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SYSTEMS MICROSCOPY ANALYSIS OF CELL MIGRATION

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SYSTEM MICROSCOPY ANALYSIS OF CELL MIGRATION

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DEDICATED TO MY HUSBAND & PARENTS FOR THEIR UTMOST CARE, LOVE AND GUIDANCE

ABSTRACT

Single cell migration is heterogeneous and a complicated process. It arises from a hugely complex network of multi-scale interactions between molecular and macromolecular entities. Though the full, spatiotemporally resolved molecular complexity of the cell migration system is currently inaccessible, two macromolecular entities, namely cell-matrix adhesion complexes (CMACs) and F-actin, provide a means to abstract this complexity to a level that is tractable with imaging approaches, while also enhancing the significance of information captured from the cell migration system. Based on this rationale, we combined quantitative imaging and acquisition of multi-scale quantitative data, describing simultaneously both cell behavior (migration) and organization (e.g. CMAC and F-actin status) on a per cell basis, thereby leveraging a natural cellular (spatial and temporal) heterogeneity. This was then further combined with multivariate statistics and mathematical modelling, resulting in an approach referred to as systems microscopy. Subsequently, we employed this approach to interrogate several biological aspects.

In the first study, we used a systems microscopy approach, including use of the Granger causality concept, to map pairwise causal (directional) relationships between organizational and behavioral features of the cell migration system, advancing on the commonly used correlative (non-directional) relationships. This way, we were able to leveraging the natural cellular heterogeneity to better understand the cell migration system. We found that organizational features such as adhesion stability and adhesion F-actin content causally determined the cell migration speed. Contrary to previous findings, we observed that cell speed also acted upstream of organizational features, including cell shape and adhesion complex location. A comparison between unperturbed and modulated cells provided evidence that Granger causal interaction patterns are in fact plastic and context dependent rather than stable and generalizable.

In the second study, we employed a systems microscopy approach to separate the regulatory associations underlying either cell migration or its membrane dynamics. We introduced a new measure of relative membrane dynamics, corrected membrane dynamics (CMD), which is independent of cell speed. We found that F-actin features (e.g. F-actin concentrations at CMACs and F-actin concentrations per cell) were strongly associated with membrane dynamics while cell migration was more strongly correlated with adhesion-complex features (e.g. variance in CMAC age and CMAC shape). Moreover, these correlative linkages were often non-linear and context-dependent, changing dramatically with spontaneous heterogeneity in cellular behavior.

In the third study, cellular plasticity was studied, using the Nuclear-Golgi positioning as a model system addressing the coordination between cell migration and cellular asymmetry. We systematically analyzed these processes over a two-dimensional experimental array wherein intracellular tension and matrix ligand density were progressively co-varied. We found plastic responses of cellular behaviours, e.g. for the cell motion angle, cell polarity

angle and the polarity and motion alignment. Moreover, polarity and motion alignment and cell motion angle dynamics displayed non-linear and non-monotonic relationships to cell speed and the correlative relationship between them were context-dependent. Some of these relationships were susceptible to decoupling with a reduction in tension or attachment strength. Moreover, we found that the forward polarity of the Golgi is an ordered cellular state, in contrast to backward polarity. More broadly, we found that in the majority of cases, motion and asymmetry were coordinated and that the different types of coordination coincide with specific cellular behaviors.

In the fourth study, we employed the systems microscopy approach to demonstrate the existence of two divergent modalities of mesenchymal cell migration, spontaneously emerging in parallel under a uniform environmental condition. The discontinuous migration acquires faster and less persistent migration and is characterized by a dramatic cell rear-retraction events that are temporally decoupled from protrusion. Quantification of cell-matrix adhesion, F-actin and cell morphological features in each mode revealed that the cell speed within each mode is controlled by the unique assemblage of organizational features, suggesting the differential mechanism of regulating cell speed within each mode. We also demonstrated that the cell adaptive response is mediated by an adaptive switching rather than a progressive adaptive stretching, rendering adaptive switching as a dominant mechanism. We also provided evidence of important molecular regulators involved in adaptive switching, involving the sub-cellular systems of actomyosin contractility and cell-ECM interactions in this regulation.

LIST OF SCIENTIFIC PAPERS

I. John G. Lock, Mehrdad Jafari Mamaghani, **Hamdah Shafqat-Abbasi**, Xiaowei Gong, Joanna Tyrcha, Staffan Strömblad. Plasticity in the macromolecular-scale causal networks of cell migration. *PLOS ONE*; 2014; 9; e90593.

II. Jacob M. Kowalewski, **Hamdah Shafqat-Abbasi**, Mehrdad Jafari-Mamaghani, Bereket Endrias Ganebo, Xiaowei Gong, Staffan Strömblad, John G. Lock. Disentangling membrane dynamics and cell migration; Differential influences of F-actin and Cell-matrix adhesions. *PLOS ONE*; 2015; 10; e0135204.

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CONTENTS

LIST OF ABBREVIATIONS

ECM	Extracellular matrix
CMACs	Cell-matrix adhesion complexes
WASP	Wiskott-Aldrich syndrome protein
Cdc42	Cell division control protein 42 homolog
MLCK	Myosin light chain kinase
ROCK	Rho-associated protein kinase
MTOC	Microtubule organizing center
aPKC	Atypical protein kinase C
MT	Microtubules
MMPs	Matrix metalloproteinases
2D/3D	Two/Three dimensional
NCAM	Neural cell adhesion molecule
EMT	Epithelial to mesenchymal transitions
MAT	Mesenchymal to amoeboid transitions
CAT	Collective to amoeboid transitions
FC	Focal complexes
FA	Focal adhesions
FAK	Focal adhesion kinase
iPALM	Interferometric photoactivated localization microscopy
VASP	Vasodilator-stimulated phosphoprotein
PAD	Patch morphology dynamics
KS	Kolmogorov-Smirnov
CVA	Canonical vector analysis
PCA	Principle component analysis
EM	Expectation maximization

FN	Fibronectin
Arp2/3	Actin related protein 2/actin related protein 3
GEFs	Guanine nucleotide exchange factor
ARHGAP22	Rho GTPase activating protein 22
DOCK3	Dedicator of cytokinesis 3
NEDD9	Neural precursor cell expressed, developmentally down-regulated 9
RhoA	Ras homolog family member A
Par6	Partitioning defective protein 6
Tiam2	T-cell lymphoma invasion and metastasis 2
mRNA	Messenger RNA
CMD	Corrected membrane dynamics
Y-27632	Rho-kinase inhibitor

1 INTRODUCTION

1.1 CELL MIGRATION

1.1.1 Physiology

Cell migration is a key cellular process, spanning from simple unicellular organism such as Amoeba, to more complicated multicellular organisms, such as mammals. Cell migration plays a crucial role in the physiology of multicellular organisms, during processes such as embryonic development, wound healing and immune responses. In embryonic development, cells during gastrutration migrate in groups from inside the blastocyst and constitute the three germ layers such as ectoderm, endoderm and mesoderm. Later, cells within each germ layer move to their specialized location and form skin, brain, the nervous system, skeletal muscle, inner lining of a gut and the circulatory system [1, 2]. Similarly, fibroblast and vascular endothelial cells migrate to the injured area in wound healing. In immune responses, immune cells such as lymphocytes and leukocytes migrates into lymph nodes and inflamed tissue to destroy intruding microorganisms, thus providing defence to an organism [2].

1.1.2 Pathophysiology

Cell migration is not specific to embryonic development, but rather takes place throughout the entire life span of an organism. Nonetheless, deregulated cell migration during embroyonic development can lead to many congenital diseases e.g. Heart septation defects, DiGeorge syndrome, craniofacial abnormalities and severe retardations are associated with impaired migration of the neural crest cells [3, 4]. Abnormal cell migration contributes to many inflammatory diseases (e.g. asthama, rheumatoid arthritis) [5, 6], vascular diseases and also underpins cancer metastasis [7].

1.2 THE MIGRATORY MACHINERY

Cell migration emerges from a diverse and interconnected system of molecular interactions. This includes links between integrins and extracellular matrix (ECM) proteins, integrins and mechanical or signaling adaptor proteins, adaptor proteins and actin etc. These interactions combine progressively with each other to produce macromolecular systems of intermediate complexity, such as cell-matrix adhesion complexes (CMACs); the cell polarity system; the microfilament and microtubule systems; vesicular trafficking machinery; the plasma membrane; the extracellular matrix etc. Each of these sub-cellular systems interact with each other, ultimately constituting and regulating the cell migration system [8].

1.2.1 Cell-matrix adhesion complexes

Cell-matrix adhesion complexes (CMACs) are present in the plasma membrane at the interface between cell and the extra-cellular matrix (ECM), thereby mediating cell attachment to the ECM. Through integrins and adaptor proteins, they also provide a mechanical linkage between the ECM and microfilaments. CMACs are central players in regulating the cell migration process [8] and are discussed in more detail below (sections 1.6).

1.2.2 The extracellular matrix

The extracellular matrix (ECM) is a crucial non-cellular component of all living tissues and organs. It acts as a substratum and mediates cell anchorage, establishing a tissue scaffold. Beside its function as providing support to the cell, the ECM also sends chemical and mechanical signals to cells, thereby influencing many cellular processes, including cell migration, proliferation and differentiation [9].

The ECM is composed of fibrous proteins (glycoproteins), water, minerals and proteoglycans [10].There are several types of fibrous proteins including collagens, fironectin, vitronectin, elastin, fibrillins and laminins [11]. The ECM is extremely heterogenous and tissue specific [12]. Collagen type I is the most abundant protein, present in several tissues [13]. Fibronectin is secreted by fibroblasts, and constitutes a protein family produced by the alternative splicing of a single gene. Fibronectin is secreted in two different forms. The water soluble form is present in blood plasma, while the water insoluble form generates fibrils that assemble together by di-sulphide bonding to form a meshwork in the ECM [14].

The ECM provides adherence to cells, which is mediated by several receptor types present on the plasma cell membrane, including integrins, syndecans and discoidin domain receptors [15, 16]. Thus, cell adherence to ECM transmits informational cues from outside to the cell interior, thereby controlling the cell migration process [17, 18]. In addition to the molecular composition of ECM, several other factors including ECM dimension, density, stiffness and orientation also affect the efficiency and method of cell migration [19].

Abnormalities in the ECM arise in part due to mutations in the genes coding for ECM proteins. These may lead to various congenital diseases e.g. osteogenesis imperfecta, caused by a mutation in the collagen type I gene and is usually associated with low bone density [20], kniest dysplasia caused by mutations in the collagen type II gene and is characterized by abnormal bone growth [21] and Alport syndrome caused by the collagen type IV mutations and is associated with malfunctioning of kidney [22].

1.2.3 Microfilaments

Cell-matrix adhesion complexes associate with the microfilaments, and involve in regulating organization, dynamics and of microfilaments. The bi-directional interaction between CMACs and microfilament induces cellular tension which effect both structures [8] and are discussed in more detail below (sections 1.6.1.3).

1.2.4 Cell polarity

Establishment of front-rear cell polarity is crucial in directed cell migration. Small GTPases such as Cdc42 is implicated in regulating cell polarity by confining the formation of the lamellopodium [23], localizing the MTOC (Mitochondrial organization center) and Golgi complex at the cell front, mediating the Microtubule (MT) growth as well as MT targeted delivery of vesicles towards the cell leading edge [24, 25]. Cdc42 forms a complex with Par proteins (Par-3, Par 6) and atypical protein kinase C (aPKC), which mediates the MTOC positioning in the direction of migrating cell [26].

1.2.5 Microtubules

Microtubules (MT) are polymers of α and β tubulin dimmers. MT are implicated in modulating cell migration by regulating adhesion turnover. MT grow in the direction of focal adhesions and contribute to the disassembly of focal adhesions. Studies have shown that Tiam2, an activator of small GTPases is involved in microtubule-dependent regulation of focal adhesion disassembly. Moreover, MT deliver membrane vesicles, post Golgi carriers [27, 28], recycling endosomes carrying membrane associated molecules small GTPases, Rac, Cdc42 and βPIX towards the leading edge, necessary for effective cell migration [29, 30]. Microtubule associated motors also deliver mRNAs encoding β-actin and proteins of the Arp2/3 complex, thereby facilitating actin polymerization. Microtubule-dependent delivery of mRNAs to the leading edge also plays a crucial role in cell migration [31-33].

1.2.6 Vesicular trafficking

Vesicular trafficking is implicated in transporting integrins and contribute to turnover of adhesions and thereby regulating cell migration [34]. Integrins can be internalized by three different pathways such as macropinocytosis [35], clathrin-dependent and -independent endocytic pathway [36]. Internalized integrins are trafficked to endosomes where they are assorted for either degradation or recycling. Integrin recycling is mediated either by Rab4- [37] or Rab11-and Arf6- dependent pathways [38]. Moreover, integrin trafficking pathways are implicated in regulating intracellular signalling e.g. Rab-21 mediated recycling of α5β1 is involved in the direct activation of RhoA [39]. A recent study has shown that Rab21 mediated integrin endocytosis, positively regulates adhesion-induced Focal adhesion kinase (FAK) [40]. Additionally, vesicular trafficking also deliver lipid molecules to the cell leading edge which is required for the establishment of the lamellopodium, as well as receptors for soluble ligands [41].

1.2.7 Membrane dynamics

Cell migrates by producing thin plasma membrane extensions or protrusions at the leading edge and retractions at the rear. Thus, protrusions and retractions occur in a coordinated manner that drive the cell forward. The level of coordination between them determines the efficiency of cell migration. Cell protrusions and retractions differ in actin organization. Protrusions, e.g. lamellopodium and filopodia, are formed by the growth of branched or linear actin filaments. In contrast, retractions are formed by anti-parallel actin growth together with contractile arrays of myosin II [42].

Actin polymerization is implicated in protrusion formation [43]. There are two main categories of actin nucleators: the Arp2/3 complex and the mDia/mDia2 formins. Arp2/3 is involved in nucleating branched actin filaments or "Dendritic filaments" [44], regulated by WAVE/Scar, WASP and N-WASP proteins [45]. Formins attach to the barbed ends of F-actin and enhance a linear extension of actin filaments. The formins are regulated by small GTPases (RhoA and Cdc42) [46], requiring also profiling for promoting actin polymerization [47].

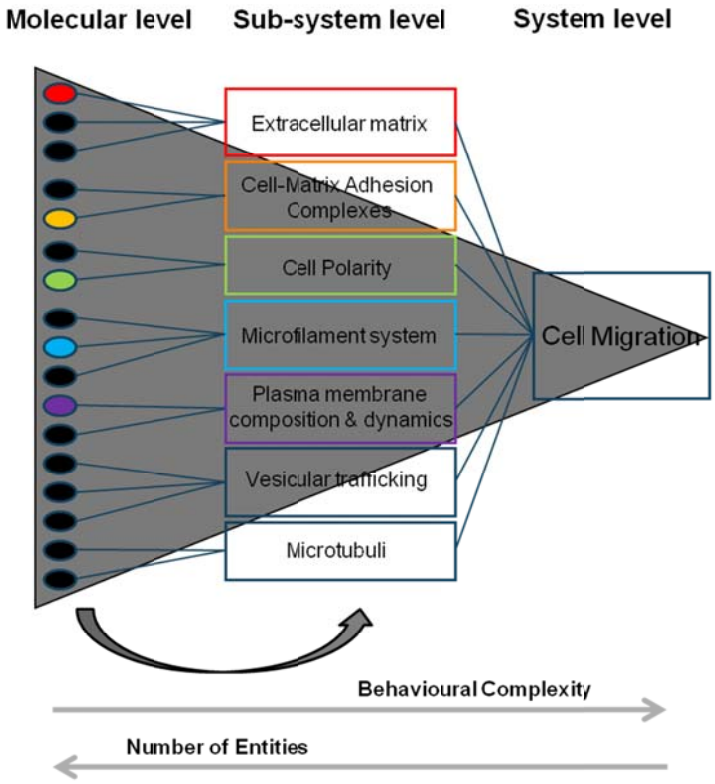

Figure 1: Cell migration as a multi-scale process (Adapted from Lock et al., 2007)

1.3 POLARITY IN CELL MIGRATION

Front to rear planar cell polarization is crucial for directed cell migration [48], which is impelled by the asymmetric distribution of intracellular organelles between the Nucleus and cell motion axis e.g. the Golgi complex, microtubule organizing center (MTOC) and centrosome. [49, 50]. Both the microtubule and actin cytoskeleton are key players in determining the cell asymmetry and their regulation is mediated by CMACs binding to the extracellular matrix (ECM) proteins and subsequent activation of a cell specific signalling cascade. The activation of Rho GTPases including RhoA, Rac and Cdc42 is implicated in cell polarization by indirectly modulating actin cytoskeleton [48]. A downstream effector of Rho GTPases such as Rho-associated protein kinases (ROCK) is a serine-threonine kinase with two isoforms: ROCK I and ROCK II, is involved in regulating actomyosin contractility by phosphorylating Myosin light chain (MLC) phosphatase [51]. Rho GTPases are also involved in regulating microtubule dynamics and indirectly affecting cell polarization through recruitment of the Cdc42-Par6-PKCzeta complex towards the cell front. Moreover, microtubule targeted delivery of Golgi-derived vesicles to the cell front containing proteins and nutrients, which are needed for the formation and sustaining membrane protrusions in migrating cells [52].

1.4 MODES OF CELL MIGRATION

Cell migration is heterogenous, as cells do not migrate uniformly. The migratory modes were initially categorized based only on their morphogical properties. Later, more molecular descriptors have been used to define each mode more precisely and make the mode discernable from each other. These molecular descriptors include cytoskeletal organization, adhesiveness and proteolytic remodeling of the tissue microenviroment [53-57]. Cells move either individually or in a collective manner as cohort, highly coordinated without breaking their contacts with neighbouring cells. Single cell movement is futher categorized into Amoeboid and Messenchymal [53].

1.4.1 Amoeboid migration

This type of cell migration is the most primitive and resembles the crawling behavior of the Amoeba *Dictyostelium discoideum*. Amoeba are ellipsoidal in their morphology and move from one place to the other with rapid changes (in seconds) in morphology, forming membrane extensions and contractions. In eukaroytes, this type of migration relates to migrating cells with rounded or ellipsoid morphology, devoid of focal adhesions and stress fibers [55, 58]. Ameboid cell migration is further divided into two subtypes. The first type involves migrating cells with rounded morphology, forming blebs with very low adherence with the substratum and capable of propelling. The second type of cells are more elongated and renders actin rich filopodia at the leading edge and engage in weak attachment with the substratum [59, 60]. Leukocytes are extremely deformable, generating weak and unstable focal contacts which renders them to move rapidly with high velocities (2-30 μm/min) and

disseminate among connective tissues, organ and blood circulation. They lack stress fibers, but possess cortical filamentous actin, which mediates rapid shape deformation while providing stiffness to the cell body [61, 62]. Small GTPases such as RhoA and its effector ROCK are involved in regulating the cortical actin dynamics, thus maintaining the rounded morphology [63, 64]. On the other hand Rac and Cdc42 mediate actin polymerization through adaptor proteins such as WASP, thereby regulating membrane dynamics and sustaining cell polarization and elongation [65]. Ameboid cell migration can also utilized by tumor cells, such as lymphoma, small-cell lung carcinoma and carcinoma. Such tumor cells migrating in an amoeboid manner disseminate very rapidly even at their early stages and thus contribute to systemic disease [53].

1.4.2 Mesenchymal migration

Mesenchymal migration is also termed as Lamellopodial migration. It is characterized by slow speed, irregular morphology, strong cell-substratum adherence, prominent stress fibres and actin containing structures such as lamellopodia and filopodia at the cell front [66]. Cells exibiting this migratory mode usually acquire spindle-shaped morphology in 3D ECM [67] and move through tissues by forming focal contacts containing integrins, adaptor proteins and localized actin. Mesenchymal migration is characterized by slow speed in 3D (0.1-2 μm/min) due to slow adhesion formation and turnover [54, 68-70]. In messenchymal migration, small GTPases such as Rac and Cdc42 are involved in actin polymerization, forming membrane extensions in the form of either pseudopods or lamellipodia enabling the formation of integrin mediated cell contacts with ECM (2D or 3D) [68]. Conversely, Rho is involved in adhesion maturation, stress fiber formation and thus reducing the speed of mesenchymal migration in 2D. While in 3D, its role with regard to cell shape and adhesion dynamics is more complex and ambiguous [68, 71, 72].

1.4.3 Collective migration

In collective cell migration, cell retains their adherence junctions between cells and move in a highly coordinated manner, as interlinked multicellular chains or cords, tubes or sheets [53]. Collective cell migration can be observed both in 2D e.g. in wound scratch assays [72], and in 3D ECM [73]. Cells at the leading edge form actin-mediated membrane ruffles and generate integrin-mediated traction forces [74, 75], while the rear of the leading cell(s) maintain contacts with the other cells by means of adherence junctions that may be mediated by cadherins, NCAM or leukocyte adhesion molecules [76-78]. The trailing edge retractions of the leading cell(s) exerts pulling forces on neighboring cells and drag them along the already existing migratory track while retaining cell-cell contacts [73, 76, 79]. Additionally, cortical actin is also involved in retaining the collective integrity [74, 75, 80]. Collective cell migration is necessary for tissue regeneration and modeling such as epithelia, ducts, glands and vessels, but also cancer cells often utilize collective migration leading to tumor cell dissemination [81, 82].

1.5 PLASTICITY IN CELL MIGRATION

Each differentiated cell most often uses a pre-defined migration pattern such as leukocytes that employ ameboid migration, stromal cells move using mesenchymal and epithelial cells move in a collective manner. However, it has become evident in recent years that naturally or experimentally induced changes in either the environment or cell components can induce adaptive switching between different migration strategies. This phenomenon is named plasticity or transition [19].

When the cell-cell adherence junctions are weakened by modulations, individual cells attain separation from multicellular sheets or cohorts and disseminate in a mesenchymal manner. Epithelial to messenchymal transition (EMT) is implied in several developmental processes as well as in invasive cancers, separated cells acquire spindle shaped morphology that employs integrin-mediated force generation for tissue cell invasion in the form of single migrating cells or by multicellular cohorts [57, 83] . Collective to amoeboid transitions (CAT) occurs when the separated cell disseminate in an amoeboid manner [74, 84], alternatively up-regulation of cell-cell adhesion molecules can contribute to a transition from individual to collective migration [57].

Similarly, weakening of cell-ECM interactions can cause mesenchymal to amoeboid transitions (MAT). A fundamental pathway ascertaining the interconversion of MAT is the balance between RhoA/ROCK and Rac [85]. MAT interconversion is triggered by pathways that either directly or indirectly weakens Rac and strengthens RhoA/ROCK [56, 86, 87]. Inhibition of ARHGAP22, a GTP-ases activating protein (GAP) [87], guanine nucleotide exchange factors (GEFs) DOCK3/NEDD9 [87], the E3 ubiquitin ligase, the Smurf1 [88] and Rab5 mediated endocytosis all downregulate Rac and induces MAT interconversion [30]. Pathways that triggers RhoA also induce MAT interconversion, including inhibition of negative regulators of Rho such as p90RhoGAP [89].

Studies have shown that plasticity is also induced when cell-ECM interaction is manipulated by modulating integrin activation that contribute to the transition from amoeboid to messenchymal migration in myeloid cell [90]. Alternatively in 3D, the transition from mesenchymal to amoeboid migration occurs when the adhesion formation is inhibited by the downmodulation of β1 integrin or tyrosine kinase c-Src [91, 92]. Plasticity is also induced by the changes in the ECM dimensions, such as cells with the more spread morphology in 2D adopt a spindle shaped mesenchymal phenotype and migrate vertically in a 3D matrix [93]. Similarly, inhibition of surface protease activity also contributes to the transition from mesenchymal interstitial cell to ameoboid migration [84, 94]. A recent study has shown that adhesion strength and physical confinement can also influence the plasticity of cell migration and induce messenchymal- to- amoeboid transitions (MAT) [95].

Thus, modulation of cell-cell, cell-matrix adhesion, cytoskeletal and mechanics, proteases and physical confinement are the key determinants of plasticity or adaptive switching between modes.

1.6 CELL-MATRIX ADHESION COMPLEXES

Cell-matrix-adhesion complexes (CMACs) contain a core composed of clustered, transmembrane integrins bound extracellularly to extracellular matrix (ECM) proteins, thereby providing cell-to-ECM adhesion. The intracellular domains of integrins connect indirectly to the F-actin cytoskeleton via numerous adaptor proteins, providing a link between the ECM and F-actin and allowing the transmission of mechanical forces. This facilitates force sensing and the application of mechanical forces necessary for cell migration. In addition, integrin cytoplasmic tails also link to diverse pathways controlling a variety of cellular functions such as cell proliferation, survival and apoptosis. Aditionally, CMACs function as hub, which detect, coordinate, transmit, adapt to and generate various signals, necessary to control many cellular functions [8].

CMACs vary in size, morphology, location, componentry and linkage to F-actin. CMACs are categorized in to different types [96, 97]; focal points or nascent adhesions are small in size and form near the leading edge in protrusions and linked to F-actin; focal complexes (FC) are larger than nascent adhesions, linked to the cortical actin or F-actin and reside in the lamellipodium [98]; focal adhesions (FA) are large and mature adhesion that are linked with actin stress fibres [99, 100]; fibrillar adhesions are elongated in size, and physically link extracellular fibronectin fibers with actin stress fibers [70]; Podosomes are different from FA and FC, form a ring shape and contain diffuse membrane domain of integrins and component proteins surrounding the dense actin core [101]; Invadopodia are actin rich structures present on cancer cells and closely resembles to podosomes [102]. Importantly, CMACs are highly adaptive, they respond to changes in the environmental stimuli by modifying size, shape, localization, composition and dynamics [8].

1.6.1 Composition of cell-matrix adhesion complexes

1.6.1.1 Integrins

Integrins are the main cell surface receptors for ECM proteins. Integrins belong to the family of transmembrane proteins. Integrins are heterodimeric receptors generated by the non-covalent pairing between alpha and beta subunits. Each alpha and beta subunits have a relatively large extracellular domain and a short cytoplasmic tail. In mammals, there are 18 alpha and 8 beta subunits, which pairs with each other in varying combinations to generate 24 distinct integrin receptors. Different integrins bind to different ECM components, thereby the integrin variety enable the cells to sense the local microenvironment [103]. Each integrin subunit has a distinctive expression patterns. For example integrin β1 is ubiquitously expressed, the β6 subunit is expressed in during wound healing in adult stages [104].

The extracellular domain of the integrin receptors binds with different ECM protein ligand and control cell adhesion, while the intracellular cytoplasmic domain form complexes with and adaptor proteins [105]. They are involved in bi-directional signal transmission. In outside signaling, cell attachment to the ECM protein ligand transmit information via integrin receptor to the inside of the cell, which is important in many cellular processes such as cell proliferation, differentiation, growth, cell survival and cell migration. Conversely, intracellular signaling is mediated by various proteins that can induce changes in the integrin affinity and clustering. This process is called inside out signaling [106].

1.6.1.2 Adaptor and signalling proteins

CMACs vary in size, morphology, localization and contain variable composition of cytoplasmic proteins. Small nascent adhesions are associated with few component proteins while large, mature focal adhesions are associated with a large number of component proteins. There are about 2412 CMACs component proteins identified so far [107]. The CMACs components are further sub-divided based on their function [108]. 1) Adaptor/scaffolding protein which lack enzymatic activity 2) signaling proteins.

Integrin mediated binding to the ECM initiates clustering of CMAC component proteins including signaling and adaptor/scaffold proteins and is associated with initiating signaling cascade. Adaptor proteins provide physical linkage between integrin and actin e.g. talin, tensin, filamin, plectin and α-actinin. Adaptor proteins also associated with other proteins and can behave as a scaffold. For example vinculin, paxillin, α-actinin and zyxin function as scaffold proteins. Focal adhesion kinase (FAK) is an example of a signaling protein with an intrinsic kinase activity which modifies the integrin downstream effector proteins and potentially involve in initiating signaling cascade. c-Src also modifies several proteins such as FAK, paxillin, tension and p130Cas, permitting them to transmit to the other components [15, 108-110].

Moreover, a super resolution fluorescence microscopy such as interferometric photoactivated localization microscopy (iPALM) has revealed that CMACs component proteins are organized in different layers and possibly linked to distinct function of each protein. For example focal adhesion kinase (FAK) and paxillin are integrin cytoplasmic tail are present in lower integrin signaling layer, talin and vinculin are present in middle force transduction layer and zyxin, vasodilator-stimulated phosphoprotein (VASP) and α-actinin are present in an upper actin regulatory layer [111].

1.6.1.3 Microfilaments

Polymerization of G-actin monomers to form filamentous actin at the leading edge is a crucial step in cell migration [112]. The cell leading edge protrudes owning to the addition of G-actin monomers at the barb ends of filamentaneous actin and thus driving cell migration [113, 114]. The filaments are coordinated in two different ways depending on the type of

protrusions such as lamellopodia contain branched filamentous actin also called “Dendritic network” while filopodia contain a bundle of parallel filaments [115].

The Rho GTPases, RhoA, Rac1 and Cdc42 are involved in regulating the actin dynamics in protrusions [116-118]. RhoA activates profilin that upon binding with actin monomers enhances the rate of actin polymerization. RhoA acts on mDia1 and elevate actin polymerization. Arp2/3 is involved in branching of filamentous actin in the lamellopodium and it is regulated either by the Cdc42/WASP or Rac1/WAVE-protein complexes [119].

Myosin II is a family of actin binding proteins that crosslinks with actin filaments and is involved in generating contraction forces [120, 121]. The actomyosin contractility exerts pulling forces at the cell rear and resulting withdrawal of cell-substratum interactions. Myosin II activity is regulated by phosphorylation of the myosin light chain (MLC) by several kinases such as myosin light-chain kinase (MLCK) and Rho-associated protein kinase (ROCK) [120].

1.7 CHALLENGES IN UNDERSTANDING CELL MIGRATION

The cell migration process arises from a hugely complex network of molecular interactions. Most studies performed so far have used reductionist approaches to understand the cell migration process, by focusing on a few biological components. While reductionist approaches provide a useful knowledge regarding the influence of individual components underlying the cell migration system, they are less able to provide in-depth understanding of how these components collectively interact to constitute and regulating the complex cell migration system. The emerging field of systems biology has made remarkable progress in recent year, including high throughput screening in genomics and proteomics studies that have provided extensive information on underlying components. These methods are increasingly capable of dealing with the complexity of biological systems, especially at the cellular level. However, such approaches are unable to resolve essential elements of cell system organization and behaviors, such as the high degree of heterogeneity intrinsically present in migrating cell populations. Heterogeneity arises at three different levels: intercellular, temporal and intracellular spatial heterogeneity. Cell polarization is an example of spatial heterogeneity whereby the migrating cell acquires front and rear polarity through the asymmetric distribution of intracellular machinery, including organelles such as the nucleus and Golgi [72].

To resolve such heterogeneity, alternative approaches are needed, including studies of the spatiotemporal dynamics of cell migration at the single cell level. While the full, spatiotemporally resolved molecular complexity of the cell migration system is currently inaccessible, two macromolecular entities, namely cell-matrix adhesion complexes (CMACs) and F-actin, provide a means to abstract this complexity to a level that is tractable with imaging approaches, which themselves provide vital sensitivity to spontaneous or induced heterogeneity.

1.8 SYSTEMS MICROSCOPY: AN EMERGING METHODOLOGY

Notably, imaging approaches uniquely provide the spatial (molecular to cellular) and temporal resolution scales, necessary to capture information embedded in such natural heterogeneity. However, with recent technological advances in fluorescent proteins, automated fluorescence microscopy, quantitative image analysis tools and related statistical analysis and modeling techniques, it has become possible to perform quantitative imaging in a systematic manner – allowing systems biology through single cell imaging. This loosely defines the Systems Microscopy approach. As such, systems microscopy is now an emerging research methodology in the life sciences, with the potential to potently complement existing omics approaches.

1.8.1 Components and concepts of systems microscopy

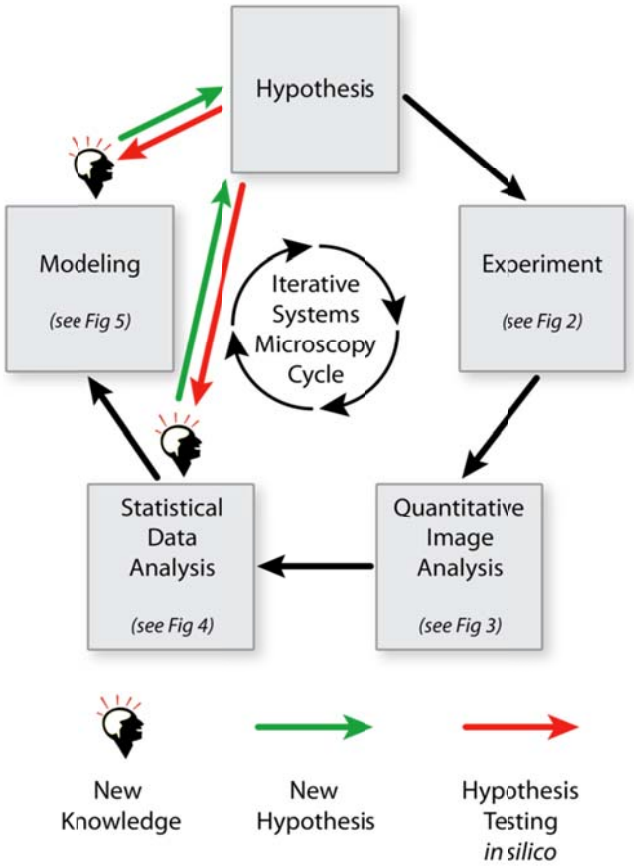

Figure 2: A schematic view of Systems Microscopy approach (Adapted from Lock et al., 2010)

2 AIMS

Specific aims for the papers included in this thesis:.

I. To explore the causal wiring underlying the cell migration system.
II. To disentangle the regulatory associations underlying cell migration and membrane dynamics.
III. To elucidate the effect of varying matrix ligand density and intra-cellular tension on cell polarity and migration.
IV. To elucidate the heterogeneity underlying mesenchymal migration and its regulation.

3 MATERIAL AND METHODS

Experimentation

The experimental conditions were standardized to reduce the experimental variability. The following parameters were given specific attention in terms of standardization: 1) Cell confluency has been implicated in influencing gene expression and proliferation in many cell lines. To overcome this potential source of experimental variability, we always performed passaging of cells approximately at 90% of their confluence. Also standardizing, how the cells were split, seeded and treated with inhibitors before the experiments and always used a same amount of trypsin and other reagents including inhibitors to ensure uniform treatment of cells. 2) A cell counter (Millipore) was used to automatically count cells to assure the same number of cells were seeded each time. 3) Robot mediated coating and washing steps were used to ensure uniform labeling of the matrix proteins, an even coating was then confirmed by conjugation of purified fibronectin with Alexa 647 fluorophore (Molecular probes, Invitrogen). 4) We tested coating of matrix proteins on 96-well glass bottom plate from several commercially available manufacturers and found that the Zell Kontakt manufacturer was best suited for coating with matrix proteins. We also found that acid treatment of a glass is not ideal for coating, as it produces more roughness of the glass surface and less absorption of the matrix protein.

Imaging

Image acquisition is a major limiting factor for performing parallel sampling and thus limiting the throughput capacity. Moreover, the required frequency of sampling is also dependent on the dynamics of the studied cellular processes. In most of our studies (paper I, paper II and Paper IV), we standardized imaging conditions by focusing on the followings: minimum exposure light, good image quality (low signal/noise ratio), improved cell viability, better autofocusing, an optimum sampling frequency to record the cell migration process, reasonable no of imaged cells. We found the best condition after extensive testing, which covered all above mentioned aspects. We then performed live cell imaging using Galvano scanner of Nikon Air confocal microscope at 5 min interval for 8 h with a pixel resolution of 0.21 μm. In one study paper III, we were unable to use a previously optimized imaging condition to perform parallel sampling of 20 conditions with a reasonable throughput, which became impossible to achieve with the low speed Galvano scanner. Therefore, we switched to high speed Resonance that enabled us to meet the requirements of this study. We found the best condition after extensive testing and performed parallel sampling of 20 experimental conditions with a reasonable throughput using a Resonance scanner, 2X zoom, 3×3 montage fluorescent images using Plan Apo VC 20x/0.75NA DIC objective with a pixel resolution of 0.63μm. Approximately 150 cells were acquired and tracked for 15 h with an interval of 5 min. CO_2 and humidity conditions were always maintained during imaging.

Image analysis

There are several software packages available for quantifying fluorescence images. For example Imaris, Volocity, NIS Elements, Slide Book, Image Pro Plus, Zen, Amira, BioImageXD, Icy, Cell Profiler, 3D Slicer, Python, Image Slicer and Fiji. Each software package contains certain modules and sometimes it is microscope specific. Thus, each package has its own strength and weaknesses. We used a custom built software called Patch morphology dynamic software (Digital Cell Imaging Laboratories, Keerbergen, Belgium). The major reason for the development of this particular software was when this project was started nine year ago, no suitable segmentation software was available that was able to segment and track CMACs with high quality. Using this software, cells and CMACs were segmented using fluorescent channels and tracked over time by using tunable algorithms. The cells touching the image border were excluded from analysis. The segmentation and tracking of cells and CMACs were affirmed again by visual monitoring. PAD also enabled us to segment and dynamically track closely associated CMACs. CMACs with an area above $0.05m^2$ could be accurately segmented [122]. Background intensity correction was employed to remove the optical noise within each CMAC per channel. PAD enables the multi-scale quantitative extraction of (organization and dynamics) features of cell and CMACs.

Image preprocessing

After PAD segmentation, image data were imported to Matlab (Mathswork) and pre-processed prior to statistical analysis. This pre-processing included the exclusion of CMAC tracks detected for less than three consecutive time points. CMACs were removed from the first and last frames of an image sequence for the life time analysis due to the incomplete life time information. Within each channel, CMACs fluorescence intensities were standardized to the control condition per experimental repeat by taking the median value of mean CMAC intensities in the size range between 0.15 and 0.2 μm^2. Cells and CMACs trajectories were smoothed via smoothing splines in order to remove a random noise which arises from the imaging system. Smoothing factor was carefully selected by visually examine the real and smoothed trajectories in several different cells.

Statistical analysis

Several univariate and multivariate statistical methods were employed to interrogate quantitative image data.

Univariate statistical analysis

Several methods were employed to visualize distributions of individual variables between groups such as probability distribution function and cumulative distribution function. We also employed the two sample Kolmogorov-Smirnov (KS) test, which is a non-parametric test and compares the distributions between groups by calculating distances between them.

Variable correlation based heat maps

Heat maps were employed to visualize pairwise associations between variables, wherein individual correlation values were represented as colors. Pearson's correlation coefficient was used to measure a linear correlation between two variables, while Spearman's correlation coefficient, a non-parametric approach was used for non-linear and monotonic relationships

Correlation values range from +1 (strong positive correlation), 0 (no correlation) and -1 (strong negative correlation).

Multivariate statistical analysis

Canonical vector analysis

Canonical vector analysis (CVA) involved the linear combination of variables which maximizes differences between groups or within a group. It was calculated using eigenvalues decomposition of covariance data matrix within group or between group.

Principal component analysis

Principal component analysis (PCA) is another dimension reduction method. The PCA involved the linear combination of original variables which the maximizes variation between the multivariate observations. The first principal component PC1 provides maximum variation between multivariate observations and PC2 is the second and so on. PCA was calculated using singular value decomposition of a normalized data matrix.

4 RESULTS AND DISCUSSION

4.1 PAPER I

Plasticity in the macromolecular scale causal networks of cell migration

This study entailed two parts; first the development of a unique Systems Microscopy approach, incorporating single cell imaging, quantitative image analyses, and the adaptation of statistical methods for the analyses, then employing this approach for making biological interpretations.

Though the full, spatiotemporally resolved molecular complexity of the cell migration system is currently inaccessible, two macromolecular entities, cell-matrix adhesion complexes (CMACs) and F-actin, provide means to abstract this complexity to a level that is tractable with imaging approaches. CMACs and F-actin represent a regulatory hub as well as a dominant interface across which information is transmitted bi-directionally between cells and their environment, an interface that is critical for cell adhesion and migration. It is a basic premise of our Systems Microscopy approach that quantitative, imaging-based analyses of these structures provide a rich source of information regarding the status and mechanisms of the cell migration system as a whole.

In this paper, we performed live confocal imaging of single H1299 (metastatic non-small cell lung carcinoma) cell migration on fibronectin-coated glass-bottomed plates. These cells have been selected for stable expression of EGFP-Paxillin (CMAC marker) and RubyRed-LifeAct (F-actin marker) to allow detection of these critical macromolecular assemblages implicated in cell migration. Image analysis involved automated extraction of quantitative information from the macromolecular- (CMACs, F-actin) to the cellular-scale (e.g. cell shape, CMAC number), including both static (morphology, position, intensity etc.) and dynamic features (CMAC stability, cell speed, etc.). We quantified 88 variables and these can be further divided into two additional categories: those variables that define the System behaviour, e.g. migratory behaviour of individual cells; and those variables that define the System organization, such as the status of all recorded molecular and cellular-scale features underlying cell migration. Crucially, the simultaneous recording of System organization and -behaviour features enable an integrated analysis of their inter-relationships under various experimental conditions.

We deployed various statistical techniques (principal component analysis (PCA), canonical vector analysis (CVA), expectation-maximization (EM) algorithm, hierarchical clustering and elastic net regression) to test if there is a correlative linkage between the System organization and behavior. We found that the measured features defining the System organization are biologically relevant to the process of cell migration (System behavior) and thus that our approach is useful. To extend beyond purely correlative analyses, we employed a new implementation of the Granger causality concept, based on auto-regression, to examine the

causal (directional) rather than correlative (non-directional) interactions between system features. This analysis was initially performed using control cell data only, thereby leveraging the natural heterogeneity of these cells to define the "unperturbed" status of the cell migration system. Using Granger causality, we identified organizational features that either causally influenced cell migration or were influenced by cell migration speed. Notable among these features are adhesion stability and adhesion F-actin content, which both causally influenced cell migration speed. Previous studies used simply correlations to assume causality upstream and downstream of cell migration.Thanks to our causality approach, we found that cell speed can also exert causal influence over organizational features, including cell shape and adhesion complex location.

We also identified a chain of causative interactions that extend both up- or down-stream of cell migration speed. We next assessed the sensitivity of this putative causality chain to perturbation. We found that this chain of causative interactions was preserved following inhibition of ROCK, while no evidence of the described causal interactions could be detected following Rho activation. We therefore concluded that the wiring of cell migration is differentially sensitive to perturbation and therefore is contextually dependent. As a result, this very comprehensive study provides both technical advances and unique contributions to the biological understanding of cell migration, principally by being the first to outline the macromolecular-scale causal wiring of cell migration.

4.2 PAPER II

Disentangling membrane dynamics and cell migration; Differential influences of F-actin and cell-matrix adhesions

Cell migration and the dynamics of membrane protrusions and retractions are overlapping processes, making it difficult to assess the regulatory mechanisms that differentiate these processes. Interestingly, a recent study showed that cancer cell invasion in 3D may be better predicted by 2D membrane dynamics rather 2D cell migration [123]. This implies that the regulatory mechanisms underlying these processes are distinct from each other, irrespective of the fact that cell membrane dynamics and cell migration processes are highly overlapping process in highly motile cells.

In this study, we used a combination of multi-scale single cell imaging, quantitative image analysis and systematic statistical analyses to separate the regulatory associations underlying either cell migration or membrane dynamics. In this study, we introduced a new measure of relative membrane dynamics, corrected membrane dynamics (CMD), which is statistically independent of cell migration speed. This enabled us to compare the variation in cell speed or CMD in relation to corresponding changes in the features of core migratory machinery such as cell-matrix adhesion complexes and the F-actin cytoskeleton.

Importantly, this approach leverages natural heterogeneity to expose such correlative links, rather than using experimental perturbations. We found that F-actin features were strongly associated with membrane dynamics while cell migration was more strongly correlated with adhesion-complex features. Moreover, these correlative linkages were often non-linear and context-dependent, changing dramatically with spontaneous heterogeneity in cell behaviour.

4.3 PAPER III

Plasticity and context-dependent regulation of the relationship between cell migration, morphology and nuclear-Golgi polarity

Cellular plasticity is defined as the ability of cells to change in response to the varying environmental conditions. In this paper, we developed a systematic experimental and analytical approach to study cellular plasticity associated with a complex adaptive process of cell migration and polarization, using a model system addressing the coordination between cell migration and cellular asymmetry, i.e. the Nuclear-Golgi positioning.

To achieve this we performed live imaging of single randomly migrating H1299-PO cells (human non-small cell lung carcinoma) stably expressing a polarity sensor construct consisting of β-1,4-Glactosyltransferase-GFP (Golgi marker) and Histone 2B-mCherry (nuclear marker) previously described [124] and labeled with a far-red membrane dye (Membrane marker). We used a 2-dimensional (2D) condition array composed of twenty conditions wherein Fibronectin (ECM ligand) and Y-27632 (Rho-Kinase-Inhibitor) were progressively co-varied to modulate extracellular ligand density (adhesion strength) and intracellular tension.

We quantitatively measured the adaptive responses of nine key cellular features (morphological and dynamic). We analysed changes in feature values, inter-feature relationships, as well as temporal coordination between nuclear-Golgi and cell motion dynamics. We found plastic responses of cellular behaviours e.g. cell motion angle, cell polarity angle and polarity and motion alignment. Moreover, polarity and motion alignment and cell motion angle dynamics displayed non-linear and non-monotonic relationships to cell speed and correlative relationship between them are context-dependent. Some of these relationships were susceptible to decoupling with the reduction in tension or adhesion levels. Moreover, we found that the forward polarity of the Golgi is an ordered cellular state in contrast to backward polarity with respect to coupling of motion, polarity and and morphological features . We report that in the majority of cases, motion and asymmetry are coordinated and that the different type of coordination coincide with specific cellular behaviors.

4.4 PAPER IV

Heterogeneity in mesenchymal motility reflects adaptive switching between two distinct migration modes

Mesenchymal cell migration is profoundly heterogenous. However, it is not clearly evident whether this heterogeneity results either due to progressive cell variability (i.e. the single "stretchable phenotype") or transitions between distinct migratory modalities ("switching" between distinct phenotypes).

In this study, we performed confocal live cell imaging of H1299 (non-small cell lung carcinoma) cells, stably expressing EGFP-paxillin (CMAC marker) and RubyRed-LifeAct (F-actin marker) using Fibronetin coated glass bottom plates.

We found that in fact mesenchymal migration can be divided into two divergent modalitites, termed "Continuous" and "Discontinuous", which emerge spontaneously, in parallel and alternating with each other, within a range of cell types and conditions. Moreover, Discontinuous migration produces faster but less persistent migration characterized by reduced temporal coordination of membrane protrusions and retractions.

We then explored the underlying organization of each distinct mode. We exploited a system microscopy approach and quantified 55 organizational features determining cellular-scale morphology or state (e.g. size, position, number, density, morphology) and dynamics (e.g. motion, stability, rate of area/density change) of core migratory machineries including CMACs and F-actin. This multi-scale quantitative data is acquired on per cell and per time point. This permitted us to inquire the organizational states which produce particular migratory behaviour. We found that each migratory mode comprises distinct underlying macromolecular configurations that undergo unique patterns of remodelling in correlation with changing cell speed, employing fundamentally divergent migratory strategies.

As each mode has distinct underlying macromolecular organization, we next evaluated if the effect on organization feature values of the perturbations could potentially cause corresponding changes in the frequency equilibrium between modes. We found that the frequency equilibrium between Continuous and Discontinuous modes could be shifted by targeting each of three core mechanisms at the intersection between cell-matrix adhesion and the actomyosin system, implicating Talin, Rho kinase and Fibronectin as key molecular regulators of these migration modes. Interestingly, while each mode shows some capacity to adapt to experimental perturbation ("adaptive stretching"), switching between modes provides access to an extended adaptive range and is the dominant response to applied perturbations ("adaptive switching"). Overall, we comprehensively characterize cell migration behaviours, macromolecular organization, regulation and adaptive capacity, thereby defining two distinct forms of mesenchymal migration.

5 CONCLUSIONS AND FUTURE PERSPECTIVES

Reductionist approaches have been widely applied in the cell biology field, typically examining cellular processes - including cell migration - by piece-wise targeting of individual molecular components, to establish a catalogue of component functions. However, it is arguable whether assembling such a functional catalogue of all the components underlying a complex process is sufficient to acquire a complete understanding that process.

Accordingly, to achieve a broader view of the cell migration process likely demands a simultaneous recording of systemic information related to both cellular organization and dynamics. We therefore developed such an alternative approach, termed "Systems Microscopy", involving multi-scale quantitative imaging to simultaneously evaluate the organization of core machineries (CMACs and F-actin; macromolecular scale) and associated cell processes (membrane dynamics, cell migration; cellular scale) on per cell basis. Through this approach, it becomes feasible to exploit natural cellular heterogeneity (between cells and over time), so as to define relationships between features of the system, and the structure of variation at any accessible scale. By leveraging natural rather than artificially induced heterogeneity, this approach provides particular benefits, given that perturbations (e.g. molecular targeting) can impinge on numerous cellular processes unintentionally, making it difficult to infer functional connexions.

In paper I we employed a systems microscopy approach combined with the Granger causality concept, which is introduced for the first time in the cell biology settings, to examine the causal (directional) rather than correlative (non-directional) relationships between organizational and behavioral features of the cell migration system. A comparison between unperturbed and /modulated cells provided evidence that causal interaction patterns are in fact plastic and context dependent rather than stable and generalizable. Thus the systems microscopy approach - together with Granger causality analysis - have the capacity to provide new insights regarding the complex cell migration process as well as other complex dynamic cellular processes.

In paper II, a systems microscopy approach is applied again to disentangle the regulatory associations and dependencies between two highly inter-connected cellular processes, namely cell-migration and cell–membrane dynamics. This is again based on utilizing natural cellular heterogeneity. This study showed that membrane dynamics are most closely correlated with F-actin features, while cell migration corresponds more strongly with CMAC features. We also observe that, most often, these linkages are non-linear and context-dependent, changing with spontaneous changes in cell behaviour. This study has provided a novel exploratory approach, for the perturbation-independent interrogation of complex and highly inter-connected cellular processes.

In paper III, we introduced a systematic experimental and analytical strategy to study cellular plasticity associated with the composite adaptive processes of cell migration and cell

polarization. In this study, we described the plastic responses of cellular behaviours to two, simultaneously varied sources of regulation, thereby revealing non-monotonic and context-dependent inter-relationships. We showed that these relationships are susceptible to decoupling by reducing intracellular tension and adhesion levels. Therefore, this systematic approach provides a powerful method to explore cellular plasticity. The findings made therein have broad implications for future studies, especially concerning the emergence of cellular heterogeneity.

In paper IV, we employed the systems microscopy approach to detect and define two divergent modalities of mesenchymal cell migration. Each mode is quantitatively distinct in organization, behaviour and regulation. We are the first to show the spontaneous and the parallel emergence of these distinct modes. This determination will discourage the aggregation of heterogeneous cells with dissimilar behaviours and different dependencies on underlying machineries. In this context, we also demonstrated that the adaptive response of migrating cells to perturbation is principally mediated by adaptive switching (between modes) rather than progressive adaptive stretching (i.e. remodelling of individual modes). This highlights migration mode switching as a dominant adaptive mechanism. We also illuminate key molecular regulators involved in the control of adaptive switching, thereby more broadly implicating subcellular systems in this regulation, including the actomyosin- and cell adhesion-systems. Given these various studies, we consider the system microscopy approach to be extremely useful in studying the cell migration process. Such an approach may also contribute to the understanding of similarly complex and heterogenous cellular processes.

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