-type: none"> <li>• ECM concentration (pore-size, stiffness, ligand density)</li> <li>• ECM layer thickness</li> </ul>	<ul style="list-style-type: none"> <li>• Commercially available matrices (e.g. BD Biosciences)</li> </ul>	[39]

Assay	Key advantages / disadvantages	Applications	Important parameters that can be controlled	Implementation guidelines	References
Micropatterned	<ul style="list-style-type: none"> <li>+ Cell-cell signaling</li> <li>+ Easy to use</li> <li>+ Live imaging</li> <li>- low cell numbers</li> <li>- gradient control requires modifications</li> </ul>	<ul style="list-style-type: none"> <li>• ECM topography</li> <li>• Cell-cell interactions</li> </ul>	<ul style="list-style-type: none"> <li>• Pattern dimensions</li> <li>• Pattern coating</li> </ul>	<ul style="list-style-type: none"> <li>• Microstencil, custom designs require wafer master (Stanford Microfluidic Foundry) and nanoimprint lithography (Scivax)</li> </ul>	[29]
Microfluidic	<ul style="list-style-type: none"> <li>+ Fluid flow</li> <li>+ Gradients</li> <li>+ Cell-cell signaling</li> <li>+ Live imaging</li> <li>- complex to use</li> <li>- low cell number</li> <li>- biochemical assays</li> </ul>	<ul style="list-style-type: none"> <li>• Microenvironment</li> <li>• Temporal control of input signals</li> </ul>	<ul style="list-style-type: none"> <li>• Flow-rate</li> <li>• Gradients</li> </ul>	<ul style="list-style-type: none"> <li>• Custom designs require wafer master (Stanford Microfluidic Foundry)</li> <li>• Special equipment (syringe pumps: e.g. Harvard Apparatus.; plasma cleaner: e.g. Harrick Plasma)</li> <li>• Commercial microchannels (Ibidi, Millipore,)</li> </ul>	[28, 32, 147, 206]

Table 2

Approximate values for parameters governing tumor cell migration.

Parameter	Value	Notes	Source
Stiffness			
Normal stromal ECM stiffness	200 Pa	Unconfined compression with electromechanical indenter	[12]
Mammary adenocarcinoma ECM stiffness	$4 \times 10^3$ Pa	Explants of tumor tissue from MMTV- <i>Her2</i> neu, <i>Myc</i> , and <i>Ras</i> transgenic mice measured by unconfined compression	[12]
Colon adenocarcinoma stiffness	$4 \times 10^3$ Pa	Explants of tumor tissue from L5147T mouse xenografts measured by confined compression	[207]
Glioblastoma stiffness	$26 \times 10^3$ Pa	Explants of tumor tissue from U87 mouse xenografts measured by confined compression	[207]
Reconstituted basement membrane (Matrigel)	175 Pa	Unconfined compression with electromechanical indenter	[12]
Tissue culture plastic	$10^9$ Pa	Elastic modulus	[208]
Collagen gel elastic modulus	8 Pa	0.5 mg/ml collagen density, storage modulus measured by parallel plate rheology	[108]
	328 Pa	2 mg/ml measured with electromechanical indenter	[12]
Collagen fiber extensional modulus	$9 \times 10^9$ Pa	Force applied to fiber, strain measured by x-ray crystallography	[109]
Cell Stiffness	$1 \times 10^0 - 1 \times 10^3$ Pa	Highly dependent on cell type, culture substrate stiffness, and mechanical testing method. Lower stiffnesses measured by particle tracking microrheology for cells embedded within collagen gel [96]. Higher stiffnesses measured by AFM on metastatic cancer cells isolated from pleural fluids [209].	[209], [96]
Length Scales			
Basement membrane pore size <i>in vivo</i>	50–100 nm	Measured by electron microscopy	[210]
Tumor-associated matrix pore size	20–130 nm	Measured by electron microscopy of human melanoma implanted subcutaneously. Two distinct regions of matrix were observed. Bundles of aligned collagen fibrils with interfibrillar distances of 20–42 nm and bundles of poorly organized fibrils with interfibrillar distances of 75–130 nm.	[211]
	<150 nm	Herpes simplex virus (HSV, ~150 nm in diameter) did not penetrate into collagen-rich areas of human melanoma implanted into mouse skinfold chambers. Treatment with collagenase increased transport of HSV into the tumor.	[212]
Collagen gel pore size	$2 \times 10^3$ nm	4.0 mg/ml collagen gel polymerized at 37°C	[110]
	$11 \times 10^3$ nm	1.0 mg/ml collagen gel polymerized at 22°C	[110]
Tumor cell dimensions 3D	$50 \times 10^3$ nm	Major axis length of MDA-MB-231 in fibroblast derived ECM, measured from image provided by reference	[213]
Mesenchymal migrating cell microtrack	$10 - 15 \times 10^3$ nm	Diameter of defect in ECM generated by proteolytically active migration	[4]
Collagen fiber bundle diameter	$2 - 10 \times 10^3$ nm	Measured <i>in vivo</i>	[214]
Collagen fibril diameter	35 nm	Collagen type I gel polymerized at pH7 measured by emission scanning electron microscope	[215]
	75 nm	Measured by electron micrograph from <i>in vivo</i> tissue samples	[214]

Parameter	Value	Notes	Source
Transport Properties			
Chemokine and growth factor diffusivity	130–160×10 <sup>-8</sup> cm <sup>2</sup> /s	CCL5, CCL17, CCL21 in dilute solution	[117]
	10×10 <sup>-7</sup> cm <sup>2</sup> /s	EGF in dilute agarose gel	[216]
Oxygen diffusivity	5×10 <sup>-5</sup> cm <sup>2</sup> /s	In cell culture medium	[217]
Collagen gel permeability	10 <sup>-13</sup> m <sup>2</sup>	Measured in a 2 mg/ml collagen gel in a microfluidic device with a modified PIV technique	[32]
	10 <sup>-13</sup> –10 <sup>-14</sup> m <sup>2</sup>	2.5 mg/ml	[171]
Colon adenocarcinoma permeability	3.4×10 <sup>-16</sup> m <sup>2</sup>	Explants of tumor tissue from L5147T mouse xenografts measured by confined compression and assuming a poroelastic model for deformation	[207]
Glioblastoma permeability	4.8×10 <sup>-16</sup> m <sup>2</sup>	Explants of tumor tissue from U87 mouse xenografts measured by confined compression and assuming a poroelastic model for deformation	[207]
Interstitial flow speed	0.1–4.0×10 <sup>-6</sup> m/s	Measured <i>in vivo</i> by FRAP	[167], [168]
Computational model for IF in solid tumors	< 20×10 <sup>-6</sup> m/s	The magnitude of IF speed depends on ratio of transvascular to interstitial flow resistance. Larger resistance to flow in the interstitium creates high IF speed at the tumor periphery.	[57, 166]
Forces			
Myosin II motor force	1.4–3.5 pN	Measured using an optical trap	[218], [219]
Force generated by actin polymerization	5 pN/filament	Dependent on experimental method and platform	[13]
α <sub>s</sub> β <sub>1</sub> /fibronectin bond strength	0.1–100 pN	Dependent on loading rate and method. Lower strengths measured with magnetic tweezers, higher strengths measured with AFM	[13]
Cellular contractile force	5×10 <sup>3</sup> pN	Epithelial cell edge measured on fibronectin coated pillars	[220]
	300×10 <sup>3</sup> pN	MDA-MB-231 total magnitude of contractile force measured on compliant substrate	[221]
	2×10 <sup>6</sup> pN	Fibroblast whole cell on collagen coated compliant substrate	[222]
Intratumoral IFP	4–38 mmHg	Mean IFP measured for various tumors measured <i>in vivo</i> . Brain tumors are characterized by the lowest IFP with a mean of 4.6 mmHg, and renal cell carcinomas are characterized by the highest IFP with a mean of 38 mmHg. Individual mammary carcinomas varied from 4.0–53.0 mmHg, with a mean of 23.7 mmHg. IFP is a function tumor size and of position within the tumor.	[223], [166]
Normal IFP	0–0.4 mmHg	Control measurement within rat thigh muscle.	[223]