Durotaxis: the hard path from *in vitro* to *in vivo*

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Running title: Durotaxis

Abstract

Durotaxis, the process by which cells follow gradients of extracellular mechanical stiffness, has been proposed as a mechanism driving directed migration. Despite the lack of evidence for its existence *in vivo*, durotaxis has become an active field of research, focusing on the mechanism by which cells respond to mechanical stimuli from the environment. In this review, we will describe the technical and conceptual advances in the study of durotaxis *in vitro*, discuss to what extent the evidence suggests durotaxis may occur *in vivo*, and emphasise the urgent need for *in vivo* demonstration of durotaxis.

Keywords: durotaxis, mechanics, chemotaxis, cell migration, stiffness

Introduction

Cell migration, in which cells translocate from one distinct location to another, is a fundamental biological process that underpins development, physiology and disease (Yamada and Sixt, 2019). The basic mechanisms of cell motility are well understood. Classical cell migration involves cell polarisation, in which leading and trailing edges are defined (Fig 1). Polarised intracellular signalling permits actin polymerisation near the front (Fig 1A). Myosin motors pull the actin rearward to create retrograde flow. When this flow is resisted by attachment to the underlying substrate, such as by focal adhesions, rearward movement of actin is impeded, and polymerisation drives the leading edge forward (Fig 1B). At the rear of the cell, focal adhesions are disassembled, and the cell retracts thanks to myosin II-dependent contraction (Fig 1C). These polarised events often lead to asymmetric morphology. These processes occur in many migratory cells, stereotypically on 2D surfaces (Ridley, et al., 2003), and often transpire simultaneously and in a highly complex inter-regulatory fashion (Case and Waterman, 2015; Krause and Gautreau, 2014).

However, research *in vivo* and using more complex methods *in vitro* has led to an appreciation that cell motility is highly flexible: cells can exhibit a wide variety of migratory modes (Yamada and Sixt, 2019). Cells can migrate collectively, whereby movement is achieved while retaining intercellular adhesions (Mayor and Etienne-Manneville, 2016). Cells can also migrate in the absence of direct attachment to the substrate. This amoeboid mode of migration uses intracellular propulsive forces based on retrograde flow of the cortex and friction forces to coordinate movement (Paluch, et al., 2016). Alternatively, cells using lobopodial migration use their nucleus as a mechanotransducer to propel the cell forward. In this case, myosin II-based contractility pulls the nucleus forward to pressurise the front of the cell and provide a mechanism for front-directed lobopodial protrusions that uses different machinery to that which is involved in generating lamellipodial protrusions (Yamada and Sixt, 2019; Petrie, et al., 2014). Thus, migration is highly flexible and adaptive, using different mechanisms depending on the situation.

These basic principles of cell migration can be driven intrinsically but persistent directional migration is more often regulated extracellularly (Shellard and Mayor, 2020). In this review, we first describe intrinsic cell motility and the best understood

means for directing cell movement, chemotaxis. We then examine how environmental stiffness gradients may coordinate cell movement: a process called durotaxis. We evaluate the *in vitro* and *in vivo* data of durotaxis and assess the current mechanisms and future challenges for studying durotaxis.

Directional cell migration

Motile cells can move randomly, which allows them to explore their local environments. Cells can also move in a directional fashion with high persistence and without turning (Petrie, et al., 2009). The mechanisms by which random or directional cell migration are achieved relay on cell polarity, in which leading and trailing edges of the cell are distinguished. Cell polarity can arise intrinsically, which leads to either randomly oriented motility or to directional migration (Petrie, et al., 2009). The strongest evidence for intrinsic directional motility in the absence of external signals has come from *in vitro* studies, whereas it is impossible to rule out the role of external signals for directional movement *in vivo* because of the enormously complex cell microenvironment. This *in vitro* research has shown that whether a cell migrates straight or in randomly changing directions is the result of small intracellular molecular changes (Petrie, et al., 2009).

The small GTPase Rac drives actin polymerisation by activating WAVE and the actin nucleator Arp2/3 complex, which causes membrane outgrowth. Protrusions are stabilised by anchoring to the substrate via cell-matrix adhesions, through which traction forces can be applied for movement. Competition between protrusions determine cellular persistence and direction of movement (Trinkhaus, 1969). High levels of active Rac supports random motility by promoting multiple peripheral lamellae that are oriented in different directions, which permits cell turning; whereas intermediate levels of active Rac support directionally persistent migration using front-directed lamellae because such cells do not exhibit competitive peripheral protrusions (Pankov, et al., 2005). Thus, Rac activity is a regulatory switch between intrinsic random motility versus directional motility. Mechanistically, the polarity protein Par3 targets TIAM1 to the front, which promotes local restriction and activation of Rac to the leading edge for directional migration (Pegtel, et al., 2007). Caveolin-1, a principle component of caveolae membranes, at the rear locally regulates the small GTPases RhoA, Rac1 and Cdc42 (Nakayama, et al., 2008), and

the polarisation of these components is reinforced by mutual inhibition between Rac at the front and RhoA at the rear (Petrie, et al., 2009). Hence, intrinsic polarity and motility arises from coordination of small GTPases that regulate the cytoskeleton.

Whilst cells have the inherent propensity to move persistently, it is more common for cell polarity to be generated and sustained by extracellular signals, such as chemical, mechanical or electrical signals (Shellard and Mayor, 2020; Haeger, et al., 2015). The best described mode of directional migration by external cues is chemotaxis, in which cells migrate along gradients of soluble chemoattractant. Chemotactic signals enhance and stabilise polarity through signalling pathways that converge on the same downstream components that regulate intrinsic motility. Typically, chemoattractant binds to receptors presented on the cell surface, activating signalling pathways like PI3K and Akt, that ultimately leads to activation of Rac and Arp2/3 at the leading edge, thereby generating front-directed protrusions. Alternatively, the chemotactic signal can bias the direction or retention of pre-existing, randomly oriented cellular protrusions (Insall, 2010; Weber, 2006). In both cases, chemoattractant signalling regulates Rac, Rho and Cdc42 to further polarise the cell, and coordinate its mechanical activities for sustained directional migration.

Chemotaxis is very well characterised and well documented for many cells types *in vitro* and *in vivo* including immune cells and neurons (Weber, et al., 2013; Marin, et al., 2010). However, chemotaxis may not explain all directional migration in complex environments such as those found *in vivo*. In this review, we will explore the potential for environment mechanics to dictate directional cell migration.

Durotaxis

Cells respond to physical variables of their environment, including confinement, adhesion, topology and rigidity (Janmey, et al., 2020), which profoundly impact cell function, including migration (Charras and Sahai, 2014). The idea that extracellular stiffness modulates cell movement originated from evidence that the actin cytoskeleton is modified by extracellular stiffness and that cells can sense and respond to applied forces. This led the to the proposal that matrix rigidity might serve as a guidance cue (Choquet, et al., 1997). Soon after, fibroblasts were discovered to directionally migrate from soft to stiff substrates, a process that was named durotaxis (Latin *durus*, hard; Greek *taxis*, arrangement; Box 1), in which cells are guided by

gradients of extracellular rigidity; the cells moving in the direction of increasing stiffness (Lo, et al., 2000). Durotaxis *in vitro* has since been described for various other cell types, including smooth muscle cells (Wong, et al., 2003), immune cells (Bollmann, et al., 2015; Choi, et al., 2012), cancer cells (DuChez, et al., 2019; McKenzie, et al., 2018; Lachowski, et al., 2017), and others (Evans, et al., 2018; Lachowski, et al., 2018; Tse and Engler, 2011).

Durotaxis has also been described for cell groups: MDCK cell monolayers, A431 carcinoma spheroids and keratocyte sheets (Martinez, et al., 2016; Sunyer, et al., 2016). Like observations in chemotaxis and electrotaxis (Zhu, et al., 2020a; Malet-Engra, et al., 2015; Theveneau, et al., 2010), collective durotaxis is more efficient than individual durotaxis of its constituent cells (Martinez, et al., 2016; Sunyer, et al., 2016), which suggests gradient sensing of different types of cues is an emergent property of cell groups. Despite these *in vitro* data, evidence of durotaxis *in vivo* has remained elusive.

Criteria to define durotaxis

Although durotaxis has been shown for many cell types *in vitro*, studies have been relatively simplistic, primarily due to the challenges of fabricating substrates with precise and reproducible stiffness gradients upon which cells can be cultured and studied (Sunyer and Trepat, 2020) (Box 2). Partly for this reason, there have been attempts to infer how cells operate during durotaxis without strict durotaxis experiments. Durotaxis is the process by which cells respond to a gradient of extracellular stiffness, and therefore any experimental approach to test it should expose cells to a stiffness gradient; unfortunately many reports that claim durotaxis are based on comparing the behaviours of cells exposed to stiff or soft substrate or by analysing cells at the interface between a stiff and soft substrate, and not to a gradient. Although many cells are more motile, persistent and morphologically polarised along the front-rear axis on stiffer substrates than softer substrates this is not sufficient to conclude that they will move along a stiffness gradient. Durotaxis requires the sensing of local stiffness differences, followed by an integrated response across the cell that can happen only when the cells are exposed to a stiffness gradient. On that basis, some attributes of durotaxis are summarised as follows.

The migratory cells should express receptors to transduce environmental stiffness. Integrin is the best described mediator of this, but mechanosensory proteins like Piezo are also involved. *In vitro*, the cells should follow the stiffness gradient, and impairment of sensing the stiffness gradient should lead to a failed directional response in cell migration *in vitro*. The cells should not become non-migratory; rather, non-directional movement can be expected. These criteria should also be evident *in vivo*, in addition to extra features: *in vivo*, the ECM should be modified such that it produces a quantifiable stiffness gradient, for example, by differential cross-linking of ECM fibres (see *Generating a stiffness gradient in vivo*). Alternatively, there may be a difference in cell density to produce such a gradient of rigidity. Additionally, ectopic stiffness gradients *in vivo* should divert cells from their normal path and durotaxis should be rescued by exogeneous stiffness gradients when the endogenous gradient is lost *in vivo*.

Migratory response to a stiffness gradient

Some evidence supports the hypothesis that durotaxis depends on the strength of the gradient (Koser, et al., 2016; Sunyer, et al., 2016; Bollmann, et al., 2015; Vincent, et al., 2013; Isenberg, et al., 2009), whereas other data indicate that absolute stiffness of the gradient that the cells sense also influences the durotactic response (DuChez, et al., 2019; Sunyer, et al., 2016). The durotactic response may be cell type specific; Schwann cells undergo durotaxis equally well on steep and shallow stiffness gradients (Evans, et al., 2018). Durotactic index (a measure of durotactic movement along a stiffness gradient with respect to its entire movement), velocity and cell orientation positively correlate with increasing magnitude of the gradient, and for most examples of durotaxis, these parameters are independent of the absolute modulus on a stiffness gradient (Joaquin, et al., 2016; Isenberg, et al., 2009). By contrast, cell elongation seems to better correlate with the absolute modulus (Isenberg, et al., 2009). Thus, gradient strength and absolute stiffness both may regulate durotaxis.

Interestingly, collective cell durotaxis is evident for cell types that are less efficient of individually undergoing durotaxis, suggesting that collective durotaxis is an emergent property of cell clusters (Martinez, et al., 2016; Sunyer, et al., 2016). Clusters can sense a larger difference in stiffness between its front and back than a single cell can

because a cluster covers a larger region of the gradient, thereby allowing the cluster to sense and response to the stiffness gradient more effectively. Biophysically, the cell group probes ECM rigidity at its front and rear, communicating the information via a long-range supracellular actin cytoskeletal network (Sunyer, et al., 2016).

Molecular mechanisms of durotaxis

All our knowledge on the molecular mechanisms of durotaxis has come from *in vitro* studies. Cells that are initially unpolarised interpret graded extracellular stiffness. To explore the microenvironment, cells form actin-rich protrusions, including filopodia and lamellipodia, that are biased in the direction up a stiffness gradient, where they are mechanically reinforced by a positive feedback loop (Wong, et al., 2014; Kawano and Kidoaki, 2011; Galbraith, et al., 2007; Lo, et al., 2000). When filipodia extend, they establish nascent adhesions that are pulled upon by myosin II-mediated forces (Fig. 2A, i) (Wong, et al., 2014; Giannone, et al., 2004). The resulting strain determines the cellular response. Protrusions that land on stiff regions receive strong feedback through high resistance, causing nascent adhesions to mature into focal adhesions (FAs) (Fig. 2A, ii) (Wong, et al., 2014; Kawano and Kidoaki, 2011), thereby stabilising the protrusion and anchoring it to the substrate. Mechanical feedback from high substrate rigidity causes protrusions to expand via Arp2/3- and Rac1-mediated actin polymerisation (Fig. 2A, iii) (Wong, et al., 2014), which develops lamellipodia that further probes extracellular rigidity (Plotnikov, et al., 2012). Indeed, Rac-mediated rigidity sensing is crucial for durotaxis; inhibition of the GTPase activating protein, cdGAP, maintains Cdc42 and Rac activity at the leading edge (Fig. 2A, iv), thereby encouraging adhesion maturation, whereas suppression of cdGAP abrogates the durotactic response by preventing the generation of asymmetric traction forces in response to graded ECM rigidity (Wormer, et al., 2014). The Ena/VASP family member, EVL, also regulates durotaxis by polymerising actin at FAs (Fig. 2A, iv), which reinforce cell-matrix adhesion and mechanosensing (Puleo, et al., 2019). Additionally, stiff matrix causes dephosphorylation of myosin IIA, leading to its accumulation on stress fibres, and a rear polarisation of myosin IIB (Fig. 2A, v), which may be essential for durotaxis (Chao, et al., 2014; Raab, et al., 2012). By contrast, low strain arises from soft substrates, causing nascent adhesions to disassemble and protrusions to retract via a myosin-II-dependent process (Fig. 2A, vi) (Wong, et al., 2014; Pelham and Wang, 1997). Thus, cells measure the local

rigidity by contracting attachments and sensing the response (Pelham and Wang, 1997). On stiffness gradients, this results in an asymmetric substrate adhesion (Fig. 2A, vii), which contributes to durotaxis (Breckenridge, et al., 2014).

Durotactic mechanisms operate at both the local and global level. Mechanosensing occurs by individual FAs, which act as sensors to guide durotaxis by independently applying traction forces that dynamically fluctuate spatially over the FA unit (Plotnikov, et al., 2012). ‘Tugging’ of the ECM in this way relies on components of the FA: focal adhesion kinase (FAK), phospho-paxillin and vinculin (Plotnikov, et al., 2012), and their interplay may locally strengthen the molecular clutch (Fabry, et al., 2011; Mierke, et al., 2010). Disruption of FAK activity, paxillin phosphorylation or interactions between vinculin and paxillin reduces traction stresses and lowers the threshold of rigidity that promotes tugging to softer ECM, preventing durotaxis (Plotnikov, et al., 2012; Wang, et al., 2001). Rigidity sensing during durotaxis is not only locally driven by FA growth, because equally sized FAs can generate different forces depending on substrate stiffness (Trichet, et al., 2012). Tension mediated through the actin cytoskeleton polarises actin fibres that orient along the direction of applied force in response to substrate stiffness, meaning stress fibre contractility involves a large-scale mechanical feedback that involves reorganisation of actin stress fibres that is essential for durotaxis (Trichet, et al., 2012).

At adhesion complexes positioned along the stiffer regions of ECM, traction will generate the most intracellular tension, forming an intrinsic mechanical sensor at the plasma membrane. Membrane tension polarises cells for migration (Houk, et al., 2012). Differential membrane tension is exhibited during durotaxis, where it is lower at the rear of the cell than at the front, which causes accumulation of caveolae at the trailing edge (Fig. 2B, i) (Hetmanski, et al., 2019). Caveolae recruit the RhoA GEF, Ect2, to activate RhoA to promote cell retraction by controlling local F-actin organisation and actomyosin contractility through Rock1 and PKN2 signalling, which positively feedback to keep local membrane tension low (Hetmanski, et al., 2019). High local membrane tension, which is observed at the front, transiently opens mechanosensitive channels, such as Piezo and TRPM7, leading to ion influx, with local calcium flickers (Fig. 2B, ii) that can steer directional turning by facilitating local cytoskeletal modelling and force generation (Wei, et al., 2012). Thus, polarised cell function is mechanochemically regulated by differential membrane tension resultant

from graded substrate stiffness. Indeed, mechanosensing through Piezo1 dictates axon growth along stiffness gradients *in vivo* (Koser, et al., 2016).

Durotaxis is believed to operate from pre-existing cellular molecules rather than via genetic regulation. However, stress-sensitive activation of FAK is required for the nuclear translocation of YAP on uniformly stiff substrates, and knockdown of either protein results in disrupted durotaxis *in vitro* (Lachowski, et al., 2018), suggesting gene expression may also dictate durotactic response. Although it has been proposed that asymmetric FAK activity regulates YAP on stiffness gradients, it is unclear how YAP would function differentially as a consequence of locally active FAK compared to globally active FAK.

External factors may also regulate a cell's durotactic response. Fibroblasts undergo durotaxis on fibronectin but not laminin, even though they can migrate on uniformly stiff gels with either substrate (Hartman, et al., 2016). When the substrates are combined, they act antagonistically on the cell's ability to recognise the stiffness gradient: laminin inhibits the durotactic response to fibronectin-coated stiffness gradients (Hartman, et al., 2017). Thus, stiffness-independent ECM composition regulates durotaxis.

Physical models of durotaxis

Because durotaxis is the result of a mechanical response to extracellular rigidity, there have been attempts to understand the physical principles by which cells move along stiffness gradients. Instead of looking at the molecular details, such physical models focus on the size and distribution of focal adhesions and forces, and the response by the substrate, to inform how forces might generate durotaxis.

There are three primary physical models of durotaxis (Fig. 3). A persistent random walk model, where the cell polarises, moves a certain distance then turns and continues in a new direction (Fig. 3A), has been used to simulate durotaxis. Based on some experimental data (Missirlis and Spatz, 2014; Raab, et al., 2012), which suggests cells move more persistently on uniformly stiffer substrates than softer ones, when this model is employed with varying persistence of cellular motion on a stiffness gradient, it is sufficient to reproduce durotaxis (Novikova, et al., 2017; Yu, et al., 2017). This may work because cells become more morphologically polarised (elongated) on stiffer substrates, which restricts the spatial distribution of their focal

adhesions (Yu, et al., 2017). If this is true, then cells would be incapable of sensing rigidity gradients without moving around (Yu, et al., 2017); instead, cells must explore the environment to achieve directional migration, and stiffness gradients act more as a durokinetic cue than a durotactic one (Novikova, et al., 2017). However, such a model may produce migration that is extremely inefficient (Doering, et al., 2018) and there is no experimental evidence that cells become more persistent in their motion as they migrate up a stiffness gradient. Also, axons *in vivo* are guided toward softer substrates (Koser, et al., 2016) and human fibrosarcoma cells are able to undergo reverse durotaxis (Singh, et al., 2014), an experimental validation of a computational prediction (Ni and Chiang, 2007), which suggests that stiffness gradients provide directional information. Indeed, most experimental data shows that durotaxis relies on the steepness of the gradient rather than the absolute modulus (Hadden, et al., 2017; Vincent, et al., 2013; Isenberg, et al., 2009).

A second physical model is an evolution of classical models of migration that emphasise adhesion strength (Dimilla, et al., 1991). These classical models are based on asymmetry in adhesion strength that a migrating cell possesses, and the fact that adhesions at the front are mechanically coupled with those at the rear (Dimilla, et al., 1991). It predicts a biphasic migratory response to increasing adhesion strength: when adhesion is low, contraction pulls FAs from the substrate; when adhesion is high, contraction cannot overcome adhesion strength; whereas it is optimum at intermediate adhesion, when traction at the front is balanced with – and coupled to – adhesion retraction at the rear. The prediction that cells migrate fastest at intermediate adhesion strength has been experimentally validated (Goodman, et al., 1989). Such models were developed by considering thermodynamics: focal adhesions self-assemble and elongate upon application of pulling forces and dissociate when these forces are decreased (Bershadsky, et al., 2003). For example, contractile forces of stress fibres cause focal adhesions to mature and grow, with its chemical potential reduced, making it thermodynamically favourable (Shemesh, et al., 2005). Under these circumstances, durotaxis would be a phenomenon of stress fibres, in which focal adhesions become more stable on stiffer substrates than on softer ones (Fig. 3B) because mechanical stress is higher at the front of the cell than the rear (Rens and Merks, 2020; Lazopoulos and Stamenovic, 2008; Shemesh, et al., 2005). When stress is above a critical threshold,

the adhesion assembles; when it is lower, the adhesion disintegrates. Thus, this model proposes durotaxis is driven by differential adhesion between the front and the rear of the cell, similar to the classical models of cell migration (Ron, et al., 2020; Tanimoto and Sano, 2014); indeed, adhesion is often stronger to stiff substrates, compared to soft substrates (Plotnikov, et al., 2012). Importantly, this asymmetric substrate adhesion does not involve an imbalance of traction forces, as it is sometimes misinterpreted in the literature. Since cells migrate at small scale in dissipative circumstances, the inertial forces are negligible and therefore the summation of tractions stresses on the substrate must equal the viscous stress applied by the medium on the cells, which is also negligible.

A third physical model of cellular force transmission is based on the motor-clutch hypothesis (Mitchison and Kirschner, 1988) in which intracellular molecular motors, like myosin II, transmit force to the ECM through rigid actin filament bundles and compliant transmembrane molecular clutches like integrins (Chan and Odde, 2008). A generalised clutch model simulating the dynamics of cell-matrix adhesions suggests that when stress fibres apply an equal force to the substrate at the front and rear, it differentially deforms an ECM that is graded in its stiffness: deforming more at the soft edge than the stiff (Fig. 3C'), thereby causing the cell centre to shift toward stiffer regions of the substrate (Sunyer and Trepat, 2020; Escribano, et al., 2018; Sunyer, et al., 2016; Bollmann, et al., 2015). Myosin-powered contractility produces a flow of actin called retrograde flow. Tilted expansion to the stiff edge works alongside actin polymerisation which protrudes the membranous edges of the cell to cause continual movement forward: at the leading edge, the speed of polymerisation exceeds actin retrograde flow whereas at the trailing edge, polymerisation speed is less than retrograde flow, resulting in retraction at the rear and membrane expansion at the front. This model has been used to explain stiffness-dependent cell spreading and *ex vivo* cell migration as a function of adhesion molecule expression (Klank, et al., 2017; Chaudhuri, et al., 2015), because higher stiffness is optimal for force transmission when the expression of molecular motors and clutches increases (Bangasser, et al., 2017; Bangasser and Odde, 2013; Bangasser, et al., 2013).

The motor-clutch model has been extended to explain collective cell durotaxis (Sunyer, et al., 2016). Indeed, many of the molecular components involved in

collective cell durotaxis of epithelial monolayers are the same as those required for single cell durotaxis, which suggests that the mechanisms of collective cell durotaxis and single cell durotaxis may be similar. Cell clusters generate actomyosin-based traction forces of equal magnitude at its edges, which probes the underlying substrate through integrin-based FAs (Sunyer, et al., 2016). The primary physical difference compared to single cells is that tensile forces are propagated through the monolayer via cadherin-dependent intercellular junctions (Fig. 3C'') (Martinez, et al., 2016; Sunyer, et al., 2016). These mechanically propagated forces are evident through the assembly of actin filaments, more robust vinculin-containing cell-cell junctions and FAs, actomyosin contraction, and cell morphology. Accordingly, perturbed contractility or force transmission through cell-cell contacts via α-catenin inhibits collective durotaxis (Sunyer, et al., 2016).

Generating a stiffness gradient *in vivo*

To study durotaxis *in vitro*, cells are cultured on stiffness gradients that are artificially generated by differential polymer crosslinking (Sunyer and Trepat, 2020) (Box 2). However, there are only a few examples of well characterised stiffness gradients *in vivo*, and the mechanisms by which the gradients are produced remains largely unknown. Cells can detect the stiffness of their surroundings, which is primarily ECM, and ECM stiffness can be modulated by many different processes: degradation, fibre alignment, density, deposition, crosslinking, as well as the type of ECM components of which it is comprised and their spatial arrangement (Janmey, et al., 2020). Thus, it is likely that cells may move along stiffness gradients generated by the cells surrounding them i.e. the gradient is externally generated, because any cell that can modify the ECM may be able to contribute to forming short- or long-range stiffness gradients. Indeed, the extracellular microenvironment is mechanically anisotropic; at the level of the tissue, stiffness can be soft and deformable or very stiff and non-deformable, such as brain and bone, respectively (van Helvert, et al., 2018). At the other end of the scale, single collagen fibres are much stiffer than fibrillar collagen networks (Doyle, et al., 2015), meaning the stiffness a single cell can sense around it may be highly heterogeneous.

Growing tumours modify adjacent matrix in terms of density, crosslinking and anisotropy (van Helvert, et al., 2018). Cancer-associated fibroblasts (CAFs) produce

collagen and its crosslinker, lysyl oxidase, which stiffens ECM and remodels its composition and architecture (Kawano, et al., 2015; Levental, et al., 2009). Myofibroblast and tumour cells jointly rearrange tissue topology, by alignment and bundling collagen (van Helvert, et al., 2018), which may facilitate routes of invasion that have stiffness gradients. CAFs can pull, stretch and soften the ECM leading to the formation of gaps through which cancer cells can migrate (Glentis, et al., 2017), potentially by durotaxis. Similar mechanical modifications of the extracellular environment through collagen deposition and stiffening by various cell types underlies fibrosis in atherosclerosis (Lan, et al., 2013). The migratory response of cells in diseases like these may therefore be a durotactic outcome of many cell types in the tissue generating a highly anisotropic mechanical microenvironment, with short-range stiffness gradients that can guide motile cells.

Migrating cells can also regulate environmental stiffness (Matsubayashi, et al., 2020; van Helvert and Friedl, 2016), so it is conceivable that they may be able to self-generate a stiffness gradient. In this way, cells may be able to produce a gradient *de novo* or sharpen a pre-existing gradient. Gradient-like fibre realignment, densification and stiffening has been observed ahead of the leading edge of migrating cells *in vitro*, and collectively moving cells may stiffen the matrix more than individually migrating cells (van Helvert and Friedl, 2016), likely as a consequence of the higher forces a cell group can exert compared to a single cell. However, there is no evidence that cells can continually generate an increasingly stiffer ECM as they move, and for the cell to locally stiffen the ECM in front of it, there would need to be strong contractility initially generated in a specific direction, meaning the cell is modifying the matrix in a specific spatial manner rather than responding to it. Alternatively, because many migratory cells express metalloproteinases like MMPs and ADAMs, which degrade the ECM and cause matrix softening (Liang, et al., 2017), the migratory cells may locally degrade ECM, thereby generating a stiffness gradient for the cells outwards. Local softening can also be achieved independently of MMPs (Glentis, et al., 2017). However, on a uniformly stiff ECM, it is unclear which direction the cells would durotax because the gradient is in all directions away from the cell. Nonetheless, the fact that ECM modification is a ubiquitous process suggests gradients may be produced by any cell capable of modifying the microenvironment. These ideas of gradient generation are similar to that described in

chemotaxis, in which gradients can be externally generated by a source and sink; or self-generated by migratory cells by actively modifying the shape of the gradient (Tweedy, et al., 2016; Roca-Cusachs, et al., 2013).

Other than the ECM, stiffness also depends on cell density. High cell density produces a stiffer environment than low cell density. Indeed, cell density correlates with – and contribute to – tissue stiffness *in vivo* (Thompson, et al., 2019; Barriga, et al., 2018; Weber, et al., 2017; Koser, et al., 2016). Graded cell density can be theoretically produced by differential cell migration, apoptosis, cell size, or by other mechanisms. For example, differential cell proliferation along an axis can generate a stiffness gradient, like in the developing *Xenopus* brain (Thompson, et al., 2019).

Durotaxis *in vivo*

It is unclear whether durotaxis is relevant *in vivo.* Apart from a small number of recent studies, the potential role for durotaxis *in vivo* (Fig. 4) has only been inferred from *in vitro* experiments. For example, the fact that microglia and epithelial cell sheets undergo durotaxis on stiffness gradients mimicking physiological and pathological conditions, respectively, suggests that durotaxis could have a role in the immune response and wound healing (Sunyer and Trepat, 2020; Sunyer, et al., 2016; Bollmann, et al., 2015); however if these cells really move following a stiffness gradient *in vivo* remains to be demonstrated.

There are two main challenges that have limited research into durotaxis *in vivo*. The first is the ability to measure tissue rigidity with sufficient resolution to characterise stiffness gradients spatially and temporally. Recent technological developments have provided tools to address the challenge of probing mechanical forces *in vivo* (Box 3). Such techniques have shown that the *in vivo* environment is dynamic in which elasticity may change over space and time. For instance, various tissues in embryogenesis and disease progression, like the epicardium of fibrotic scars, become stiffer over time (Barriga, et al., 2018; Berry, et al., 2006). Elasticity also varies naturally at interfaces: hard, calcified bones are connected to soft cartilage (Engler, et al., 2006; Guilak, 2000). Such heterogeneities in stiffness are inevitably encountered by moving cells. For example, as mesenchymal stem cells egress from bone marrow and migrate through tissue (Katayama, et al., 2006), they may encounter stiffness gradients. Indeed, long-range stiffness gradients have been

shown for the developing limb bud (Zhu, et al., 2020b), embryonic *Xenopus* brain (Koser, et al., 2015), elongating zebrafish tailbud (Serwane, et al., 2017) and fibrotic muscle tissues in myocardial infarction (Berry, et al., 2006).

The second challenge is the ability to manipulate tissue rigidity to functionally probe the role of stiffness gradients in cell movements, and thereby detangle correlation from causation. This has proved a significant barrier to demonstrating durotaxis *in vivo*; an important criterion as described earlier. Mechanical approaches to ablate a gradient may be technically difficult, and genetic methods are likely to have effects other than just a change in the rigidity properties of a local ECM.

The collective movement of mesodermal cells into the limb field from the lateral plate during early limb budding (Fig. 4A) corresponds with an anteriorly biased stiffness gradient, and is mirrored by the expression domain of fibronectin, which is Wnt5a-dependent (Zhu, et al., 2020b; Mao, et al., 2015; Gros and Tabin, 2014; Wyngaarden, et al., 2010). This is the best evidence to date that durotaxis may be orchestrating cell movements *in vivo*, but a lack of functional experiments to test whether durotaxis might occur *in vivo* leaves open the possibility that chemotaxis to Wnt5a, or another mode of cell guidance, might be the mechanism to coordinate cell movements for directional migration. This recent finding is the first to show cell migration that correlates with a stiffness gradient *in vivo*, and follows a previous study which showed that retinal ganglionic cell axons grow along a stiffness gradient in the developing *Xenopus* brain, turning away from the stiff regions in preference of softer regions (Thompson, et al., 2019; Koser, et al., 2016), demonstrating the physiological relevance of stiffness gradients *in vivo* for embryonic morphogenesis. In the *Xenopus* brain, the gradient arises as a result of differential cell proliferation (Thompson, et al., 2019). Although this can’t be classed as reverse durotaxis, because the axon grows and turns rather than the cell migrating, this study elegantly shows how stiffness gradients can control cell function, reproducing *in vivo* the same observations from *in vitro*.

Apart from limb budding, there are other good prospects for potentially discovering durotaxis *in vivo*. Cranial neural crest migration in *Xenopus* is triggered by stiffening of the underlying mesoderm (Barriga, et al., 2018), highlight the role of mechanics in their movement. It is currently thought that the neural crest are directed by

chemotaxis to SDF1 (Shellard, et al., 2018; Theveneau, et al., 2010) but recent evidence has questioned whether SDF1 works as a neural crest chemoattractant; after SDF1 inhibition directional neural crest migration *in vivo* can be rescued by simply enhancing cell-matrix adhesions, suggesting that SDF1 only promotes adhesion to the substrate (Bajanca, et al., 2019). Furthermore, ectopic SDF1 sources do not totally divert neural crest away from their normal route and loss of SDF1 does not seem to result in randomly oriented migration, as expected if SDF1 was a chemoattractant (Theveneau, et al., 2010). A similar observation is seen in the chemotaxis of cranial neural crest to VEGF in chick (McLennan, et al., 2010). These results suggest a durotactic gradient may be a supportive or alternative means for providing directionality information to neural crest cells (Fig. 4B).

The progression of cancers may also be linked to durotaxis: cancer cells *in vitro* undergo durotaxis (DuChez, et al., 2019) and can exert traction on the ECM to stiffen their local surroundings (van Helvert and Friedl, 2016). Malignant cells *in vivo* encounter a diverse microenvironment that changes over time and the stroma of the surrounding tumour is known to stiffen (Butcher, et al., 2009), thereby potentially offering an escape route for metastasis. Moreover, cancer-associated fibroblasts (CAFs) remodel the ECM, promoting tumour invasion by pulling on cancer cells and exerting traction on the substrate (Labernadie, et al., 2017), which might change local environmental rigidity, potentially causing a short-range self-generated stiffness gradient which the cells can follow. CAFs also pull, stretch and soften the ECM leading to the formation of gaps through which cancer cells can migrate (Glentis, et al., 2017), potentially by durotaxis. Durotaxis may therefore be a good candidate for the guidance of cancer metastasis *in vivo* (Fig. 4C). Fibroblasts, which are the best described cell type to undergo durotaxis *in vitro* (Lo, et al., 2000), are also active at wound sites, depositing material that stiffens local sites, and stiffness gradients have been observed at fibrotic lesions *in vivo* (Berry, et al., 2006). Thus, wound healing may be driven by durotaxis *in vivo* (Fig. 4D); indeed, epithelial sheets can undergo collective cell durotaxis (Sunyer, et al., 2016).

Concluding remarks

Work in recent years has made it evident that environmental mechanics has a functional role in regulating cell migration (Charras and Sahai, 2014). With new

technologies to study mechanical properties, it is tempting to speculate that spatially organised mechanical stimuli may contribute to our understanding on how cells decide on migratory routes *in vivo*. However, fundamental outstanding questions first remain, which urgently need to be addressed. Most importantly: does durotaxis exist *in vivo*? How are stiffness gradients generated and is there a single mechanism to explain durotaxis, perhaps based on an intrinsic mechanical response like the motor-clutch model, or can cells undergo durotaxis using different mechanisms, analogous to the many ways in which cells can migrate by chemotaxis? And is the mechanism for reverse durotaxis different? Can ameboid cells, which have very low cell-matrix adhesion, exhibit durotaxis, or is this a phenomenon specific to epithelial and mesenchymal cells, which are able to directly bind the matrix and have stronger cell-matrix forces? Cells *in vivo* are likely to simultaneously encounter all kinds of extracellular cues, such as chemical, mechanical, electrical, among many others. How are these integrated to control cell migration; do they work on common or distinct cellular components? We are just starting to unveil the mechanics of cell migration *in vivo*, but the development of new tools to measure and modify stiffness *in vivo* offers a promising future for this fascinating area of research.

Box 1. Definitions of durotaxis terms

Durotaxis – cell migration from soft substrate to stiff substrate following a stiffness gradient. Reverse durotaxis refers to cells moving along a stiffness gradient in the opposite direction: from stiff to soft substrate. The idea that matrix rigidity could guide migration was originally proposed to be named mechanotaxis, but this word was instead mainly adopted to describe migration in response to fluidic shear stress.

Stiffness – the extent to which an object resists deformation to an applied force. The term is interchangeable with rigidity, and inversely proportional to flexibility, which is the extent to which a material deflects under a given load. Tissue stiffness is measured by the Young's modulus, also known as elastic modulus. Stiff materials have a high Young's modulus and requires high loads to elastically deform it. Cells sense stiffness by deforming their surroundings through cell generated forces.

Young's modulus – measures the resistance of a material to elastic (recoverable) deformation under load. Thus, it is a measure of stiffness in a material. Elastic modulus varies from a few hundred pascals in the brain to a few gigapascals in cortical bone and is determined by both ECM architecture and cellular properties.

Box 2. Fabricating stiffness gradients *in vitro*

To culture cells on stiffnesses that approximate the native ECM *in vivo*, solid hydrogels produced from hydrophilic polymer chains are replacing plastic and glass petri dishes that have traditionally been used for *in vitro* research. Hydrogels can be made of various materials; the most popular is polyacrylamide. The ratio of polymer chains to crosslinking agent dictates the elastic properties of the hydrogel; the more crosslinked acrylamide is, the stiffer it becomes (Pelham and Wang, 1997). Fabricating hydrogels of varying gradient strength and stiffness range in a manner that is precise, reproducible and can be varied according to the user's needs is a major challenge (Sunyer and Trepat, 2020).

Initial studies of durotaxis were performed using 'step gradients' whereby drops of soft and stiff acrylamide solutions are polymerised adjacent to one another, creating a sharp transition (Lo, et al., 2000). Only cells spanning the soft-to-stiff interface are analysed, but this method does not generate a long-range continuous gradient of stiffness and *in vivo* gradients are much less steep. A more precise method, in which

a microfluidic device splits and recombines polyacrylamide solutions (Zaari, et al., 2004), produces substrates with well-defined, smooth rigidity gradients from discrete inputs, although the achievable range is limited to gradients in the pathological range, which is relatively large. Also, the technique is costly, labour intensive and time consuming.

The most accessible method to generate stiffness gradients utilises differential diffusion between a pre-polymerised gel of varying thickness and a second gel polymerised on top (Hadden, et al., 2017). The stiffness range is somewhat small, and the large thickness of the gel limits observation by microscopy, but stiffness gradients are reliable. A variation of this technique, in which sharp thickness variations are introduced in the pre-polymerised gel using microfabricated moulds, generates steeper gradients, although the gel can swell, and given topological cues are known to influence directional migration (Pieuchot, et al., 2018), it is difficult to attribute results solely to durotaxis.

The most advanced technique is one in which the stiffness gradient is photolithographically patterned, either by using a photomask that is graded in transparency (Tse and Engler, 2011; Wong, et al., 2003) or with a sliding mask (Sunyer, et al., 2016; Marklein and Burdick, 2010). Photoinitiator reactions are suboptimal due to impaired transmission of light, light diffraction and lateral diffusion of radicals, which limits the resolution of gradients. However, photopolymerisation is a versatile and flexible method to obtain wide stiffness ranges of physiological and pathological relevance.

Box 3. Approaches to measure *in vivo* tissue stiffness

Durotaxis *in* vivo has remained unexplored, in part, due to a lack of appropriate tools to map tissue stiffness in 3D within bulk tissues. Several new techniques have been developed and employed to measured forces and stresses in living tissues (Sugimura, et al., 2016). Mechanical properties can be derived from stress-strain curves obtained by these methods, whereas only a few methods can directly measure stiffness.

The most popular tool to measure elasticity of tissues *in vivo* is atomic force microscopy (AFM) in which a tissue is indented with a cantilever, which bends in response. The applied force can be measured from the bending of the cantilever, and the force-indentation curve can be fitted to a model to extract material stiffness. AFM has been used to map the mechanical properties of superficial tissues; however, to measure deeper tissues, the surface layers must be removed, which may alter the properties of the underlying tissue, or requires mechanical modelling and mathematical deconvolution (Tao, et al., 2019; Thompson, et al., 2019; Barriga, et al., 2018; Koser, et al., 2016; Lau, et al., 2015).

Magnets have been used in a variety of ways to investigate elasticity properties of tissues *in vivo*. Magnetic resonance elastography measures the propagation of non-invasive low frequency waves applied to tissues to generate a stiffness map, called an elastogram (Sack, et al., 2008). Owing to the requirement for an MRI machine, this technique is used to measure stiffness of diseased tissues, such as liver fibrosis, but is inappropriate for most experimental biology. Magnetic tweezers, which can manipulate microinjected magnetic beads, have been used to measure tissues in *Drosophila* and mouse embryos, but this technique has a small workspace and forces are low and non-uniform, which makes producing spatial stiffness maps difficult (Sugimura, et al., 2016). A recent development of this approach led to a magnetic device that generates a uniform magnetic field gradient within a large workspace, where the magnetic force is sufficient to displace many magnetic beads simultaneously to quantify spatial stiffness distribution in tens of loci, allowing for measurements of stiffness in three dimensions and across entire tissues or embryos (Zhu, et al., 2020b; Zhu, et al., 2020c). Another recent novel technique has involved using magnetic force to displace and deform microinjected ferrofluid droplets *in vivo*

(Mongera, et al., 2018; Serwane, et al., 2017; Huang, et al., 2002). The ferrofluid approach requires prolonged periods to assess droplet deformation due to their slow dynamic response and delivers limited spatial resolution.

Brillouin optical microscopy is a form of imaging that takes advantage of a physical phenomenon known as Brillouin light scattering (BLS), whereby light scatters when it interacts with material. Solid components experience greater BLS than liquid materials, and thus, although Brillouin optical microscopy imaging is hard to setup and slow in acquisition, it can infer viscoelastic properties of tissues *in vivo* in a non-invasive and label-free way, like which has been performed for the human eye (Scarcelli and Yun, 2012) and mouse embryos (Raghunathan, et al., 2017), giving it the potential to probe mechanical properties that other techniques cannot access. However, BLS is influenced by hydration, and because biological materials are composed largely of water, Brillouin measurements may not necessarily correlate with stiffness in hydrated materials, like biological tissues (Wu, et al., 2018).

Figure legends

Figure 1. Cell motility. A, Polymerisation of monomeric G-actin into a filamentous (F-)actin network at the leading edge causes forward-direction plasma membrane protrusions (dotted line to full line). **B**, Membrane protrusion facilitates the binding of transmembrane cell surface receptors to the underlying substrate, thereby sticking the membrane extension to the substrate. New adhesions (pale pink disk) are rapidly linked to the network of actin filaments, coupling the actin treadmill – a phrase that describes the intrinsic rearward flow of the actin network – to the substrate. Adhesions serve as traction sites for migration as the cell moves forward over them. The source of this force is myosin II (pink ovals) that contracts the actin cytoskeleton, which pulls on adhesions (purple disks). **C**, The combined activity of retrograde actin movement and intracellular contractile forces produced by stress fibres generate tension to pull the cell body and nucleus forward. Actin filament and focal adhesion disassembly result in focal adhesion detachment (grey disk). These processes result in directional cell migration (black arrows).

Figure 2. Molecular mechanisms of durotaxis. Various molecular mechanisms are at play when cells sense and respond to stiffness gradients, which leads to their durotactic movement (black arrows). The top panel, **A**, represents mechanical sensing and response through focal adhesions; the bottom panel, **B**, represents changes in membrane tension and its consequences. **A, i**, The cell connects to the ECM via focal contacts. Myosin motors of stress fibres generate contractile forces that pull on these anchors to probe ECM rigidity and determine cellular response. **ii**, Focal contacts mature into focal adhesions in the region of the cell in contact with stiff substrate. **iii**, This mechanical feedback promotes the activity of Rac1 and Arp2/3, leading to actin polymerisation and local membrane protrusions. **iv**, Inhibition of cdGAP, an activator of Cdc42 and Rac act the leading edge, is vital for durotaxis. **v**, Myosin II-B is polarised at the cell rear. **vi**, Low stiffness at the cell rear causes adhesions to disassemble and rear-directed membrane extensions to retract (grey arrow). **vii**, The mechanical response of cells to a stiffness gradient is rearward directed traction forces at the front and forward-directed traction forces at the back. Traction forces are balanced. **B, i**, A stiffness gradient causes a gradient of cell membrane tension. The low tension at the rear results in the accumulation of caveolae, that activate Ect2 causing rear polarisation of RhoA, and actomyosin

contractility. **ii**, Mechanosensitive ion channels also regulate the cellular response. As an example, Piezo causes influx of calcium ions which has many effectors, including organisation of the cytoskeleton.

Figure 3. Physical models of durotaxis. There are three main proposed physical models for durotaxis. **A**, Because cells are more polarised and morphologically elongated on stiffer substrates than softer ones, it has been proposed that this affects the distribution of focal adhesions, causing cells to become more persistent in their migration on stiffer substrates than softer ones. This ultimately results in migration (black arrow) up the stiffness gradient. In this model, the cell is incompetent at sensing rigidity gradients without moving around (black lines with arrowhead indicating direction of migration), and therefore stiffness gradients are not a directional cue in of themselves, but rather act as a durokinetic cue by modulating cell persistence. **B**, Based on adhesions and thermodynamics, larger, more stabilised, mature and plentiful focal adhesions (purple disks) are exhibited by cells at the leading edge than the trailing edge thanks to the feedback loop between the cell and underlying local stiff substrate. The myosin motors pull on the adhesion anchor, which has greater mechanical feedback at the front than the rear due to the differential stiffness, thereby allowing adhesions to grow at the front compared to the rear. Thus, cells exhibit greater attachment to the substrate at the front than at the rear, although traction forces (purple arrows) are balanced. This polarised attachment results in net forward movement of the cell. **C'**, In the molecular clutch model, equal forces by the actomyosin cytoskeleton (actin, red; myosin, pink) are transmitted from focal adhesion complexes at either end of the cell. Substrate displacement (indentations under focal adhesions) is larger on the soft edge than the stiff one, meaning equal contraction shifts the cell centre toward the stiff side. Combined with equal polymerisation at each end of the cell, this results in durotaxis. The motor-clutch model is experimentally supported by the durotaxis of both individual cells as well as clusters, **C''**. In this case, the cytoskeleton at the front and rear is still connected by a supracellular actomyosin network that spans cell-cell junctions via cadherins (green rectangles). The mechanism is the same as in C'. The cluster senses a bigger difference in substrate stiffness at its front and rear, compared to a single cell, making it more efficient at durotaxis.

Figure 4. Prospects for *in vivo* durotaxis. A, Mesodermal cell migration (purple arrow, right) shapes the early limb bud. The direction of the movement correlates with a (fibronectin) stiffness gradient that is dependent on Wnt5a. No functional tests on the durotactic gradient have been performed yet. **B**, Neural crest cells migrate long distances in the developing vertebrate embryo. Their migration requires the underlying mesoderm to increase its stiffness, however whether there is a stiffness gradient, and whether durotaxis helps direct neural crest migration, is unknown. **C**, Cancer cells metastasise away from primary tumours toward secondary sites. Stiffness changes during tumour development, and cancer-associated fibroblasts promote cancer invasion by guiding cells out of the primary site. Fibroblasts are known to undergo durotaxis *in vitro*, CAFs produce traction forces that guide cancer cell migration, and CAFs can facilitate breaching of the basement membrane by softening it and forming gaps, suggesting a self-generated durotaxis gradient may direct cancer invasion *in vivo*. **D**, During wounding and fibrosis, tissue stiffness increases, creating a gradient away from the fibrotic site, and fibroblasts can undergo durotaxis *in vitro*. Durotaxis may therefore be relevant in wound healing and fibrosis *in vivo*.

Data accessibility

This article has no additional data.

Competing interests

We have no competing interests.

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Fig 1

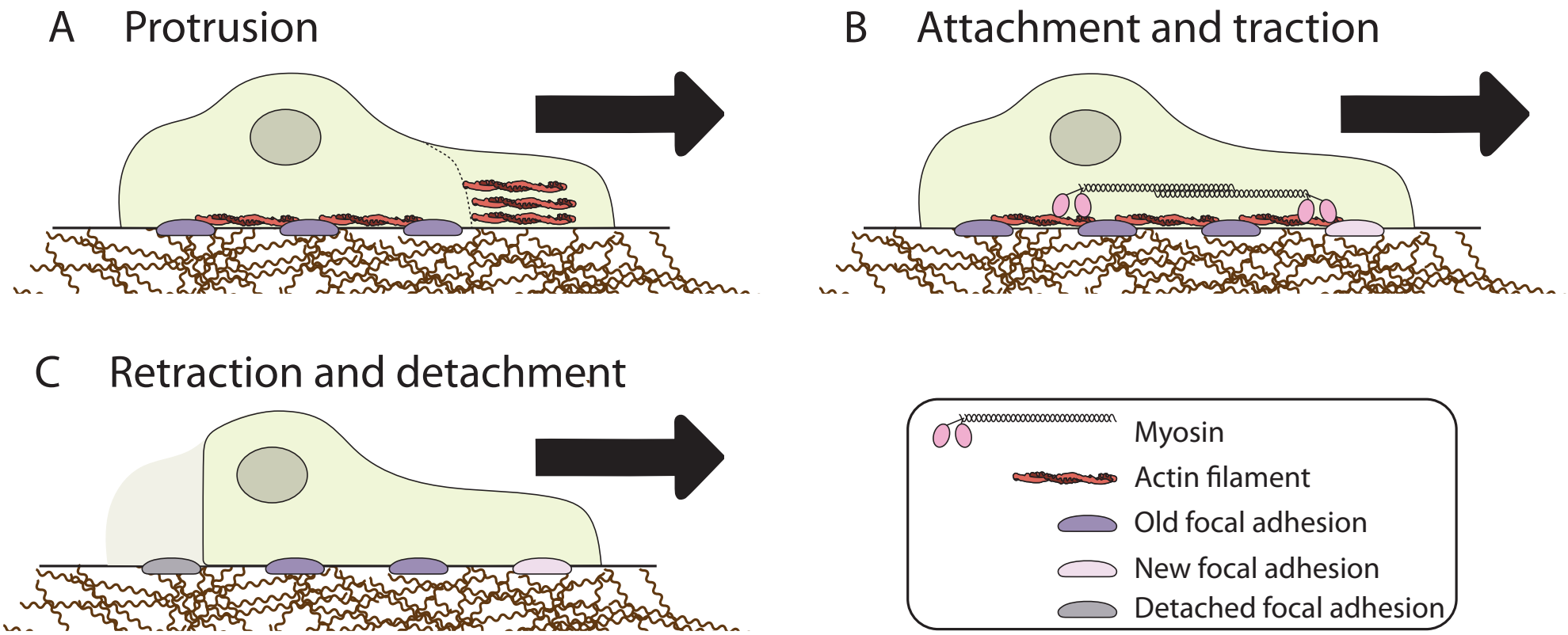

A Protrusion
B Attachment and traction
C Retraction and detachment
Myosin
Actin filament
Old focal adhesion
New focal adhesion
Detached focal adhesion

Fig 2

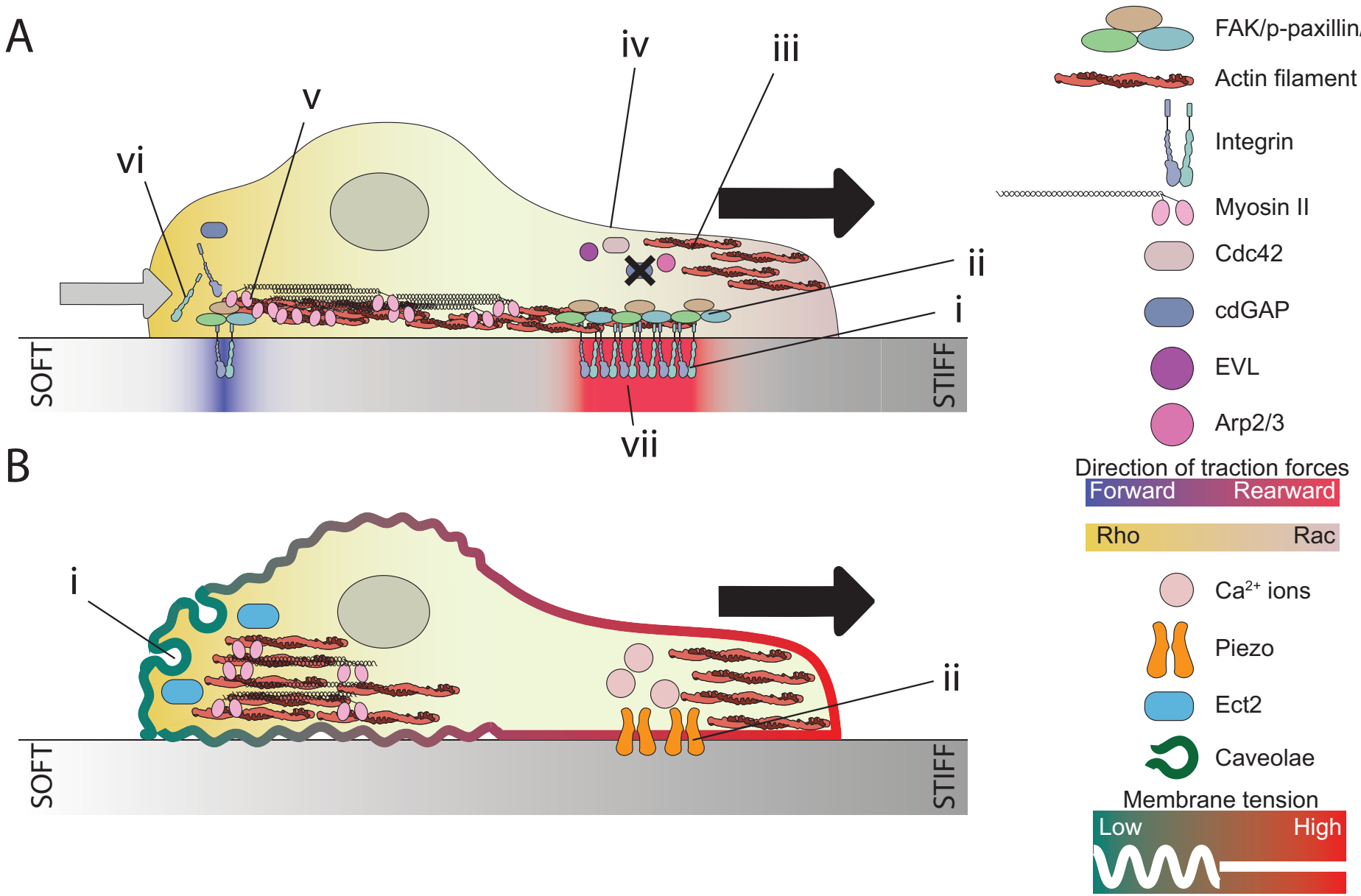

A
iv
iii
v
vi
ii
i
SOFT
STIFF
vii
B
i
ii
SOFT
STIFF
FAK/p-paxillin/vinculin complex
Actin filament
Integrin
Myosin II
Cdc42
cdGAP
EVL
Arp2/3
Direction of traction forces
Forward
Rearward
Rho
Rac
Ca2+ ions
Piezo
Ect2
Caveolae
Membrane tension
Low
High

Fig 3

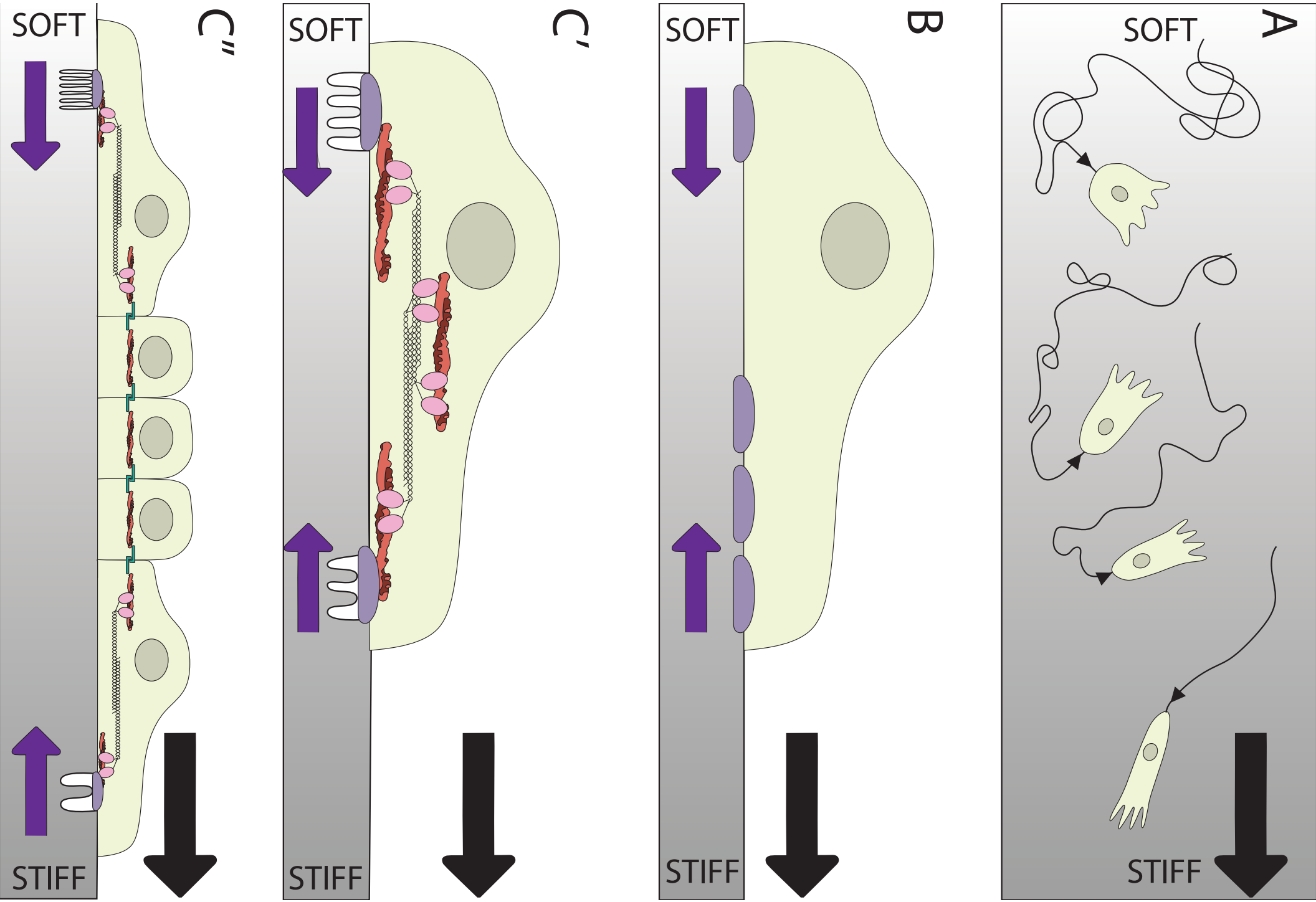
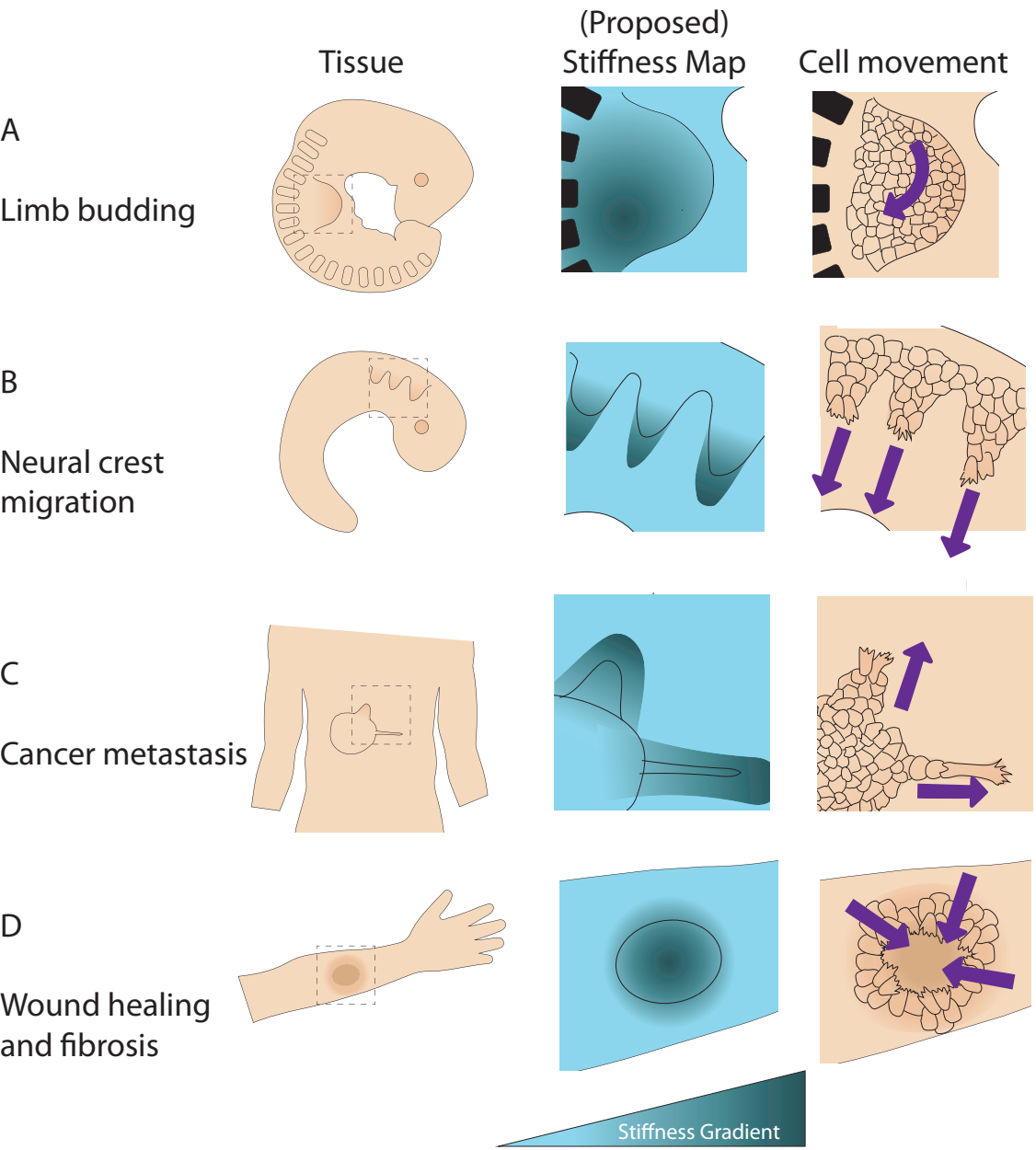


Fig 4


Tissue
(Proposed)
Stiffness Map
Cell movement
A
Limb budding
B
Neural crest
migration
C
Cancer metastasis
D
Wound healing
and fibrosis
Stiffness Gradient