Computational Methods to Quantify and Model Collective Cell Migration

Thesis submitted for the degree of Doctor of Philosophy

By

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ABSTRACT

Collective cell migration plays a major role in many essential biological processes. Understanding the molecular and cellular mechanisms of this unique migration mode is limited despite decades of extensive investigations. Numerous computational measures, methods and software tools exist for microscopic single-cell dynamics analyses, but collective-migration setting has been neglected. The current explosive growth in the volume of such data requires high throughput analyses to enable applications such as deciphering molecular and cellular metastatic mechanisms, drug-screening and personalized medicine.

Based on live cell imaging, this Thesis presents a set of quantitative measures for collective cell migration that surpasses current methods. The toolbox includes multi-cellular segmentation, motion estimation and tracking, motion in clusters and measures for cells’ individuality, indirect morphological features, spatial-temporal visualization etc. The measures are validated by comparing performance with current state-of-the-art methods in the field, and exploited for deeper understanding of multi-cellular processes and high throughput quantification.

The research focuses on the induction of collective tumor cell migration in tumor and normal cells by a specific molecular signaling, Hepatocyte Growth Factor / Scatter Factor (HGF/SF)-Met, master regulators of cell motility in normal and malignant cultures. It revealed that HGF/SF-Met activation is sufficient to induce a directional cue triggered by an acceleration wave that traverses the monolayer. The analysis is exploited to study this enhanced mode of collective motility by finding the relations between different characteristics of cell motion, morphology and intercellular coordination. Our finding offers new insight on long-term cell guidance and intercellular communication during collective cell migration.

Additional applications of collective cell migration high-throughput phenotyping are presented. We studied the interplay between tumor cell glucose metabolism and HGF/SF-Met signaling on collective cell migration, by applying spatiotemporal dynamics phenotyping, and demonstrated that HGF/SF-Met activation is more prominent in inducing collective migration than any tested metabolic treatment. We investigated the initial stages of metastasis formation by different activating- and inhibitory-Met mutations in a culture of normal cells, by applying a measurement that quantifies cell motion in respect to its neighbor cells, and revealed that HGF/SF-Met activation is the main factor in inducing individual behavior. These applications validated that our approach can have immediate implications for diagnosis and prognosis, drug screening and development of personalized treatment.
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Chapter 1

INTRODUCTION

The vast amounts of microscopic images acquired in high throughput studies preclude reasonable manual or semi-manual analyses, and hence automatic computational tools become indispensable. Bioimage Informatics, the application of image-processing and computer vision techniques to analyze cellular and molecular images, is thus an important emerging field. However, development of automatic and objective measurements for collective cell migration, a vital mode of cellular motility in many biological processes, has been neglected. Follows, we present our approach to address this challenge and demonstrate its high quality performance in an important biological system.
1.1 BACKGROUND

In vitro collective cell migration is a model system for development, wound repair and cancer invasion (1). Despite many efforts, the mechanisms underlying this mode of motion are obscure and incomplete.

Recently, increased attention has been devoted to study collective migration of cancer cells (2), while focusing on advanced communication, social networking and cooperation to proliferate, spread within the body, colonize new organs, relapse and develop drug resistance (3), (4). Understanding collective cell migration is crucial since the vast majority of cancer deaths are caused by progression from a localized lesion to distant metastases (5). Revealing cellular and molecular mechanisms that promote metastasis invasion may lead to new therapeutic paradigms to target intracellular information processing, cell-cell communication, and cooperation.

Live imaging becomes the standard in collective cell migration investigations. With the advent of high-throughput and high content imaging systems, objective and fully-automated extraction of measurements by means of image-processing and computer-vision have turned out to be indispensable for processing the vast amounts of microscopic images acquired (6), can reveal phenotypes that are invisible to the naked eye (7), and may have immediate implications for diagnosis and prognosis, drug screening and development of personalized treatment (8), (9), (10), (11).

In this Thesis, I present a unique and comprehensive computational approach to quantify in vitro collective cell migration, validate it by comparing to alternative methods and apply it to study specific biological questions. These investigations focus on understanding how Hepatocyte Growth Factor/Scatter Factor (HGF/SF)-Met signaling, master regulators of cell motility in normal and malignant processes, affect collective cell migration using live imaging.

HGF/SF-Met was found to regulate a wave of enhanced motility, directionality, persistent motion, cell stretching, morphological transition and intercellular coordination. This unique phenotype of collective migration is thoroughly studied by applying image-analysis and basic simulations. The quantitative approach is further exploited for high throughput phenotyping of various experimental data, revealing the role of HGF/SF-Met activation under different metabolic conditions and mutations.

1.2 CONTRIBUTIONS

This inherently interdisciplinary dissertation combines computation analysis, biophysical modeling and wet biological experiments, with achievements in the
interface between these tangential fields. It contributes to the following three disciplines: discovering new phenotypes during collective cell migration and deciphering their underlying mechanisms; revealing cellular mechanisms of tyrosine kinase-induced collective migration in both tumor and normal cells; designing, developing and assessing of Bioimage Informatics methods and tools to quantify collective cell migration, that surpass available alternative. More specific achievements are summarized below.

1. **New measures and approaches to quantify collective cell migration:** The team pioneers by directly addressing the problem of quantifying *in vitro* collective cell migration. We offer a rich portfolio of objective measures to enable effective investigations of diverse phenotypes on various experimental and imaging settings. The approach is suitable for in-depth biophysical investigations of general questions in collective cell migration, as well as for high throughput quantification for applications such as drug screening that requires the ability to discriminate between subtle phenotypic perturbations.

2. **A broad and detailed description of an acceleration-wave phenotype:** Waves of accelerating cells were recently reported in several studies beside ours (12), (13), but this enhanced mode of collective migration was not described and studied thorough before. Relations between cell deformation rate, directional velocity, acceleration, directionality, and persistent migration were discerned to propose a general model of this phenomenon. In short, we reveal a directional cue that is triggered by cells acceleration and stretching, suggesting that mechano-sensitive elements in the cell culture convert shear forces to directional motility. Hence, our contribution considerably enhances the knowledge and understanding of collective migration and offers a new insight on long-term cell guidance and intercellular communication during such migration.

3. **The effects of HGF/SF-Met signaling on collective cell migration:** Met activation by HGF/SF is sufficient to induce a wave in tumor and normal cells, and is thus a potential inherent "activation switch". It increases persistent migration and enhances intercellular coordination in both cell lines with similar spatio-temporal patterns. Met-inhibition prevents propagation of the waves in the presence of HGF/SF, suggesting that the complex HGF/SF-Met plays a major role in intercellular communication. HGF/SF is more prominent than (a) glucose-metabolism in inducing collective migration and (b) specific Met-variations in inducing individuality of cells. Our contribution may guide
future investigation on the molecular and cellular mechanisms of tyrosine kinase-induced coordinate cell motility and morphogenesis in metastasis.

1.3 Thesis Outline

Substantial work has been performed in the important field of collective cell migration. Chapter 2 reviews existing, related studies, as well as research performed on HGF/SF-Met-induced cell motility. We focus on collective cell migration in general, its experimental settings and quantification methods.

The methodology used for this research is described in Chapter 3. A set of quantitative measures for collective cell migration is presented based on live cell imaging. The toolbox includes multi-cellular segmentation, motion estimation and tracking, motion in clusters and measures for cells' individuality, indirect morphological features, spatial-temporal visualization etc. Superiority of our approach over alternative methods is demonstrated. Some results described in this chapter appear in (14), (15), (16), (17), (18).

In Chapter 4 we study HGF/SF-induced collective cell migration. The toolbox is applied to carefully investigate the effects of HGF/SF-Met on collective migration of tumor and normal cells. Special attention is devoted to reveal a directional cue triggered by an acceleration wave that traverses the monolayer. By finding the relations between different characteristics of cell motion, morphology and intercellular coordination we offer new insights on long-term cell guidance and on intercellular communication during collective cell migration. Chapter 4 is mainly based on (15), (17).

Chapter 5 presents several high-throughput phenotyping applications to the methods described above.

- High-throughput analysis of wound healing time-lapse experiments is applied to study the cross-talk between glucose metabolism of cancer cells and Met tyrosine kinase growth factor receptor signaling. Preliminary results indicate that Met-activation by HGF/SF is more prominent than glucose metabolism in inducing collective migration of tumor cells.
- Low concentrations of Met mutated variants were transfected to cells and spread in the monolayer to investigate individual-motion of cells. These experiments simulate the phenomenon of cells that maintain amoeboid-like motility while traversing in a tissue consisting of epithelial cells, initial stages of metastasis formation. A novel quantitative measure is presented to examine the motility of transfected cells in relation to their vicinity of
"normal" cells, denoted *individuality*. Preliminary results indicate that Met-signaling plays a major role in cells' individuality.

Chapter 6 proposes future avenues for research in this emerging field and preliminary results. It includes suggestion for computational and biophysical extensions of the work described herein.

The Thesis is summarized in Chapter 7.
Chapter 2

**Related Work**

Collective cell migration is a broad research field ranging from different biological processes, experimental settings, modeling and analysis. We focus on in vitro collective cell migration, specifically in relation to cancer. Our main contribution is development and application of Bioimage Informatics tools, specifically to quantify HGF/SF-Met-induced collective cell migration. Follows, we summarize the current state in each of these research areas, with respect to the research presented in this dissertation.
2.1 COLLECTIVE CELL MIGRATION

Cell migration is defined as the motion of individual cells, strands, clusters or cell sheets from one location to another (19), (20) and takes place in a variety of pathologic and physiologic processes (21), (22), (1). Cell invasion is the 3-dimensional migration of cells that penetrate an extracellular matrix (ECM) and is typically associated with cancer cell metastasis (20).

Collective cell migration plays an essential role during many and diverse biological processes and is the most common motility pattern in living organism (23), (1). In morphogenesis, large clusters of cells travel long distances to reach their ultimate biological destination (24). In tissue repair, sheets of cells move coordinately to repair damaged tissue (25). In cancer, groups of cells invade the extracellular matrix and traverse across normal tissue with extreme efficiency to form metastases (26), (27), (2).

The cellular and molecular regulation mechanisms underlying this process are not well understood. Investigating in vitro models may reveal important phenomena to enlighten generic mechanisms behind this mode of cellular motility.

2.1.1 IN VITRO COLLECTIVE CELL MIGRATION ASSAYS

In studying molecular mechanisms of cell motility and drug screening, high-throughput and high content imaging systems are gaining popularity (10). Traditionally, migration assays are applied to measure the change in migration or invasion rate caused by various environmental conditions or cell types. Several prevalent in vitro assays exist to quantify and study collective cell migration, leading by wound healing assay, and cell exclusion zone assays, which have been widely adapted by experimental and theoretical investigations to reveal the effects of different physiological, physical and chemical conditions on cell migration (10), (28), (29), (30), (31), (32), (33), (34), (35), (36), (15), (12). An artificial wound is usually created by scratching a cell monolayer or using a microfabricated-mask, and the healing is then followed by monitoring the cell migrating towards the center of the wound.

The in vitro model of tumor cells moving collectively studied herein does not consider important parameters that maintain a crucial role in biological processes that include collective motility such as 3D motility and the tissue’s microenvironment (e.g., extracellular matrix resistance (37), (38), (39)). These parameters have prominent effects on collective cell migration with crucial roles in e.g., embryogenesis (1), tumor invasion (1), (40), and tubulogenesis (37), (38), (41). However, the benefits of using in vitro models to study cellular and
molecular mechanisms override: controlling starting-point definition and performing high-throughput screening and analysis (1).

In the standard *in-vitro* wound healing assay (also denoted scratch assay), collective migration of cells toward the wound is induced by a sudden injury created by removal of a sheet of cells from a confluent monolayer (42) (Fig. 2.1), and can be performed by utilizing multi-well plates, with up to 384-wells (43).

Wound healing assays provide several distinct advantages: it is a simple and straight-forward assay that can be performed in any readily available multi-well plate configuration; cells move in a predefined direction to close the wound; the assay surface can be coated with an ECM of choice prior to the experiment; and cell dynamics can be tracked through live time-lapse microscopy. Its main drawbacks are: accuracy reproducing the degree of confluence and the wound size and geometry; scratching methods vary between different laboratories; scratching may damage the underlying ECM and cell debris might affect migration properties (10).

Poujade *et al.* originally suggested a technique to manufacture reproducible model wound with no cell damage (44) by microfabrication-based an elastomeric micro-stencil mask that is placed in contact with a patterned well bottom to adhere cells in parallel lines. Upon reaching confluence, the micro-stencil mask is removed allowing the cells to collectively migrate on the newly revealed surfaces.
Alternative cell exclusion zone assays (also denoted free surface assays) include the commercial Platypus’ Oris™ Cell Migration Assay, and a pillar stencil approach to produce gaps in a monolayer with high reproducibility in terms of size and geometry (32).

Comparing wound healing with free-surface assays demonstrated high correlation (45) with improved reproducibility for the free surface assay (46), (32).

Transmembrane assays (e.g., Boyden chamber (47)) and microfluidic devices (48) are more complicated to perform and less suitable for high throughput collective migration.

In the experiments described here, the wound healing assay was selected as the model experimental setting mainly due to its simplicity and the easy performed in a high-throughput manner. Since wound healing assay is inferior to free-surface assays in terms of reproducibility, being able to achieve statistically significant discrimination between different experimental conditions using wound healing assay is sufficient to prove phenotypic separation.

### 2.1.2 MECHANISMS BEHIND COLLECTIVE CELL MIGRATION

Extensive research has been carried out for many years in various experimental model systems to investigate, describe, analyze, model and simulate collective cell migration. There are several theories concerning the mechanisms behind collective motility (49). "Follow the Leader (50), were cells at the leading edge are assumed to produce force to pull passive followers among those located further away from the front was considered as a common theory (44), (51), (52), (28), (53). For example, Inaki et al. (53) recently demonstrated that directionality can be encoded within a group of cells by the constituents attaining different signaling levels.

However, accumulating evidence implies that the behavior is more complex. Modern microscopy (54) revealed that distant cells extend in what is referred to as ‘cryptic’ lamellipodia against the substratum beneath their preceding cells, evidence that the cells further behind the leading edge do not simply act as naïve followers. Recent measurements of distributions of traction- and intercellular-forces within the monolayer also suggest that the "follow the leader" paradigm is too simplistic (35), (55), (56), (13) and argue that cells farther away from the front are also self-propelled in the collective motility process. Recently, Tambe et al. reported a strong correlation between individual cell’s migratory direction within a monolayer and the local orientation of the maximal principal stress (57), (49), (36). Several mathematical models have been devised to describe collective
migration based on single cell motility and cell-cell interactions (58), (59), (60), (33), (34).

Another theory assumes that cell proliferation expands the colony and thereby generates pressures that cause the leading cells to move (44), (61), (62), (63), but earlier studies demonstrated that migration in mucosal healing is largely independent of proliferation (63). Moreover, proliferation can occur almost exclusively in the void regions and hence cannot provide complete explanation for the general phenomenon (44).

It is likely that intercellular coordination takes a crucial role in normal processes (e.g., (64)) and in most steps of metastatic formation (e.g., (65), (3), (66), (67)). Some explanatory ideas have been suggested, e.g., cells produce substrate traction due to polarized lamellipodia which tend to polarize neighboring cells in the same direction, eventually forming long-range polarization and coordination (55). However, the analysis of the underlying mechanisms by which cells coordinate in space and time is still at an early stage (2).

Waves of accelerating cells that gradually propagate inwards a monolayer of cells, is a unique phenomenon during collective migration that incorporate long-term and long-range coordination. This phenomenon was reported in several cell lines (12), (15), (13), however, was not described and studied thorough before.

We investigate general and specific phenotypes that emerge during collective cell migration, including the waves described above. Advanced analysis is applied to report and find the relations between different characteristics of these phenomena.

### 2.2 Quantify Collective Cell Migration

Characterizing and quantifying collective migration phenotypes of confluent or non-confluent cell monolayer in vitro is important step to understand physiological processes such as development, wound repair and cancer invasion. The prevalent approach is to acquire still or time-lapse cellular images using bright field microscopy, followed by manual or automated extraction of quantitative measures of cellular morphology or dynamics (e.g., (29), (28)).

Wound healing assay, the traditional method to study collective cell motility and migration (68), (69), in Medicine and Biology, is performed by following the closure of a wound formed by scratching a confluent cell culture. The scratch is imaged and measured periodically during the healing process, and rate of change in the wound’s area is recorded and can be compared with other cells and treatments (Fig. 2.2).
(43) adapted the wound healing assay to a 384 wells plate, providing mass data that allows high-quality quantitative analysis which has not been available before. Manual analysis becomes unfeasible when large volumes of data must be processed. The high variability in imaging conditions and cells’ appearance requires robust algorithms that can deal with imaging-diversity automatically, accurately, and (preferably) without the need for parameters-tuning. To achieve these ends, various computational tools and algorithms have been developed in recent years to tackle the problem of automatic quantification of the wounded area, and more generally segmentation to cellular and non-cellular region in bright field images (70), (71), (72), (73), (74), (75), (76), (77), (78), (14). This is a foreground-background segmentation task: no explicit cell segmentation is performed; rather each pixel is assigned a binary label as being part of a cellular or non-cellular region. These tools are limited to quantify global motion of complete colonies or confluence monolayers (more details are in Subsections 3.2.1-3.2.3).

Live imaging is the standard in collective cell migration investigations. Objective and fully-automated extraction of measurements by means of image-processing and computer-vision have become indispensable to process the vast amounts of
microscopic images acquired (6), and can reveal phenotypes that are invisible to the naked eye (7). Several tools and measures have been reported in recent years to exploit the rich temporal information, exploiting local motion-estimation to quantify dynamic intercellular phenomena (e.g., (9), (8), (28), (31), (30)).

The basic common computational step in most approaches is segmentation of an image to cellular and non-cellular regions, the accuracy of which is crucial for further analysis. The next step is local motion estimation: the original image is partitioned to a grid of sub-windows, the maximal cross correlation of each at time \( t \) to its vicinity at time \( t + \Delta t \) is identified as the local motion estimate. Bright field images of confluent cells contain enough textural details to allow using these algorithms with no requirement for fluorescent markers (28), (14). Particle Image Velocimetry (PIV) is the prevalent technique used for this step (79). Once the velocity fields are measured, many quantities can easily be extracted; the simplest is the average velocity of cells within the monolayer, used by (28) to compare velocities of two different cell lines. Other measurements that rely on local velocity estimation are described below.

Particle tracking is a more direct approach than correlation-based methods to measure cell migration. However, automatic cell tracking using bright field imaging in a monolayer is considered a difficult computational challenge as the boundaries between cells are not always well defined (80), (81). Moreover, high similarity between true cell trajectories and the extracted motion fields have been reported (9), (15). Fluorescent staining of cell nuclei enables automatic cell tracking (30), but requires a more complicated experimental setting, do not always perform well on dense populations, and limits the number of fluorescent markers available for molecular investigations.

Cell migration persistence is the ratio between the distance covered (translation) and the total distance traveled in a given cell trajectory. This measure was previously used in (30) and is similar to "chemotactic index" calculation, albeit without predefined direction (82). The range of persistence score is 1 (ballistic motion) to 0, intermediate scores imply zigzag motions.

Quantifying persistence during collective cell migration has been performed manually, allowing tracking of only a few cells at the leading edge per experiment (29). Recently, Ng et al. (30) applied single-cell tracking that relies on nucleus-fluorescent staining to measure the affects of substrate stiffness of persistence. Very few investigations tackled the problem of light-microscopy cells tracking in a setting of collective migration (e.g., (83)). Others have tried to implicitly measure persistence without tracking (9), or measured the angular velocity distribution as an alternative (8). A simple algorithm can perform reasonable but not perfect
tracking. Averaging many non-perfect trajectories can result in powerful phenotyping capabilities ("wisdom of the crowds") (15). This approach enables to quantify persistence of two different cell lines over different imaging conditions during collective cell migration.

The common measure for intercellular coordination is the velocity correlation length and the order parameter quantifying the extent at which distant cells’ velocity correlate (28). This measure is robust as it takes into account all cells, is easy to interpret, and thus widely used in the literature (e.g., (52), (9), (62), (84), (31)). Another similar measure is the velocity angle correlation (8). Ng et al. (30) measured velocity correlation as a function of time and distance from the wound edge: correlation increased over time, and is higher for cells near the wound edge than for those located deeper; high velocity correlation propagates into the cell sheet. In the same study, normalized cell pair separation distance was applied as a measure for long-term intercellular coordination and demonstrated higher coordination for stiffer substrates. Another alternative to quantify the common size of swirls in the monolayer, and compare it for different cells’ densities was proposed by (31). (85) suggested using finite-time Lyapunov exponent (FTLE) analysis as an implicit measure for cell rearrangement. FTLE is a measure of spatial separation between two adjacent points with time (86). The average FTLE reflects how chaotic the flow is within a finite time, and can be used to measure intercellular coordination.

All of the above mentioned measures report values that quantify the general cellular coordination in the monolayer (or in parts of it). The important attribute of grasping explicit groups of cells that move coordinate is missing. Similar ideas, previously proposed for detection of groups of individuals in crowded scenes (e.g., (87)), are based on clustering of local motion estimation or long term tracking (addressed e.g. in (88)). We detect explicit clusters of cooperatively moving cells by applying image segmentation on motion-fields (temporal-), or full trajectories (long-term-coordination). Spatiotemporal distributions of cells moving coordinate are quantified (17). Promising future avenues open up by having these clusters at hand, quantifying cellular phenotypes and molecular markers within and outside these clusters.

Tortuosity is defined as the ratio between the contour length of the edge and the distance between the two extreme points at the top and bottom boundaries of the image (for vertical monolayers). This measure was suggested by (9) to quantify the contour’s roughness. We have previously tried to apply a similar measure (89), which proved to be uninformative for our data (not shown). AVeMap's (9)
tortuosity was also proven to be a "weak" measurement for discrimination purposes.

Morphological features and dynamics are important in cell phenotyping (e.g., (90), (91)), but accurate segmentation of single cells in collective migration is considered a tough computational task, even when cells are marked with fluorescent markers. Several attempts have been made to tackle this problem directly (80), (81), but not proven robustness to various cell lines and imaging conditions. We (14), (15) used image texture as an implicit measure for cell morphology to quantify scatter and wound healing assay. Strain rate is defined as the spatial-derivative of the directional velocity toward the wound, \( \frac{\partial v_x}{\partial x} \), where \( x \) is the direction toward the wound, and \( v_x \) is the corresponding average motion component. This was defined by (92) as an implicit measure for cellular deformation rate. Several studies have used it to quantify dynamics of cell morphological stretching (13), (17). Another measure is FTLE (85) that combines intercellular coordination and morphological changes.

Cell density is important in many studies (52), (62), (84), (31), (28), (35), (36): a strong relation was found between density, velocity and coordination. However, density is usually measured manually (44) or semi-automatically by cell-counting (62). Some algorithms have been proposed for automatic cell counting in a monolayer (93), (80), (94), but these algorithms never proved themselves on diverse experimental data, and their source code is not publicly available. Fluorescent staining is the only feasible current approach to this end (e.g., (62)), but this method has its limitations. Another important measurement that is currently not feasibly performed is proliferation events. Manual marking of mitosis events demonstrated that more cell divisions occur closer to the monolayer edge during tissue expansion (44). The only attempt that we are aware of to tackle this task in a direct manner is by (95). Measuring cell density and proliferation automatically can serve and advance this field considerably. In future work (Chapter 6) we suggest specific ideas that might enable quantification of these measurements.

Until recently, measuring the forces that cells apply on the substrate during collective cell migration was not feasible computationally-wise (96). (35) was the first to devise a method to report by direct measurement the explicit maps of those physical forces and their distribution. This was done by using Fourier-transform traction microscopy together with a balance of forces that is demanded by straightforward application of Newton’s laws of motion. The experimental setting includes embedding fluorescent beads within polyacrylamide gel substrates, followed by measuring their displacements in relation to a reference
frame. Later, (36), (97) used traction forces to measure stresses within and between cells in an expanding monolayer. This technique, denoted Monolayer Stress Microscopy, was exploited to reveal Plithotaxis, cell guidance by physical forces imposed by adjacent cells (49). Recently, (56) suggested a method to infer the mechanical state of each cell on the basis of image-derived geometric data from a 2D cell sheet, thus enabling force-estimations without the need for special experimental settings. In Section 4.5 we perform preliminary analysis, using traction and stress measurements, to investigate symmetry breaking during monolayer expansion experiments, using data from (13).

Several mathematical models have been suggested to explain mechanisms that govern collective cell migration (e.g., (33), (13), (34)). The juxtaposition between simulated and experimental results is usually performed qualitatively by visual comparison. The type of measures presented above can provide a uniform infrastructure for such comparisons.

Visualization is important to understand dynamic processes. (13) used spatiotemporal strain-rate kymographs to define X-waves, and symmetry. AveMap+ (9), allows extraction of spatiotemporal kymographs of velocity and order parameter. We used kymographs of directionality and acceleration to reveal a directionality wave that follows the acceleration wave (17). Along with heat maps and dimensionality reduction tools, fundamental 2D visualization methods can be powerful approaches to multivariate data (98).

The only two freely available tools that are designated for collective migration quantification were published last year. The first is Cell Image Velocimetry (CIV) (8), a wound interface tracking algorithm that integrates contrast enhancing filtering techniques for microscopy images. It is based on semi-automated, segmentation algorithm that detects the progression of the migrating cell front in consecutive frames, and incorporates local motion estimation (PIV) with the segmentation to provide a set of temporal measurements: the wound edge speed, the mean cell layer speed and orientation, the angular velocity distribution and the velocity angle correlation, a metric for coordinated cell migration. The second tool is Automated Velocity Mapping (AVeMap) (9): a fast, fully automated correlation-based software for cell migration analysis, also based on dynamic edge-segmentation and local motion estimation. More specific details on AveMap are found in Section 3.8.

Enhancing the quantification capabilities of the collective cell migration community will allow faster, elaborated, reproducible, and more effective analysis which will naturally lead to new biological and biophysical discoveries. In order to achieve
this end several fundamental steps must be taken. Firstly, making algorithmic
source code publically available for use. With some exceptions, anything less than
release of source programs is intolerable for computation-dependant results (99),
(100), (101). The community should also endorse and reward researchers making
an effort to supply user-friendly computational tools for non-experts' use (101),
(102). Second, providing benchmarks for algorithmic tasks will allow objective
validation and evaluation of computational methods. The ability to evaluate an
algorithm constitutes a major motivation for computational scientists to develop
new tools. Several repositories for image benchmarks exist (e.g., (103)), but their
number is generally very limited, and is almost non-existent for the collective
cell migration setting. The last fundamental step is making image-based
experimental data publically available. High throughput imaging is emerging,
producing mass data, allowing sophisticated analyses. The potential impact of using
this data is huge. However, image repositories and data standards for imaging
experiments is lacking, especailly when comparing to what has been established
for and by the gene expression, and DNA sequencing communities (104), (105).
In this thesis, I pay special attension to these issues by wrapping our algorithms
with a descent user-interface and making the source code publicly available
(MultiCellSeg, (14)), byy publishing benchmarks (16), and by making our
experimental data freely available (106), (107).

2.3 HGF/SF-Met Induced Cell Migration

The vast majority of cancer deaths result of progression from a localized lesion to
distant metastases (5). Collective cell migration is common in breast cancer and
many other cancer types (108). Several signal transduction pathways and
proteins that are related to collective processes in morphogenesis contribute to
metastatic formation and cancer progression, but their molecular action
mechanisms remain mostly unknown (1), (2).

Met, a tyrosine kinase receptor that is overexpressed in 20-30% of breast tumors
is a strong, independent predictor of poor prognosis (109). Much effort is
invested in targeting Met and its ligand HGF/SF, master regulators of cell motility
in normal and malignant processes (110), (111), (112), (113). In response to
HGF/SF stimulation, cells expressing Met trigger several signaling cascades that,
depending on cell type, lead to a variety of biological events, including
proliferation, migration and invasion (113). Epithelial cells respond to HGF/SF
and Met signals by scattering, epithelial-to-mesenchymal transition and increased
motility and invasiveness, and respond to a gradient of HGF/SF by chemotaxis
(114), (115), (116), (113), (117). Thus, many efforts are invested in targeting Met
and HGF/SF (118).
Single-cell amoeboid migration is characterized by the formation of membrane blebs, irregular protrusions characterized by sudden formation and gradual retraction (Fig. 2.3). Blebbing occurs during migration of several cell lines (119), (120). Induction of membrane blebs enhances the ability of noninvasive to invade three-dimensional (3D) matrices (121). 2D protrusions in response to growth factor cues are predictive of enhanced 3D migration of breast cancer cells (122). HGF/SF-Met induced blebbing plays an important role in cell detachment, amoeboid motility and invasion ability, which are utilized by cancer cells for migration and metastasis (123).

HGF/SF significantly accelerates healing of Madin-Darby Canine Kidney epithelial cells (MDCK), the most common model system for collective migration (44), human pancreatic cancer cell line L3.6pl (117), and mouse mammary adenocarcinoma tumor cell line DA3 (14). But a thorough investigation of the specific molecular or cellular mechanisms that govern this increased collective motility was never performed.
This study focuses on the effect of HGF/SF-Met signaling on collective cell migration of DA3 tumor and MDCK normal cells, to identify the intrinsic and specialized properties that are affected. Specifically, it aimed to understand cellular persistence, directional migration and intercellular coordination, and their relation to the wave of increased velocities mentioned above.
Chapter 3

QUANTIFY COLLECTIVE CELL MIGRATION

The current explosive growth in the volume of microscopic data requires high throughput analyses to enable applications such as understanding molecular and cellular migration mechanisms and drug-screening. While numerous computational methods, measures, and software tools exist for microscopic single-cells dynamics analysis, the collective-migration setting has been neglected. Thus, computational tools for automatic phenotyping are crucial to enable future progress in the field. Following, we present our contribution – a set of automatic and objective measurements, suitable for high throughput analysis. We report our approach’s superiority on available methods, and demonstrate its discrimination and phenotyping capabilities.
3.2 Multi-Cellular Segmentation

Marking the region of interest (ROI) in the wound area for each image is the basic task required to analyze wound healing assays. Automating this process would save time and effort in future studies. Correct automatic wound tagging may enable high throughput analysis, enhancing the temporal resolution of sampling, which is currently very limited. Several algorithms and tools have recently been proposed to deal with this task (e.g., (70), (124), (76), (77)) that significantly improved the ability to perform automatic analysis.

**MultiCellSeg**, a segmentation algorithm to separate between multi-cellular and background regions for bright field images is presented. It is based on classification of local patches within an image: a cascade of Support Vector Machines (SVMs (125), from the LIBSVM library (126)) is applied using basic image features. Post processing includes additional classification and graph-cut segmentation, to reclassify erroneous regions and refine the segmentation. This approach leads to a parameter-free and robust algorithm.

Comparison to an alternative algorithm on wound healing assay images demonstrates its superiority, and a diverse benchmark to compare such algorithms is supplied for the community. MultiCellSeg was applied to quantify the acceleration effect of HGF/SF on healing rate in a time lapse confocal microscopy wound healing assay and demonstrated that the healing rate is linear in both treated and untreated cells, and that HGF/SF accelerates healing rate by approximately two-fold.

This is the first attempt to apply Machine Learning to this problem and to conduct a comprehensive comparison of its performance with that of a segmentation algorithm designed for this purpose. This approach surpasses the existing algorithms in performing this task for a wide range of scales, illumination conditions, and cell types without the need to tune parameters, which is critical in such applications. It is generic and can be used alone or alongside traditional...
fluorescence single-cell processing to perform objective accurate quantitative analyses for various biological applications.

3.2.1 BACKGROUND
A variety of software tools and imaging apparatuses exist to enable high throughput studies. Cellular morphology characteristics that decipher various biological activities, obtained via bright-field imaging modalities such as Differential Interference Contrast microscopy (DIC) or Phase contrast, are considered hard to process and analyze and hence development of designated tools and algorithms for these microscopy categories has been neglected. Most of the existing work on bright field microscopy segmentation relies on some local texture descriptor followed by applying a threshold or global refinement (70), (71). Other approaches manipulate the image-acquisition to make the segmentation task easier (72), (73).

Much of the current microscopy-based cellular research focuses on the single-cell level. This approach relies on algorithmic framework with powerful image analysis tools (e.g., (127), (128), (129), review (130)) and requires single cell segmentation and tracking. However, direct segmentation of single cells in bright field images, especially of cells growing in dense populations, is an extremely challenging task and is prone to algorithmic errors. These errors are mainly derived from the difficulty to locally define the borders single cells growing in clusters, a task that is sometimes not trivial even for an expert. Several algorithms exist for this task (93), (81), (80), but their robustness is not sufficient for confluence monolayers with different cells and varying imaging conditions. We propose a specific application to analyze clusters of cells, in addition to the common fluorescent-based analyses. This approach is less susceptible to algorithmic faults and noisy data and can be performed on mass data, thus enabling a truly robust automatic analysis that is based on quantitative statistical measurements of cellular regions.

We developed a segmentation algorithm which comprises of a spatially local stage followed by a global stage - to automate the partition of a bright field image into regions of cells versus background.

3.2.2 ALGORITHMS AND TOOLS FOR MULTY-CELLULAR SEGMENTATION
TScratch (70) is freely available software that uses fast discrete curvelet transform (131) to segment and measure the area occupied by cells in an image. The curvelet transform extracts gradient information in many scales, orientations and positions in a given image, and encodes it as curvelet coefficients. TScratch selects two scale levels to fit the gradient details found in cells’ contours, and
generates a curvelet magnitude image by combining the two scale levels, which incorporates the details of the original image in the selected scales. Morphological operators are further applied to refine the curvelet magnitude image. As a final step, an automatic threshold is applied to partition the curvelet magnitude image into occupied and free regions. This approach was first applied for edge detection in microscopy images (132). However, this algorithm suffers from several drawbacks: dependence on parameter settings, shortcoming in detecting smaller wound regions and insufficient robustness to different cell types or challenging imaging conditions.

Several investigators (e.g., (74), (75)) use a combination of edge-detection or simple local texture descriptors and morphological operators. These tools can be tuned to fit specific data sets but bear difficulties in handling diverse ranges of image-acquisition conditions and different cell types. CellProfiler (127) has many useful applications, but its wound healing algorithm uses generic modules that are more appropriate for other applications; additional tools suffer from incompatibility to bright field images (127), (128), (124), or employ image processing tools that are incapable of dealing with data variability (76), (77), (78), (74), as they are mainly based on quantifying edges density or simple local texture descriptors within the image.

3.2.3 MultiCellSeg

MultiCellSeg is a new, freely-available software that is based on Machine-learning classification to segment and analyze cellular regions in bright field images, similar to the general framework described by Shamir et al. (133).

The proposed algorithm is based on statistical learning of the local appearance of cellular versus background (non-cell) small image-regions (denoted patches) in wound healing assay images. As a classification application, it is comprised of two phases, training and testing. In the training phase, a set of (manually) tagged images are given as input to a standard classification algorithm that calculates a linear statistical model to assess the expected appearance of a background patch, the patch classifier. In the testing phase, the acquired model is applied to classify new, untagged images.

A given image (Fig. 3.1A) is partitioned into patches (typically of size 20x20 pixels). For every patch the patch classifier outputs a confidence score that represents the model's "certainty" in that patch being "cell" or "background"; the Euclidian distance of its feature-vector representation to the hyper plane defined by the linear model. This is demonstrated in Fig. 3.1B, where bright pixels are more likely to be "background", while dark are more likely to be "cellular". This is followed by applying an automatically-selected threshold on the confidence
scores to define the initial image segmentation (Fig. 3.1C). The next step is to apply another (separate) pre-trained classifier, denoted the region classifier, to reclassify cell regions previously classified by the patch classifier as background. It is designed to identify spatially connected components of patches that were originally misclassified as "background" (Fig. 3.1D); the patches' grouping introduces a substantial advantage that enables to consider a large region in the image, which contains much more image-textural information than the local patches. Graph-cut based segmentation algorithm is used to refine the spatial classification and produce the final partitioning (Fig. 3.1E-F). This algorithm is denoted MultiCellSeg and is described in detail in Subsection 3.1.7. Using this approach, a small amount of high quality, manually analyzed data can be used to produce large amounts of automated annotated data of similar quality.
Fig. 3.1 **MultiCellSeg Algorithmic overview**

(A) Initial image.

(B) Apply classification on 20 x 20 pixels patches to produce confidence score: image regions with higher intensity are more likely to be non-occupied (background), darker regions are more likely to be cellular.

(C) Discrete version, produced by applying an automatic threshold on the confidence image.

(D) The regional classifier is applied to discard cellular regions misclassified as non-occupied: non-occupied regions contours are marked in white while filtered regions are marked in black (some are pointed with yellow arrows). The union of the black and white contours is the output of the first phase, patches classification.

(E) Initial image's energy map for Graph-Cut refinement.

(F) Final segmentation: result of Graph Cut segmentation using the output of the regional classifier as its baseline.
Given a test image, the following steps are applied to define the background as the region of interest:

- Create patches’ grid, extract texture-based features (such as patch’s gradient histogram), apply cascade of SVMs to classify all patches as cell/background (visualized in Fig. 3.1C, and in more detail in Fig. 3.2);
- Discard cellular regions that were marked as background by the patches classifier and reclassified as cellular by the region classifier (Fig. 3.1D);
- Apply graph-cut and output the region of interest (Fig. 3.1F).

Details of MultiCellSeg are in the Supporting text below.

Utilization of several types of features on several scales makes MultiCellSeg robust for varying conditions. In contrast to other approaches that tend to refrain from fine details to avoid gross mistakes or use data-specific assumptions, our algorithm operates in higher spatial resolution, detects small regions of interest and then decides whether to keep or to discard them via post processing (regional classification), in a fully automated manner. As a result, in many images where the wound is almost healed, our algorithm performs satisfactorily, whereas other algorithms fail to mark open regions, as exemplified in Fig. 3.4.

To further enhance the proposed segmentation performance, one can suit a model to fit a specific experiment, cell type or imaging conditions. This can be exceedingly useful nowadays, when high-throughput experiments are performed, each with hundreds of images (69). To this end, one (or more) image(s) should be manually marked to apply the training phase in our algorithm. This process is only partly automatic, but it requires no-parameter setting and may result in notable improvement in performance with minimal effort.

The automatic, accurate zero-parameters MultiCellSeg may serve as a tool for various biological analyses. MultiCellSeg’s Matlab source code is freely available as standalone software to allow others to use it for wound healing analyses, multi-cellular bright field cells segmentation, and for other applications yet to evolve. The source code and accompanying graphical user interface (GUI) can be found at http://www.cs.tau.ac.il/~assafzar/MultiCellSeg.zip, it is recommended to carefully read the README file (http://www.cs.tau.ac.il/~assafzar/MultiCellSeg_README) before applying it. In the future, we plan to add training capabilities to enable specific designated models for different cell lines and imaging conditions and/or to integrate it as part of a larger project (e.g., (127), (128)).
3.2 Multi-Cellular Segmentation

Fig. 3.2 Patches Classification
(A) Initial image is divided into patches (20x20 pixels per patch for the wound healing application).
(B) Five sets of basic image-processing features are extracted per patch.
(C) Five pre-trained Support Vector Machines (SVM) are applied to classify the feature sets.
(D) A confidence score is produced for each combination of patch and features set. An additional pre-trained SVM is applied on the assembly of the confidence scores.
(E) The final confidence map, brighter patches correspond to high probability of non-occupied regions.
3.2.4 Direct Comparison with TScratch

To the best of our knowledge, the only freely available software for automatic analysis of wound healing that performs reasonably well on bright field images without specific parameter setting is TScratch (70). The quality of MultiCellSeg was therefore compared with it.

Both MultiCellSeg as well as TScratch can be seen as composed of two parts. First, the original image is used to create a new one, in which the intensity of each pixel represents the algorithm's confidence in its classification. Then, this image is used to define the final ROI.

The first phase in TScratch is the construction of the curvelet magnitude image, whereas in our approach, it is the generation of the classifier's confidence image. The second phase in TScratch is the automatic setting of a threshold and then the application of morphological operators. In MultiCellSeg, the second phase includes removal of erroneous tagged regions and contour refinement.

Thus, the comparison of these algorithms is performed in two steps. The robustness of the first phase is measured by examining the Receiver Operating Characteristic (ROC) which plots true-positive versus false-positive classification rates of the pixels in each image across the entire range of possible thresholds of the confidence threshold, encoding the true potential of the underlying approach. The second measure is a direct comparison between the algorithms' final tagging.

To evaluate our segmentation algorithm, we used wound healing images available from TScratch website, images received with the courtesy of Dr. S. Izraeli and Prof. I. Witz (personal communication), and new images that were acquired in our lab. These 126 images were manually marked to quantify our algorithm's performance. Twenty arbitrary images from all data sets were selected to train the patches- and the regional-classifiers.

The 126 images were partitioned to the following four data sets:

- Only 24 images were available from the TScratch website. The imaging configuration is detailed in (70): "Two crosses were scratched in each well, and these were instantly center-imaged at 5× magnification, using a Zeiss Axiovert 200 M microscope equipped with a Zeiss AxioCam MRm camera with maximum contrast (Carl Zeiss AG, Feldbach, Switzerland)". This data set was denoted TScratch;
- 20 images of cell populations of brain metastatic melanoma were acquired in the I. Witz lab using an inverted microscope (Eclipse TE 2000-S; Nikon, Enfield Enfield, CT, USA) fitted with a digital camera (DXM1200F; Nikon).
Ten of these over-produced CLDN1, the other 10, infected with a mock plasmid. This data set was denoted Melanoma;

- 28 DIC images (pixel size 0.625x0.625µm), denoted Init, were acquired using LSM-410 microscope (Zeiss, Germany) in non-confocal mode from 2 single-well experiment in our lab: DA3 cells were plated on glass-bottom 35 mm diameter microwell plates (MatTek, Ashland, MA) and imaged overnight every 3 minutes.
- A set of 54 DIC images (pixel size 1.24x1.24µm), denoted SN15, were acquired LSM-510 microscope (Zeiss, Germany) in non-confocal mode, from 27 different wells acquired in a multi-well experiment performed in our lab: DA3 cells were grown in 24 well plates and imaged overnight every 15 minutes. The position of each scratch was predefined, and a macro that repetitively positions the microscope to each point was executed. The acquired time-lapse images were used for the analysis until occurrence of the first contact between opposing borders of the wound.

Each image was manually segmented to enable comparisons between accuracies of different segmentations. These images are an initial version of the complete benchmark described in the following section.

The ROC curves comparing TScratch with MultiCellSeg are presented in Fig. 3.3. The x-coordinate represents the false-positive rate, which is the percent of pixels that were tagged incorrectly as background out of all cellular pixels of the given image. The y-coordinate is the true-positive rate, which is the percent of background pixels tagged correctly out of all image's background pixels. Each curve was produced by averaging the ROC curves of all images in the data set. The higher the threshold is, the lower the false positive classification rate will be, but this comes at the cost of true positives. When comparing the potential accuracy of several classification algorithms, an algorithm that has higher true-positive rate for any fixed false-positive rate values is proved to be the best. Thus, higher curves correspond to more discriminative algorithms.

To set TScratch's threshold, MultiCellSeg's true-positive rate was used to define TScratch's threshold such that the same true-positive rate was achieved; the total error rate (rate of false-negative and false-positive pixels out of all image's pixels) was compared after applying this threshold. It is important to note that this process actually "upgraded" TScratch; using a predetermined threshold or a dynamic threshold using Otsu's method (134) resulted with inferior performance (results not shown).
Table 3.1 compares the final segmentation results, after patch-classification, and the final segmentation (after applying region-classification and graph-cut refinement) with TScratch’s performance. Each entry is an average accuracy on all images in the designated data set. MultiCellSeg surpasses TScratch (p-value 0.001 for the TScrach dataset and < 0.000015 for the other datasets, paired Student t-test): it outperforms in 75-95% of the images for all data sets, and presents accuracy of over 3% better than TScratch’s. Since TScratche’s accuracy is over 90% for most datasets, our approach actually decreases the rate of misclassified zones from around 10% to around 7%, and this improvement may turn crucial. A qualitative demonstration of MultiCellSeg’s superiority on images with narrow background regions is presented in Fig. 3.4.
3.2 Multi-Cellular Segmentation

MultiCellSeg's local-patches classification approach significantly surpasses TScratch's in all data sets but one (tscratch, see Fig. 3.3D). This data set is TScratch's with many images that contain scattered cells. The region-classification is designed to deal with this problem. When considering the final segmentation, MultiCellSeg significantly tops the alternative in all data sets. In principle, the second phase of MultiCellSeg may be plugged in to enhance the performance of TScratch's second phase, but TScratch seems to be less sensitive to small details, which results in significantly less fine regions then with our approach.

<table>
<thead>
<tr>
<th>Dataset Name</th>
<th>Init</th>
<th>SN15</th>
<th>Melanoma</th>
<th>TScratch</th>
</tr>
</thead>
<tbody>
<tr>
<td>Number of images</td>
<td>28</td>
<td>54</td>
<td>20</td>
<td>24</td>
</tr>
<tr>
<td>Patch Classifier Accuracy (%)</td>
<td>95.5</td>
<td>94.5</td>
<td>90.5</td>
<td>89.8</td>
</tr>
<tr>
<td>MultiCellSeg Accuracy (%)</td>
<td>96.9</td>
<td>95.3</td>
<td>91.2</td>
<td>92.2</td>
</tr>
<tr>
<td>TScratch Accuracy (%)</td>
<td>92.3</td>
<td>92.3</td>
<td>87.0</td>
<td>89.8</td>
</tr>
<tr>
<td>pValue Patch Classifier vs. TScratch</td>
<td>1.9e-4</td>
<td>1.8e-5</td>
<td>4.6e-5</td>
<td>0.95</td>
</tr>
<tr>
<td>pValue MultiCellSeg vs. TScratch</td>
<td>9.1e-8</td>
<td>1.38e-5</td>
<td>3.37e-6</td>
<td>0.01</td>
</tr>
<tr>
<td>Percent of Images for which MultiCellSeg Outperforms TScratch (%)</td>
<td>95</td>
<td>85</td>
<td>90</td>
<td>75</td>
</tr>
</tbody>
</table>

Table 3.1 Segmentation results
Summary of segmentation accuracy and significance. Accuracy is defined as percent of correctly tagged pixels out of the total number of pixels in all images. Accuracy was calculated for the patches classifier (intermediate segmentation) and for the final MultiCellSeg segmentation and was compared to TScratch accuracy on the same set of images. pValue calculated as a paired t-test on the accuracy sequences: patches classification vs. TScratch MultiCellSeg vs. TScratch for each image. Percent of images for which MultiCellSeg outperforms TScratches refers to the percent of images in the dataset that are better segmented by MultiCellSeg in comparison to TScratch.
3.2.5 BENCHMARKING

A uniform framework to benchmark algorithms for the task of multi-cellular segmentation in bright field microscopy images is presented. A large set of manually segmented images from diverse origins were partitioned to 8 datasets and evaluated on 3 leading designated tools ((70), (14), (74)). All raw images, manual segmentations and evaluations are freely available at the Broad Bioimage Benchmark Collection (BBBC, [http://www.broadinstitute.org/bbbc/](http://www.broadinstitute.org/bbbc/)). The

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1 Initial version that includes published results is already available online as a draft, [http://www.broadinstitute.org/~ljosa/bbbc/draft/BBBC019/](http://www.broadinstitute.org/~ljosa/bbbc/draft/BBBC019/), a final version including all data will be available upon the publication date of 16. Zaritsky A, Wolf L, Ben-Jacob E, & Tsarfaty I (2013)
scientific community is encouraged to evaluate new algorithms on this benchmark, and to contribute additional annotated datasets.

We propose herein a uniform framework to benchmark algorithms for the task of multi-cellular segmentation in bright field microscopy images. A set of 171 manually segmented images of 5 different cell lines at diverse confluence levels, acquired in several laboratories under different imaging conditions were partitioned to 8 datasets, as follows (example images are presented in Fig. 3.5):

- TScratch: 24 bright field images of confluent cells available at the TScratch (70);
- Melanoma: 20 bright field images of confluent cell populations of brain metastatic melanoma acquired during a wound healing experiment;

Benchmark for Multi-Cellular Segmentation of Bright Field Microscopy Images. Submitted.. Until then, all raw data can be found here, http://alturl.com/a4x6j.
3.2 Multi-Cellular Segmentation

- **Init**: 28 differential interference contrast (DIC) images of confluent DA3 cells (derived from the mouse mammary adenocarcinoma cell line D1-DMBA-3) acquired during a wound healing experiments;
- **SN15**: 54 DIC images of confluent DA3 cells acquired during a multi-well wound healing experiment;
- **Scatter**: 6 DIC images of Madin-Darby Canine Kidney (MDCK) epithelial cells acquired during a multi-well scatter experiment;
- **Microfluidics**: 13 DIC images of MDCK cells grown in a microfluidic plate acquired during a scatter assay experiment with a Hepatocyte growth factor / scatter factor gradient.
- **HEK293**: 12 DIC images of confluent HEK293T cells acquired during a multi-well wound healing experiment;
- **MDCK**: 14 DIC images of confluent MDCK cells acquired in a multi-well wound healing experiment;

Twenty arbitrary images from these datasets were selected as a training set for algorithms that apply supervised learning, or for adjusting parameters' values. Two freely available tools (TScratch, (70); MultiCellSeg, (14)) and one implementation of a designated algorithm ((74)) were evaluated on these datasets (Table 3.2). All raw images, manual segmentations, and algorithms' evaluations are freely available at the Broad Bioimage Benchmark Collection (BBBC, [http://www.broadinstitute.org/bbbc/](http://www.broadinstitute.org/bbbc/), (103)). We encourage the scientific community to evaluate new algorithms on this benchmark, and to contribute additional annotated datasets.

<table>
<thead>
<tr>
<th>Algorithm / Dataset</th>
<th>Init (N = 28)</th>
<th>SN15 (N = 54)</th>
<th>Melanoma (N = 20)</th>
<th>TScratch (N = 24)</th>
<th>Scatter (N = 6)</th>
<th>Microfluidics (N = 13)</th>
<th>HEK293 (N = 12)</th>
<th>MDCK (N = 14)</th>
</tr>
</thead>
<tbody>
<tr>
<td>TScratch (Geback et al., 2009)</td>
<td>0.96 (0.96)</td>
<td>0.97 (0.96)</td>
<td>0.90 (0.88)</td>
<td>0.93 (0.94)</td>
<td>0.47 (0.47)</td>
<td>0.41 (0.42)</td>
<td>0.91 (0.90)</td>
<td>0.92 (0.93)</td>
</tr>
<tr>
<td>MultiCellSeg (Zaritsky et al. 2011)</td>
<td>0.98 (0.90)</td>
<td>0.98 (0.97)</td>
<td>0.91 (0.85)</td>
<td>0.95 (0.93)</td>
<td>0.56 (0.55)</td>
<td>0.45 (0.35)</td>
<td>0.95 (0.95)</td>
<td>0.96 (0.90)</td>
</tr>
<tr>
<td>Topman et al. 2011</td>
<td>0.98 (0.98)</td>
<td>0.97 (0.95)</td>
<td>0.93 (0.93)</td>
<td>0.75 (0.77)</td>
<td>0.59 (0.58)</td>
<td>0.63 (0.63)</td>
<td>0.86 (0.85)</td>
<td>0.89 (0.93)</td>
</tr>
</tbody>
</table>

Table 3.2 Benchmark results

Evaluation of three designated tools on the 8 available datasets. F-measure was used for evaluation in three forms: mean F-measure of images in the dataset, median, and mean F-measure after threshold adjustment on the training set.
We would like to thank all the people that took part in data collection: Dr. Sivan Izraeli from Prof. Isaac P. Witz's laboratory who acquired the "Melanoma" dataset. Prof. Petros Komoutsakos who approved publishing the images available in the TScratch website as part of this benchmark ("TScratch" dataset). Sari Natan, Dr. Doron Kaplan and Yaniv Goikhman from Prof. Ilan Tsarfay's laboratory who acquired the rest of the datasets. Doron Kaplan labeled region of interest in "MDCK" images.

3.2.6 Quantifying Healing Rate by Applying MultiCellSeg

To validate the effect of HGF/SF on healing rate, we trained models to analyze a specific time-lapse microscopy multi-wells experiment, where DA3 cells in some wells were treated with HGF/SF. An image was sampled every ~75 minutes until first contact formation between cells from opposing borders of the wound. Several arbitrary images were selected and manually marked for training. Every image was segmented and the wound area was examined as a function of time. Two measures were determined in this study: the linearity of the healing process and the change in wound closure rate under HGF/SF treatments.

The healing rate was linear in all experiments ($r > 0.978$, $p < 0.0003$ Pearson's linear correlation coefficient, for all experiments with more than 3 time points), as is visualized by the normalized wound area over time (Fig. 3.6A). The healing slope is calculated based on rate of change in wound area. HGF/SF accelerates healing about 2-fold ($p < 0.0016$ via Wilcoxon rank sum test, which has no prior assumptions on the data distribution, thus it is a strict test) (Fig. 3.6B).

MultiCellSeg was also applied to study migration of Melanoma brain metastasis. Claudin-1, a key component of tight junction complexes, was shown to suppresses the malignancy Phenotype of Melanoma brain metastasis cells (18).
3.2.7 SUPPORTING TEXT: DETAILS OF MULTYCELLSEG

As described above, the segmentation algorithm consists of a training stage that computes a linear model (classifier) designated to distinguish between background and cellular local patches (patch classifier). An additional classifier is trained on spatial clusters of local patches (regions) to reclassify cellular regions that were tagged as "background" by the patches classifier (region classifier).

Given an image at test, a grid of patches is overlaid on it; each patch is assigned to the patches classifier to produce the initial segmentation. Next, regions marked as background are reclassified by the regions classifier. Finally, graph-cut segmentation is applied to refine the wound's contours.
3.2 Multi-Cellular Segmentation

The process is visualized in Fig. 3.1, and the algorithmic details are described in the following paragraphs.

**Learning background patches:** Every image is partitioned into a grid of non-overlapping 20x20 pixels patches. The input for the training set includes the images and their corresponding pixel-wise cell-background baseline marking, performed manually by an expert's visual inspection. From each patch, five sets of features (or combination of features) are extracted; each describes the patch's statistics to be used for separating cellular patches from background patches, as follows:

- Image (global) and patch (local) gray-level mean and standard deviation;
- Patch's gray level histogram;
- Histogram of patch's gradient intensity, a measure for the amount of details in the patch;
- Histogram of patch's spatial smoothness: the absolute difference between each pixel's intensity and its neighborhood.
- Concatenation of features from a broader area surrounding the patch, including mean and standard deviation of gray-level, gradient intensity and smoothness, as well as gray-level histogram.

Five SVMs are trained, one for each set of features. The output of each SVM is a **confidence score** for every patch that measures the classifier's "certainty" in its classification. The confidence of a given patch is defined as the Euclidian distance of its feature-vector representation to the hyper plane defined by the linear model. The five confidence scores per patch, one from every SVM, are further fed, as a vector of length 5, into an additional training session that calculates the final SVM classifier\(^2\), which is used to put weights on the five classifiers' output to achieve the final classification. Such a combination of the output of multiple classifiers is referred to in the literature as stacking (135). It is important to note that the same training set is used to train all six classifiers (five classifiers and the combination of their outputs). The patches-learning phase is illustrated in Fig. 3.2.

To evaluate the contribution of each feature set to the overall patch classifier performance the following analyses were performed:

- Each of the 5 features vectors was evaluated separately in comparison to the combined patch classifier (i.e., using a single feature vector and the corresponding SVM classifier as the patch classifier) on every data set.
- The results obtained using the intensity histogram was significantly inferior to the combined classifier, while the results obtained using the other feature sets were marginally inferior to the features combination.

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\(^2\) Although each of the five feature sets is represented by a vector of different lengths, the SVM is not biased since each set's classifier outputs a single feature that is utilized in the additional training session.
Discarding the intensity histogram feature set and training a patch classifier based on the 4 remaining feature sets resulted with inferior performance compared to the classifier that was trained using all 5 feature sets.

Since the ultimate purpose of the algorithm is to produce accurate and generally robust segmentations, the combination of all 5 feature sets, producing the most accurate segmentation was used.

**Learning cellular regions:** After the classification of local patches, an additional classification of each spatially-connected component of background patches is performed to decide whether it is actually part of the background or not. An additional SVM classifier is trained to perform the global regional classification task. The original image and the patches' confidence map are used to extract the following features per candidate region: the region's size (number of pixels), a histogram based on the original image intensities and another based on the patches' confidence scores. The training session results in a regional classification model later used to discard cells that are tagged as background by the patches' classifier. An example is shown in Fig. 3.1D.

**Graph-cut segmentation:** The final step is refining the contours to fit the image's gradients map, which represent the true cellular-background edges in the original image. This allows achieving higher spatial resolution in segmentation by using the original image's resolution rather than the patch-size resolution that was enforced by the algorithm until this stage. To this end, the graph-cut segmentation approach (135), (136), (137) is applied, the implementation of which is described by Bagon et al. (138). As input, the algorithm receives a mask that represents the prior knowledge about the image and an energy term that defines the cost of different tagging of neighboring pixels.

Eroded versions of the cellular and the background regions together define the prior mask, and the image gradient is used to specify the energy term. A maximal cut is calculated using these constraints, and the result is defined as the final MultiCellSeg segmentation. Examples are illustrated in Fig. 3.1E,F and Fig. 3.4A,B.

### 3.3 Quantitative Approach to Measure Similarity Between Wound Healing Time-Lapse Experiments Under Different Conditions

Can machine-learning be used to automatically classify a time-lapse experiment to the correct treatment? The ability to "predict" the treatment based on the phenotype is stronger than just correlation; it means that the phenotype inherently captures the distinct behavior induced by the treatment.
3.3 Quantitative Approach to Measure Similarity Between Wound Healing Time-Lapse Experiments Under Different Conditions

We propose a generalization scheme that is based on machine-learning classification to quantify \textit{in vitro} collective migration assays across different experimental conditions, capturing even subtle differences by considering spatiotemporal descriptors. The general idea is to represent a full time lapse experiment by a high dimensional vector, representing varying characteristics as function of time and spatial location in relation to the free-edge. This descriptor can be later used as a rich feature vector to cluster similar phenotypes found in different experiments or to use classification for phenotypic discrimination between different experimental conditions. This approach might have immediate implications for diagnosis and prognosis, drug screening and development of personalized treatment (8), (9), (10).

3.3.1 \textbf{DATA AND TECHNICAL DETAILS}

To assess this approach we used two datasets of time-lapse wound healing assays:

- 17 wound healing experiments using tumor DA3 cell line under 3 different treatments (6 - control, 5 +HGF/SF, 6 PHA+HGF/SP), previously reported (15).
- 10 wound healing experiments using MDCK cell line (5 – control, 5 +HGF/SF) were processed.

Raw image data is freely available at "The Cell: an Image Library", (106), (107) to enable reproducibility and additional insights by others (105). Full description of the cell lines and experiments is in Chapter 4.

Given a group of time-lapse videos of wound healing experiments, each performed under known predefined conditions (e.g., treatment, substrate stiffness, cell line), machine learning classification is applied to determine the relative-relations between the different conditions based on their high-dimensional descriptor vectors.

For visualization, principal component analysis (PCA) (139) is applied; projecting the high dimensional descriptors onto a two-dimensional grid, maximizing the variance between these descriptors loci. In order to obtain a quantitative measure for the difference between different experimental-conditions we apply supervised machine learning: training a classifier on some of the experiments and measuring its success of classifying the rest. In our case, classification means prediction of the experimental settings based solely on the descriptor vector. Linear support vector machine (SVM) (125), was selected as the classification machinery. An SVM model is a projecting of high dimensional descriptors of the experiments selected
for training, so that the experiments of the separate experimental-conditions are divided by a clear gap that is as wide as possible in this space. New experiments are then mapped into that same space and predicted to belong to an experimental-condition based on which side of the gap they fall on. Since the number of experiments is small, "leave one out" validation was used; a single experiment is omitted, a classifier is trained on the rest of the experiments and is tested on the omitted experiment. This process is repeated for each experiment. In our case, classification is performed for each pair of treatments. Statistical significance (P-values) is calculated for each pair of treatment by using the Wilcoxon rank sum a-parametric test (140) on classification-confidence score extracted by the SVM for each experiment. Inherently, 2D projection of high dimensional vectors (such as PCA) usually lose some of the information, thus classification results that are superior to what seen in the PCA-visualization are not surprising.

The healing process was partitioned to three conceptual phases, as defined in (15) (Fig. 3.7). *Phase 1*, from the scratch formation until first contact between cells from opposing wound edges; *Phase 2*, from first contact until full closure of the wound, when the wound area is completely covered by a monolayer of cells; *Phase 3*, post wound closure (we limited this phase to 15 time-frames). Dividing the healing process to phases is important since the duration of each phase varies between experiments and treatments. When considering a full wound healing experiment, feature vectors were extracted for each healing phase and concatenated to define the experiment's descriptor.
3.4 Exploiting Image Texture

Single cell morphological characteristics and morphological dynamics are important to understand many biological processes (e.g., EMT (141), (142)). Quantification of cell morphology is a prevalent step in Bioimage Informatics (143), (130). There are several methods for single cell detection and segmentation in bright filed images (94), (93), (81), (80), but none has proven robustness (and also no code is made available for the public). We propose using simple image texture features for implicit quantification of cell morphology in dense populations. This textural descriptor can be used as a fully-automated, accurate, zero-parameters method to classify and score various appropriate applications as detailed below.

3.4.1 Texture as an Indirect Measure for Cell Morphology
It is hypothesized that in dense monolayers of cells, texture can serve as a rough descriptor for cell density and maybe even morphology. We used the Local-Binary Patterns (LBP), a gray-scale invariant texture known to perform well in face-recognition (144), as a texture descriptor. For every pixel, a code is generated based on the number and location of neighboring pixels with higher and lower intensities than that pixel. There are ten possible codes, and their histogram over all cellular pixels is used to describe the image’s texture (Fig. 3.8).
Given a bright field image, the "translation" to a texture-based feature vector was performed in the following manner:

- Down-sample the image’s spatial resolution such that each pixel is of approximated size of 5x5 µm;
- Apply MultiCellSeg to partition the image to cellular and background regions;
- LBP code was extracted for every pixel classified as "cellular";
  - The pixel is compared to its 8 neighbors, higher gray level is encoded as "1", lower as "0"
  - This gives an 8-digit binary number, that is converted to one of ten possible rotation-invariant codes
- The final descriptor is the accumulated normalized histogram of LBP codes for all cellular regions.

### 3.4.2 WOUND HEALING QUANTIFICATION

Each image is represented by the LBP histogram as defined above. A time-lapse experiment represented by a two-dimensional map: the x-axis represents time, whereas the y-axis is the LBP histogram.

Perfect classification is achieved by considering the average cellular texture at Phase 2 (from first contact to full healing, where most morphological changes occur (15), Fig. 3.9A), applying an SVM classifier using "leave one out" validation (control vs. HGF/SF: N = 11, p < 0.0043; PHA+HGF/SF vs. HGF/SF: N = 11, p < 0.0043; control vs. PHA+HGF/SF: N = 12, p < 0.0022, Wilcoxon rank sum test). Naturally, there is a clear visual separation between the textural patterns for treated, untreated and Met-inhibited cells (Fig. 3.9B).
3.4.3 Scatter Assay Quantification

A method to classify and score scatter-assay images was developed, and demonstrated that multi-cellular texture is an excellent descriptor to measure HGF/SF-induced cell scattering.

Cell scattering is the interruption of cell-to-cell interaction that results by dispersal of cells. It is an important phenomenon in pathological, developmental and cell migration investigations. HGF/SF induces cell scattering through the tyrosine kinase-type HGF/SF receptor, c-Met (145), (146), (147), (116). Analyses of scatter assays are almost always qualitative based. Cell scattering is scored by an expert’s manual decision, based on spreading and dispersion of epithelial colonies. Only few attempts have been made to quantify objective measures for cell scattering. Kort et al. (148) suggested a simple image-processing application that detects and counts clusters of cells and single cells based on fluorescent marking. Although proving high correlation with manual counting, they do not show a quantitative measure to describe cell scattering. Powell et al. (149) quantified scatter response of MDCK cells to HGF/SF by measuring the distances between nearest neighbors’ cell nuclei, and demonstrated that addition of low concentrations of HGF/SF resulted in cell dispersion. Loerke et al. (116) recently quantified dynamics of scattering by using cell tracking for short periods of time to defined direct scattering measurements: increase in cell motility, loss of cell-cell adhesion, and spatial dispersion of cells (her work was published after ours (14)).
Scatter Assay was carried out with MDCK cells as previously described (35), (44). Cells were seeded in 96-well plates (Corning, NY, USA) (4,000 cells in each well) and incubated overnight with or without HGF/SF (80 ng ml⁻¹), examined under a microscope (CLSM-410, Zeiss, Germany) and photographed. Each cell-scattering image was visually classified by an expert as "scattered" (10 images) or "none scattered" (22 images) and was verified by 3 independent experts. An example of "scattered" and "none scattered" images is illustrated in Fig. 3.10.

Every image was described by a feature vector of size 10, defined by LBP of cellular regions. Designated classifiers were trained on 4 scatter images for the purpose of elaborating MultiCellSeg performance of scatter assay images (Fig. 3.11A). We used "leave one out" to objectively evaluate the cell scattering measure; for each image, an SVM was trained on the remaining 31 images, and the left-out image was used to evaluate the trained model.
3.4 Exploiting Image Texture

Fig 3.11 Scatter assay quantification
(A) An example of MultiCellSeg’s performance on cell scattering example image.
(B) SVM confidence on scattered / non scattered classification. 100% accurate classification is achieved on the 32 images ($p < 0.0001$, Wilcoxon rank sum test) both in leave-one-out cross validation and in repeatedly partitioning the data to equal sized train- and test-set. Train a SVM on the training set and evaluate on the test set.
(C) Visualization of the images’ feature space. Each column represents an image’s LBP descriptor vector, which is a normalized histogram. The first 22 images are none scattered images, while the last 10 are scattered.
Evaluation of the proposed cell scattering measure succeeded in all 32 images obtained. The confidence scores of scattered and non-scattered experiments are shown in Fig. 3.11B: the confidence value zero (the default) is a perfect classifier for this dataset \((p < 0.0001, \text{Wilcoxon rank sum test})\), the confidence scores for each image was achieved using "leave one out" cross-validation, as described above. In this case, it is easy to see that the features of the extracted images’ texture are highly discriminative (Fig. 3.11C), and thus it is not surprising that the classifier works so well. As an additional validation step, we partitioned the images to equal size train- and test-sets, where the only constraint was to have more than 3 scattered images in the training set. An SVM was trained on the training set and evaluated on the test set. This process was repeated 100 times, each time selecting independently the training set, and in all executions the classification was perfect with respect to the experts’ manual visual classification.

It can be visually observed in Fig. 3.11C that the most prominent features in the LBP histogram are 6, 7 and 9. This was validated by performing the same analysis on these 3 features and showing that the classification accuracy is still 100% (Fig. 3.12). However, since LBP is a well-known general texture descriptor we decided to use it as is in the scattering application. For other cell lines or different imaging conditions, the complete histogram might be a more robust descriptor.
It was demonstrated here that texture of bright field images are sufficient to predict experimental treatment. We hypothesize that when considering large numbers of cells in a monolayer, relations between neighboring pixels' intensities represent indirectly morphological characteristics of these cells. Indeed, the average texture descriptor of image frames from Phase 2 discriminate between cells untreated, HGF/SF-treated and treated with MET inhibitor and HGF/SF together, concordant with the data indicating that the morphological changes mostly occur during this phase (details in Chapter 4). Thus, using image-texture as an indirect multi-cellular morphology descriptor can be exploited as a treatment-predictor. Further investigation should try to find a direct connection between cells’ morphology and texture.
3.5 COLLECTIVE MIGRATION SPATIOTEMPORAL DYNAMICS

Velocity magnitude map is a two-dimensional visual depiction of the spatiotemporal motility in a live wound healing experiment. It is defined as a kymograph displaying the average speed of cellular image patches as a function of the distance from the wound edge, at every time point. Below we show that this representation can provide means of visualization and quantitative measurements.

3.5.1 VELOCITY MAGNITUDE MAP

Motility measurements were extracted via a fully-automated algorithm that quantifies local motion estimation from the time-lapse bright field (DIC) channel. The algorithm includes segmentation to partition a DIC image to multi-cellular- and background- regions, followed by local-motion estimation and quantification of the extracted motion fields' magnitude (which resembles cells' local speed) at different distances from the wound edge. Continuous quantitative description of cells' velocity magnitude as a function of distance from the wound throughout the healing process is achieved by constructing a “velocity magnitude map” (briefly described here, full details in the Supporting text):

Given two consecutive DIC frames t, t+1 from the time-lapse sequence (Fig. 3.13A)

- Partition the current image (at time t) to a grid of sub-cellular sized local patches.
- Apply motion estimation to retrieve velocity fields estimations for each patch (Fig. 3.13B).
- Segment the image to cellular and background regions, and use the segmented image to define strips, mask containing all pixels at a given distance from the wound edge (Fig. 3.13C).
- For a given distance d from the wound edge, calculate the speed of the "average" cell located at d by averaging the velocity magnitude of all pixels in the corresponding strip. This step is repeated for every d.

The complete process is illustrated in Fig. 3.14

Examples of two representative velocity magnitude maps, of HGF/SF-treated and untreated cells are presented in Figure 3.15; the two vertical lines define the partition to the three healing phases.
3.5 Collective Migration Spatiotemporal Dynamics

Fig 3.13 Motion estimation
(A) Two consecutive frames from the DIC time-lapse sequence.
(B) Local-motion estimation is performed at the patch level to produce the velocity-estimation vector fields. $dx$, $dy$ are the partition to the two motion-components ($dy$ – toward the wound, $dx$ – parallel to the wound), the velocity fields are explicitly represented on the right.
(C) Left: The segmented contours over the DIC image. Right: The wounded regions are used to define strip, a mask containing all pixels in a given distance from the wound.
A compact representation for velocity magnitude maps is illustrated in Fig. 3.15 (HGF/SF-treated DA3 cells). The idea is to partition a given map into a predefined number of rectangular bins, each representing a specific spatiotemporal range. Extract the average speed from each of these rectangles and concatenate these values in a predetermined order to define a compact representation. This one-dimensional descriptor vector enables quantitative spatiotemporal comparisons between different experimental conditions.
3.5.2 "WISDOM OF THE CROWDS"
To validate the local motion-estimation, which is a fundamental component in our analysis, we compared manually-validated fluorescence-based semi-automated single cells tracking to fully-automated single-cell trajectory estimation extracted using these local motion-fields (as described in the Supporting text). These trajectories are highly correlative to the manually-validated trajectories (Fig. 3.16A). Moreover, examinations of corresponding multi-cellular versus single-cell based velocity maps (Fig. 3.17) clearly demonstrate a significant qualitative advantage of the former approach: using noisy estimation of all cells (Fig. 3.16B) enables an enhanced and a more coherent representation of the true nature of the process.
Traditionally, velocity fields are extracted by tracking individual cells during a time-lapse experiment (150). Practically, single cell tracking in a monolayer requires considerable labor and can be usually performed only for a small number of cells, providing limited statistical coherency. Our method does not require single cell tracking nor fluorescent-based imaging and is fully-automated. The proposed collective cell migration morpho-kinetic analysis is based on local motion estimation, an approach well suited for bright field images, where inner cellular regions maintain high textural information enabling accurate motion-estimation at the patch level without further processing (28). The main motivation behind it is the ability to process all cells within the monolayer; the dynamics of collective motility is complex, understanding the individual cell in
more detail does not necessarily explain the collective kinetics of a monolayer of cells (65). Another important advantage is the ability to be performed in high-throughput settings, such as suggested by Yarrow et al. (43).

3.5.3 **Wound Healing Quantification**

These descriptors were used to measure the similarity between untreated to HGF/SF-treated to Met-inhibited DA3 and MDCK cells, and cross cell lines (MDCK vs. DA3, MDCK+HGF/SF vs. DA3+HGF/SF). Thus, we tested whether cells in wound healing assays can be automatically classified based solely on the DIC time-lapse images.
Three SVM-classifiers was trained and tested using "leave-one-out" validation to separate between DA3 cells treated with the Met inhibitor + HGF/SF and (1) untreated or (2) HGF/SF-treated cells, as well as between HGF/SF-treated and untreated cells. Since some of the experiments treated with the Met inhibitor + HGF/SF did not achieve full-closure (Phase 2 was not completed), only the first two healing phases were considered (until full closure, descriptor vector of length 12 per experiment). To exclude the global healing speed and to focus on the spatio-temporal motility patterns, each experiment descriptor was normalized to 1. Two-components PCA analysis was unable to fully discriminate between untreated cells to PHA+HGF/SF cells (Fig. 3.18A). Perfect classification was achieved with each of the three classifiers (Fig. 3.18C) to conclude that the motility patterns of cells treated with Met inhibitor and HGF/SF differ inherently than untreated (p < 0.0022 Wilcoxon rank sum test) and HGF/SF-treated (p < 0.0043), and between treated- and untreated DA3 cells (p < 0.0043). Perfect visual discrimination (Fig. 3.18B) and classification (Fig. 3.18D) is achieved in
To conclude, perfect classification for every pair of conditions (cell line / treatment) was achieved, using the compact representation described above. Hence, an accurate prediction can be reached based on the DIC time lapse velocity-estimation alone. This means that given a time lapse experiment one can determine with high accuracy the experimental conditions given training experiments.

3.5.4 DISCUSSION

Velocity magnitude maps are 2-dimensional continuous and compact representation of the local motion estimation vector fields during the entire time lapse wound healing experiment. Driscoll et al. (151) recently presented a similar visualization for the spatiotemporal evolution of a cell's boundary curvature. Others generate spatiotemporal maps for several measurements for the collective migration setting (9), (13), (36), (28). This concise and coherent visualization demonstrate the alteration of collective motility patterns induced by different experimental conditions. This notion was validated qualitatively, by visual inspection of the velocity magnitude maps, and quantitatively by applying classification, treating these maps as plain images and extracting appropriate image-features. This measure allows perfect classification based solely on the motility patterns; the relative-role that cells take in collective migration as a function of their location. This means that given a full time lapse wound healing experiment, it is possible to predict with high accuracy the treatment applied to cells. This ability is a substantial improvement over the standard measures, usually only showing correlation between treatment and phenotype. Thus, we address Tambe et al.'s call (36), "...our understanding of collective cellular migration lacks predictive power and remains largely descriptive".

3.5.5 SUPPORTING TEXT:

Details of Velocity Magnitude Maps: Local-motion estimation: A grid of local patches of size ~18.5µm x 18.5µm each (15 x 15 pixels) is overlaid on each image from the time-lapse sequence. For every patch, motion estimation is computed on the consecutive frame using the Bhattacharyya distance measure, proven beneficial for tracking tasks (152): the normalized patch is searched for in the corresponding time-lapse frame in a radius of ~12.5µm (10 pixels, assuming a maximal cell speed of ~60µm/hour) from its original location, by maximizing the Bhattacharyya coefficient between the original patch and every possible shift in this radius, the inherent texture in DIC images allow to extract these measures directly. This step defines the motion-estimation vector for every patch in the
Collective Migration Spatiotemporal Dynamics

3.5 time-lapse sequence. Velocity of each patch within cellular regions (defined by the image’s segmentation) is computed from these motion estimations.

Quantification of local-motion fields at a given distance from the wound: MultiCellSeg is applied to define the current wound outlines. These contours were used to define a measure to estimate the velocity magnitude of the "average cell" located at a given distance from the wound; a strip at distance d is defined by a mask containing all pixels at a distance d from the wound edge. The average velocity magnitude of all pixels in a given strip estimates the average motility (speed) of cells at the corresponding distance from the wound edge. An illustration of the velocity magnitude estimation as function of distance from the wound is presented in Fig. 3.14.

The "velocity magnitude map", cells velocity as function of time and distance from the wound: A compact representation of a full-length time lapse experiment is defined by the velocity magnitude map, a two-dimensional depiction of the average motility of all cells at a given distance from the wound edge at a given time point. Extraction of the mean motility is accomplished for every time point and for a discrete quantization of distances from the wound to produce the velocity magnitude map. Thus, the x-axis represents time; the y-axis represents the distance from the wound. Each bin (t,d) in this map represents the average velocity magnitude (µm/hour) of cells at distance d from the wound at time t. The continuous representation of all time points in discrete distance quantization defines the complete map.

To enable automatic prediction whether a full DIC time lapse wound healing experiment was or was not treated with HGF/SF, the 3-dimensional time-lapse (image space and time) was "compressed" to a 2-dimensional representation, the velocity magnitude map described above. This compact description was further represented by a one-dimensional descriptor vector as follows: distances from the wound were partitioned to 6 intervals, and the average speed of all cells in any given interval during the 3 phases in the healing process were used to define a vector representation of a time lapse wound healing experiment (Figure 3.15).

Since the wound did not fully heal for all MDCK experiments (which were performed for shorter times than DA3 experiments) only phase 1 was considered for classification purposes. Hence, for the compact high-dimensional representation, each experiment was partitioned into 4 equal time-intervals. Distances from the wound were partitioned to 6 equal intervals of 62 µm each. The average speed of all cells in every spatiotemporal rectangle was calculated to define a vector of length 24 as a compact representation of a time lapse wound healing experiment.
**Fully-automated trajectories estimation:** Cell tracking from live bright-field light-microscopy images was performed as follows. Five iterations of bilateral filtering (153), an edge-preserving and noise reducing smoothing filter, were applied on the local motion estimation grid in each frame from the time-lapse experiment. Given an initial cell location at position (x,y), its estimated trajectory is defined by iteratively placing (x,y) by its estimated location (x+dx, y+dy) in the next frame by applying the corresponding vector (dx,dy) extracted from the filtered motion estimation.

**Semi-automated single cell tracking:** Three distances intervals from the initial wound were defined at 0-100µm, 100-200µm, and farther than 200µm. In each distance interval, several (5-21) cells were selected and tracked throughout the healing process. The tracks were built using the tracking & analysis software, ImarisTrack module, Imaris version 7.1.1. Cells trajectories were reviewed by an expert eye determining that each cell is tracked correctly using visual information both from the DIC and fluorescent images. Algorithmic tracking errors were corrected manually by severing the mistaken trajectory and manually tagging the next few correct locations before enabling the Imaris algorithm to continue tracking. The velocity component of each cell toward the wound was recorded based on a sliding window of 5 time frames (to cancel algorithmic tracking noise). The average velocity component toward the wound (Y-axes in our experiments) of cells at a given distance interval is presented in Fig. 3.19, this component was selected to emphasis the phenomenon signal.

**Velocity Magnitude Map based on Semi-Automated Single Cell Tracking:** An alternative velocity magnitude map is constructed from single cell trajectories that were tracked (Imaris) semi-automatically. An empty map is initiated, and
each tracked cell contributes to the relevant bins based on its local speed and distance from the wound (defined by the MultiCellSeg's segmentation). Two examples of single cell based velocity magnitude maps are illustrated in Fig. 3.17B.

### 3.6 Cell Migration Persistence

Automated cell tracking was performed as described above. Given a trajectory, cell persistence is defined as the ratio between its translation along the trajectory (distance covered) and the total distance traveled (Fig. 3.20A). Persistence was calculated for trajectories extracted from phase 1 as function of distance from the wound edge for DA3 and MDCK cells at spatial intervals of 37 µm (30 pixels). The distance from the edge was defined by applying MultiCellSeg for cellular-non-cellular segmentation, with respect to the cell’s starting position.

To quantify wound healing persistency, a measure was defined for a time-lapse experiment as the average persistence-score of all trajectories as a function of distance from the wound edge. A vector of length 11, representing the average persistence score of trajectories located at spatial intervals of 37 µm (30 pixels) up to 409 µm (330 pixels) from the wound edge was extracted from each experiment as the persistence descriptor. These descriptors were used to measure the similarity between 3 treatment-pairs (control vs. +HGF/SF, control vs. PHA+HGF/SF, +HGF/SF vs. PHA+HGF/SF) in DA3 cells, one pair (control vs. +HGF/SF) in DA3 cells, and cross cell lines (MDCK vs. DA3, MDCK+HGF/SF vs. DA3+HGF/SF).
It is demonstrated that cell persistence can serve as a discriminating measure for treatment-classification (Fig. 3.20B,C,D), to quantitatively conclude that HGF/SF-
Met signaling affects cell persistence during collective migration, and that persistence patterns of DA3 cells are inherently distinct from those of MDCK cells (Fig. 3.20E).

### 3.7 Intercellular Coordination

To assess and quantify the effect of HGF/SF on intercellular coordination, we devise an additional measure that is based on explicit detection of coherent groups of cells moving together as clusters within a confluent monolayer (Fig. 3.21).

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**Fig 3.21** Explicit detection of groups of cells that migrate in coherent clusters

For each frame in the time-lapse video, the input is a 2-dimensional grid, where each bin contains the motion estimation vector onto the next frame (upper left). First, bilateral filtering is applied to these vector fields, treating them as an image with two channels, x-coordinates and y-coordinates (upper right). Next, a region merging-based image segmentation algorithm is applied on the filtered motion fields, and detects larger groups of patches that maintain coherent coordinated motion. Finally, mutually touching groups are combined and all connected components of patches above a given area threshold (accounting for approximately 20 cells) define the clusters of coherent intercellular motion (lower left). The total area of cells participating in these clusters was recorded as a function of time to quantify temporal intercellular coordination.
Fig 3.22 Long term intercellular coordination
Total area of cells migrating coordinately for long-periods of time. (p-value was calculated between HGF/SF and the rest of the treatments).
(A) Visualization for DA3 cells: control versus HGF/SF-treated cells during all phases of healing process.
(B) DA3 cells (N = 17)
(C) MDCK cells (N = 10)
Explicit detection of cells' migrating in coordinated clusters within the monolayer was performed by applying image segmentation on the local motion estimation fields, after bilateral filtering (as described above). These fields can be referred as an image with two channels (motion in x- and y-coordinates). Spatiotemporal clusters of cells that maintain coordinated motion were segmented using a region-growing segmentation approach inspired by (154), followed by discarding clusters without significant motion or smaller than a predefined threshold (set to approximately the size of 20 cells). Details are in the Supporting Text.

To study long-term intercellular coordination, we measure the coordination between individual cell trajectories by applying the temporal-intercellular-coordination clustering algorithm to cell trajectories (rather than local motion fields). HGF/SF significantly increases the amount of cells migrating in a coordinated manner, for DA3 cells (visualization: Fig. 3.22A, quantification: Fig 3.22B), as well as for MDCK cells (Fig. 3.22C).

3.7.1 SUPPORTING TEXT:
Temporal Intercellular Coordination: Local motion estimation fields were referred as an image with two channels (motion in x- and y-coordinates) for clustering of coherent spatial regions – defining explicitly groups of cells that maintain coordinated motion. Bilateral filtering (153) was applied on these motion fields, followed by region-growing segmentation (154) as a clustering method.

The general idea is to start with regions containing a single pixel each, clusters grow by iteratively merging spatially adjacent pairs of region based on their motion similarity. Final clusters that are smaller than a predefined threshold (set to approximately 20 cells - 1230 µm²) or without significant motion are discarded. Finally, connected components of clusters are united to define the final results (Fig. 3.21).

The merging order is defined by the initial similarity between adjacent patches in the motion fields' image. The similarity measure is defined as the Euclidian distance between the average vectors in the spatial-regions currently examined. Two regions are merged if their similarity is lower than a given threshold. This threshold depends on the size of the regions examined (large regions are more "forgiving" and merge easily to other groups), and on a parameter Q that sets the merging sensitivity (more merging for high Q). Q is adjusted based on the data, but is not very sensitive. We have set Q to 0.04 and haven't changed it for all experiments. It is important to note that the original motion-fields image is scaled down to the resolution of the patches used for motion estimation (15x15 pixels in
our case), so there is significantly less data to process than in the original images. This algorithm was implemented in Matlab (MathWorks).

More systematically, given a motion estimation image in patches resolution, and a parameter Q:

1. Start by defining a region for each patch containing its motion-estimation vector
2. Calculate similarity for all 4-connectivity couples of adjacent motion-patches
3. Sort these couples of in increasing order
4. Traverse this order once, for any current couple of pixels \((p_1, p_2)\):
   a. Find \((r_1, r_2)\) the corresponding regions to \((p_1, p_2)\)
   b. Extract the average vector in \(r_1\) and \(r_2\), calculate their similarity, \(\text{sim}(r_1, r_2)\)
   c. Calculate the threshold for merging two regions \(\text{TH} = b(r_1) + b(r_2)\), whereas \(b(r) = \log(\text{size}(r)) \ast Q\)
   d. Merge \(r_1\) and \(r_2\) if and only if \(\text{sim}(r_1, r_2) < \text{TH}\)
5. Discard regions smaller than approximately 20 cells or were no significant motion was found
6. Unite touching-regions and report them as the final clusters

This algorithm can be implemented very efficiently, linear complexity of the number of patches. This is done by using a union-find data structure (155) to enable fast (constant complexity) operation of finding regions given an image patch (step 4a in the algorithm), extracting the average vector (step 4b) and merging two regions together (step 4d). Since only immediate spatial neighboring patches are considered, the number of couples (step 2) is linear in the number of patches, and sorting these couples is performed by applying bucket-sort, as the similarity between two regions have a discrete set of possible values (as motion estimation works at a resolution of a single pixel).

**Long-term intercellular coordination:** Long-term coordination was quantified by explicit detection of cell clusters that migrate coordinately over long periods of time. This was performed by applying the clustering algorithm described above on a dense grid of trajectories. Each image patch was tracked from the first frame until phase 1 ends, from phase 2 until it ends and during the first 15 frames of phase 3. The similarity between a pair of adjacent patches’ trajectories was
defined very similarly to the normalized cell pair separation distance measurement described in (30) (that is very similar to (156)). The idea is to quantify the difference between initially neighbored cell tracks over time. Since we process uniformly distributed patches, given two adjacent patches, their similarity is defined as ratio between the translation distance between the final position of their trajectories and the maximal trajectory path length (to make it insensitive to differences in cells' speeds). The parameters for the clustering algorithm were set to the same values as described earlier.

3.8 **COMPARISON TO STATE-OF-THE-ART IN THE FIELD**

To evaluate the performance of the proposed method, its classification capabilities were compared to those of AVeMap ("Automated Velocity Mapping"), a leading software for time-lapse collective migration quantification (9). AVeMap analyzes a strip of cells from the edge of the monolayer, and extracts the following measurements per time point based on the wound's contour and local motion estimation:

- **Velocity**: average velocity of cells in the strip along time;
- **Persistence**: calculated only for border cells;
- **Order parameter**: \(<\cos \theta >\), where \(\theta\) is the angle between the local velocity and the normal (perpendicular) to the average direction of the border and angle. Brackets ‘<…>’ denote a spatial averaging;
- **Healing rate**;
- **Tortuosity**: a measure for the wound’s contour roughness - the ratio between the contour length of the edge and the distance between the two extreme points at the top and bottom boundaries of the image (assuming a vertical wound).

AVeMap was applied on the DA3 wound healing experiments dataset (106), containing 3 treatments (control, +HGF/SF, PHA+HGF/SF), that inherently define the "ground truth" labeling. This dataset was used as a benchmark to evaluate AVeMap classifying accuracy comparing to than obtained by our approach.

Due to AVeMap restrictions, only phase 1 of the healing process was analyzed. A segmentation parameter was set up manually for each experiment, to achieve the best-possible segmentation results. The strip size was set to 100 (~124 µm). Results are based on 15 from 17 time lapse experiments. The two additional experiments were excluded because of severe errors in segmentation (including them only weakened AVeMap’s performance).
The performance was evaluated for each measure separately. A single measure is not sufficient for AVeMap to perfectly distinguish between control and PHA+HGF/SF experiments (Fig. 3.23A,B,C,D). Tortuosity is not informative at all, whereas the other measures were able to discriminate between cells treated with HGF/SF and the other two experiments. However, SVM achieved perfect classification between each pair of treatments (only) when all these measures were concatenated to a single feature-vector (Fig. 3.23E).

Our approach performed perfect classification based on velocity, persistence and indirect morphology (texture) measurements. Intercellular coordination was the only measure that was able to discriminate between HGF/SF-treated cells and the other treatments, but not between control and PHA+HGF/SF cells.

The ability to discriminate between subtle specific phenotypic changes is critical for applications such as drug screening and molecular studies. For example, testing the effect of a specific drug requires reporting increase in one phenotypic measure and decrease of another. Our approach was shown to surpass AVeMap, the state-of-the-art in this field, that did not succeed to process all experiments and required a combination of all the features to achieve perfect classification.
3.9 Summary

(A) Order parameter.
(B) Velocity.
(C) Healing rate.
(D) Tortuosity.
(E) 2D PCA visualization of the concatenation of all measurements to a single vector. Perfect classification was achieved (only) with this representation.
3.9 **SUMMARY**

In this chapter we have presented a comprehensive computational toolbox to quantify *in vitro* collective cell migration assays. The proposed measures were validated by discrimination of phenotypes induced by different treatments, and were shown to perform better than alternatives. A visual depiction of our toolbox is presented in Fig. 3.24.
Chapter 4

**HGF/SF-Induced Waves of Organized Motility during Collective Migration of Tumor and Normal Cells**

The fundamental underlying mechanisms of collective invasion in cancer cells and collective migration in normal cells are still not fully understood. When and how cells initiate persistent migration and under what conditions they cooperate with their neighbors to enhance collective migration are specific open questions in this arena. A wave of accelerating cells that propagates from the leading edge to the monolayer was recently reported by us and others as an elaborated mode of cellular collective migration. Following, we present a thorough study of the wave phenomenon for tumor and normal cells under HGF/SF-Met signaling. Our finding offer new insight on long-term cell guidance and intercellular communication during collective cell migration.
4.1 HGF/SF-Met Induces Waves of Organized Motility

We reveal that HGF/SF-Met signaling is sufficient to induce a wave of increasing velocities that gradually propagates to cells located farther in the monolayer during wound healing of DA3 and MDCK cells. Follows, we thoroughly study this phenomena, and discover the relations between acceleration, cell morphology, and oriented migration during its propagation.

4.1.1 HGF/SF Induces a Wave of Increasing Velocities and Morphological Transition

![Graph showing the induction of waves of increasing velocities and morphological transition by HGF/SF-Met signaling in DA3 and MDCK cells.](image)

Fig 4.1 HGF/SF induces waves of acceleration
(A) Control DA3 cells
(B) DA3 cells treated with HGF/SF
(C) Met-inhibited D3 cells treated with HGF/SF
(D) Control MDCK cells
(E) MDCK cells treated with HGF/SF
Qualitative comparison of the velocity magnitude maps visualization between control and HGF/SF-treated cells revealed unique motility patterns induced by HGF/SF (Fig. 4.1A,B). Front layers of untreated DA3 cells move faster than those located behind, demonstrating a homogeneous motility pattern during the wound healing process (Phases 1 and 2). During post wound closure (Phase 3), all cells decelerate regardless of their position. HGF/SF treatment leads to emergence of dramatic different cell motility patterns: at the beginning, front cells move faster than distant cells. Throughout Phase 1, distant cells gradually join the rapid motion by accelerating layer by layer. This gradual acceleration continues during Phase 2, where distant cells maintain a significantly higher motility toward the wound than cells located closer to the wound edge (data not shown). Finally, post wound closure (Phase 3), front cells halt, while distant cells gradually decelerate.

To further demonstrate these phenomena, an alternative visualization is presented (Fig. 4.2A,B), taking into account the direction perpendicular to (toward) the scratch. "Average" cells (calculated as indicated in Materials & Methods) at several locations (25-335 µm from the wound edge) were selected and "tracked" throughout the healing process. An "average" cell's velocity at a given time and distance from the wound is defined as the average velocity component toward the wound in the strip that corresponds to the relevant location. The distance that an "average" cell travels in each frame (retrieved from the velocity fields' estimation) was accumulated to define its displacement as function of time. Indeed, this measure is not exactly the actual displacement, as it contains algorithmic "noise", cells deformations and proliferation. Nevertheless, since the errors and noise occur in all "directions", we assume that this measure is an approximated representation of the true dynamics. Fig. 4.2A,B displays this displacement measure (denoted R) as a function of time until full closure (Phases 1 and 2). It is shown (Fig. 4.2A) that untreated cells express a "fan-like" dynamics, where front cells expand a physical gap from cells behind, a gap that grows steadily during healing. As for treated cells (Fig. 4.2B): during Phase 1, a gap is formed between front and distant cells, but cells from behind progressively accelerate so that this displacement-gap shrinks or at least remains constant during Phase 2 for cells located about 10 cell-layers behind the leading edge. Fig. 4.2C,D plots the average velocity component toward the wound over time. Untreated cells exhibit roughly constant velocity, front cells being faster than farther cells (Fig. 4.2C). The gradual acceleration of distant HGF/SF-treated cells throughout Phase 1, and the higher velocity maintained by distant cells compared to front cells is displayed in Fig. 4.1D. These conclusions referring to an estimation of the "average" cells' velocity over time are supported by single-cell tracking experiments as shown in Fig. 3.19.
4.1 HGF/SF-Met Induces Waves of Organized Motility

![Graphs showing the effect of HGF/SF-Met on cell motility](image)

**Fig 4.2** The displacement gap mystery

DA3 cells. x-axis represents time in all panels; the y-axis represents the “average” cell’s displacement toward the wound at several spatial locations (A, B), or average velocity (C, D).

(A) Average displacement of control cells.
(B) Average displacement of HGF/SF-treated cells.
(C) Average velocity of control cells.
(D) Average velocity of HGF/SF-treated cells.
Fig. 4.3 Single cell morphology (area) as function of time and distance from the wound. DA3 cells. Average cell area at different distances over time (A,B). Relation between cells density and speed (C,D). The cells’ density is estimated at two spatial location < 248µm (marked red), and > 248µm (marked green) from the wound edge.

(A) Control
(B) +HGF/SF
(C) Control (p < 0.0001).
(D) +HGF/SF (p <0.003).

Fig. 4.3A,B illustrates the average cell’s area as function of distance from the wound over the different healing phases for untreated (4.3A) and treated (4.3B) cells (as described in Subsection 4.8.3). In addition to the predefined three healing phases, a fourth time point was added, which represents the last frame in the time-lapse and is used to demonstrate the final stages of the healing process. The x-axis consists of 4 different distance intervals from the wound edge, the y-axis is the average cells’ area at a given phase and distance-interval. Until full closure (Phases 1 and 2) untreated cells that are close to the wound are larger than distant cells. Front cells shrink upon wound closure, and after the wound has
healed all cells in the monolayer shrink to maintain approximately the same size independently of their location. Similarly to its effect on cells’ speed, HGF/SF treatment induces dramatic changes in the dynamics of cellular morphology. At the initial stage, close cells are larger than distant cells. During Phase 2, front cells begin to shrink while farther cells grow. In Phase 3, only the most distant cells continue to grow while the rest shrink. After the wound has healed, all cells have shrunk to approximately the same size. A bar graph that compares treated and untreated cells’ area for every distance interval over time demonstrates that the main differences occur in Phases 2 and 3, when treated cells that are located far from the leading edge grow dramatically in a progressive manner (Fig. 4.4A).

Similar patterns of morphology alteration are depicted using cell eccentricity. Untreated cells that are close to the wound are more elliptical than distant cells throughout the healing, while front treated cells begin as more elliptical than distant cells, that in turn, during the later stages, become more elliptical than these front cells (Fig. 4.5).
Fig 4.4 Single cell morphology (area) as function of time and distance from the wound
(A) Average cell area at different distances over time (same data as in Fig. 4.3).
(B) Control. Estimated density as function of time for close (< 248 μm, red markers) and far
(> 248 μm, green markers) cells.
(C) +HGF/SF. Estimated density.
Fig 4.5 Single cell morphology (eccentricity) as function of time and distance from the wound DA3 cells. Eccentricity is the ratio of the distance between the foci of an ellipse and its major axis length. In our setting it is referred to the ellipse that has the same second moments as the segmented cell. The values range between 0 and 1. (0 and 1 are degenerate cases: an ellipse whose eccentricity is 0 is actually a circle, while an ellipse whose eccentricity is 1 is a line segment).

(A) Control
(B) +HGF/SF
(C) Control vs. +HGF/SF
Next, the relation between cell motility and density was examined. Cells density was estimated based on cell size measurements as detailed in Methods S1. Throughout the healing process, untreated cells that are close to the wound’s edge (< 248 µm) are consistently spread sparsely compared to distance cells (> 248 µm) (Fig. 4.4B). Treated cells maintain similar location-dependent characteristics to those described for untreated cells during Phase 1. However, upon Phase 2, treated cells ”switch” - distant cells become sparsely distributed compared to front cells (Fig. 4.4C). Investigating cells’ density and motility reveals that, as expected, sparser regions are correlated with faster velocities, as was recently shown (28), (49), independently of cells’ location, and is more prominent for untreated cells (Figure 4.3C,D).

Similar waves occur in MDCK cells as a response to HGF/SF. As in DA3 cells, these waves do not emerge upon starvation and HGF/SF-Met signaling is sufficient to induce it (Fig. 4.1D,E). HGF/SF-induced velocity waves in DA3 cells are characterized by a more rapid cellular acceleration than in MDCK cells (Fig. 4.1B).

4.1.2 HGF/SF-Met Induces Waves of Organized Motility

Waves of increasing directionality and cell deformation rate (strain rate, (92)) emerge in HGF/SF-treated starved DA3 cells (Fig. 4.6E,F), but not in starved untreated cells (Fig. 4.6K,L).

No significant relation is observed between acceleration and directional velocity toward the wound in untreated and in Met inhibited DA3 cells (Fig. 4.7A,C). However, DA3 cells treated with HGF/SF can be roughly partitioned into three general motility states: (i) weakly motile cells, moving slower than 5 µm hour-1 toward the edge (ii) intermediate motile, at 5 - 15 µm hour-1, and (iii) highly motile, moving faster than 15 µm hour-1. Both weakly and highly motile cells acceleration is minimal, whereas cells in the intermediate velocity range do accelerate (Fig. 4.7B). Acceleration increases with the directional velocity toward the wound, implying that these cells are in transition from a non-motile to a highly motile state, and then decreases at the high motility limit. This conclusion is actually expected from visual inspection of the corresponding velocity magnitude maps, as cells at state (ii) are those that are found at the high gradients regions in these images, defining the acceleration wave.
4.1 HGF/SF-Met Induces Waves of Organized Motility

Fig 4.6 Dynamics of control and HGF/SF-treated DA3 cells
A-F HGF/SF treated cells, G-L control cells. Spatiotemporal maps (kymographs).
(A) Speed, (B) Directional velocity toward the wound edge, (C) Directional velocity parallel to the wound edge, (D) Acceleration, (E) Directionality, (F) Strain rate. (G-H) Correspondingly for control cells.
Fig. 4.7 A closer look at the wave: relations between directional velocity, acceleration, directionality and strain rate in DA3 cells.

(A-C) Acceleration as function of directional velocity toward the wound edge.

(D-F) The ratio between directional velocity parallel to the wound edge and toward the wound edge. The green line in (E) is the linear fit to the data of control cells (D).

(G-I) Time delay between acceleration, directionality and strain rate for HGF/SF treated cells.

(J) Cell morphology (area and eccentricity/elongation) changes coordinately with strain rate. Example shown for cells located 248-372 μm from the edge.
A linear correlation was found between the directional velocities toward- and parallel to the wound for untreated and Met-inhibited DA3 cells (Fig. 4.7D, F); indicating a consistent slight preference to closing the wound (slope = 1.18), as anticipated. Thus, random motion of the cells in the direction parallel to the wound increases with the increase in the directional cell motility towards the wound. HGF/SF-treated cells display a different phenotype (Fig. 4.7E): Linearity for cells moving at under 15 µm hour⁻¹ toward the edge, but with significantly higher directionality, indicating a higher preference toward the wound. A dramatic transition occurs for fast cells, characterized by a significantly decreased directionality. Interestingly, the directionality of highly motile cells approaches that expected of untreated cells, as shown by extrapolating the trend of untreated cells (Fig. 4.7E, green line).

Taken together, these results demonstrate that HGF/SF-treated DA3 cells accelerate and move in a more directional manner during the transition from non-motile to motile cells (Fig. 4.7G). Moreover, acceleration precedes the enhanced directionality by a temporal window of approximately 70 minutes (Fig. 4.7H,I), and is coordinated with increased strain rate (Fig. 4.7H). Neither untreated nor PHA+HGF/SF-treated DA3 cells show these phenomena (Fig. 4.8A,B,C,D).
Similar velocity-acceleration-strain rate-directionality relationships are also evident in HGF/SF-treated MDCK cells, albeit less prominently (Fig. 4.9A,B,C,D,E,F,G,H). Similar to DA3 cells, acceleration precedes directional motion, but by a temporal window of approximately 220 minutes (Fig. 4.9H,I).
4.1 HGF/SF-Met Induces Waves of Organized Motility

**Fig 4.9** A closer look at the wave: relations between directional velocity, acceleration, directionality and strain rate in MDCK cells

(A-B) Acceleration as function of directional velocity toward the wound edge.

(C-D) The ratio between directional velocity parallel to the wound edge and toward the wound edge. The green line in (D) is the linear fit to the data of control cells (C).

(E-F) Directionality as function of acceleration.

(G) Cell morphology (area and eccentricity/elongation) changes coordinately with strain rate. Example shown for cells located 248-372 μm from the edge.

(H) Time delay between acceleration, directionality and strain rate.

(I) Delayed directionality as function of acceleration, optimal delay of 217 minutes.
In Subsection 4.7.3 we present a simplified model to test the hypothesis that strain-rate triggers a cell directionality response, i.e. determines the ratio of the magnitude of the velocities towards and parallel to the wound (Fig. 4.10).

Cell morphology changes accordingly: area and elongation (eccentricity) grows along with the strain rate, when the cells also move in a more directional manner. However, after the wave has passed these morphological features remain (Fig. 4.7J), while the directionality is lost. This indicates that the static shape (aspect ratio) of a cell is not the indication for its directional motion. Eventually the cells get squeezed by cells from the other side of the wound (Fig. 4.3).

**4.1.3 HGF/SF-MET INDUCES ENHANCED CELL PERSISTENT MIGRATION**

HGF/SF increases the persistence of DA3 cells across all distances from the wound edge, relative to untreated or cells treated with Met-inhibitor and HGF/SF, as shown in Fig. 4.11A. Cell persistence is maximal for cells located approximately 7-8 rows in the sheet, and steeply decreases for cell located in inner rows. The full distribution of persistence as function of location related to the wound can be determined through simulation.
found in Fig. 4.11C. The persistence of MDCK cells was slightly lower than that of DA3 cells but exhibited very similar patterns (4.11B,D).

4.2 HGF/SF-Met Enhances Temporal Intercellular Coordination

4.2.1 HGF/SF-Met Enhances Temporal Intercellular Coordination

It is shown that untreated DA3 cells are characterized by a low, approximately constant, number of cells that are moving in clusters throughout the healing process (Fig. 4.12A). However, in the presence of HGF/SF, the fraction of cells
moving in clusters steadily increases as wound closure progresses, and drops after the wound is healed (Fig. 4.12B). A similar effect of HGF/SF on the number of cells that move in clusters was observed in MDCK cells (Fig. 4.12C,D).

![Fig 4.12 Temporal intercellular coordination](image)

**Temporal intercellular coordination**
- total area of cells moving in coordinated clusters as function of time
- DA3 control, b, DA3 + HGF/SF, c, MDCK control, d, MDCK + HGF/SF

(A) DA3 control
(B) DA3 + HGF/SF
(C) MDCK control
(D) MDCK + HGF/SF

### 4.2.2 Wave of Long-Term Intercellular Coordination

Waves of long-term intercellular coordination propagate through the monolayer in both DA3 and MDCK cells under all treatments. Thus, this is an intrinsic property. In both cell lines the intercellular coordination wave of HGF/SF-treated cells is more prominent than the wave of untreated cells. (Fig. 4.13A - visualizes a DA3+HGF/SF wave, Fig. 4.13B,C,D,E – quantification).
Fig 4.13 Long term intercellular coordination

(A) Visualization of long term intercellular coordination for DA3+HGF/SF cells during the 3 phases of the healing process. The first row displays trajectories displacement component parallel to the wound, the second row the component toward the wound, and the third row illustrates the clustering results overlaid on the initial time frame.

Distribution of long term intercellular coordination in relation to the distance from the edge.

(B) DA3 control
(C) DA3 +HGF/SF
(D) MDCK control
(E) MDCK +HGF/SF
4.3 **Effects of Met-Inhibition**

Met-inhibition experiments demonstrating inhibition of cell motility (Fig. 4.1C), persistent migration (Fig. 4.11A) and intercellular coordination (Fig. 4.14) validated the important role of HGF/SF-induced Met activation in collective cell migration.

Perfect classification between all pairs of treatments was demonstrated for texture, motility and persistence representations (Fig. 3.9, Fig. 3.18, Fig. 3.20). Thus indicating that the endogenously over expressed Met in these cells (110) plays a role in collective cell motility thus validating the involvement of the Met-signaling pathway with induction of collective motility patterns as well as the discriminative power of our proposed measurements.

Met-inhibited DA3 cells treated with HGF/SF are characterized by similar persistence to HGF/SF-treated cells close to the sheet edge, followed by a steep drop viewed much closer to the wound compared to HGF/SF-treated cells (Fig. 4.11A, N = 7, p < 0.0007). These cells also introduce slightly reduced coordination compared to HGF/SF-treated cells at phase 1, which is reduced drastically in later phases of the healing process (Fig. 4.14B vs. Fig. 4.13C).

These results indicate that endogenously-activated over-expressed Met plays an important role in collective cell motility and further validates involvement of Met-signaling pathway in this process.
4.4 DISCUSSION

Displacement gap: The displacement gap formed between front and distant cells during untreated DA3 experiments, visualized by the fan-like dynamics (Fig. 4.2A), contradicts the fact that the monolayer is kept continuous with no visible gaps throughout the healing process. Morphology transitions alone cannot account for this phenomenon, since cells’ growth is insignificant in the gap formed by accumulating displacements between cells located closer and farther from the leading edge. On the other hand, the gap formed under HGF/SF treatment can be explained solely by morphology transitions; it is formed when cells near the wound’s edge become larger than distant cells. During the next phase, distant cells exceed the size of cells located closer to the leading edge and fill the gaps. We hypothesize that accelerated proliferation at the leading edge is the answer for the untreated cells’ "gap mystery", as shown in Poujade et al. (44). Since HGF/SF induces accelerated proliferation (113), we believe that it is spread approximately equally throughout the monolayer under treatment.

HGF/SF-Met-signaling is sufficient to induce a wave: Recent studies report that a wave of accelerating cells begins at the leading edge in both, wound healing (12), (15) and monolayer expansion experiments (13). Chemical stimulation has an important role in the wave’s characteristics: for example, acto-myosin contractility functions as a negative regulator of the number of cell rows potentially participating in collective migration (12); chelation of extracellular calcium disrupts and inhibits cell-cell junctions leading to monolayer integrity breakage destroying the wave (13). It is demonstrated here that starved cells do not exhibit this phenotype and that the activation of Met-signaling by HGF/SF is sufficient to induce the wave for both tumor (DA3) and normal (MDCK) cells.

The wave, acceleration, directionality, strain rate and persistence: We found that the reported wave is characterized by acceleration propagating through the monolayer in coordination with cellular stretching (strain rate), and followed by enhanced directionality (DA3 cells - Fig. 4.6A-F, Fig. 4.7H,J, MDCK cells – Fig. 4.9H,I). Directionality drops drastically when approaching a maximal directional velocity toward the wound, as acceleration halts (DA3 – Fig. 4.7E, MDCK – Fig. 4.9D, simulation – Fig. 4.10B). Interestingly, the directionality of these fast cells resembles the expected trend for untreated cells, where the random and directional motions are found to be proportional.

These observations lead to propose that there is a common underlying mechanism controlling directionality; it is described as a directional cue propagating with the acceleration wave to span the monolayer, triggered by the presence of HGF/SF. After the acceleration wave reaches deeper cells, the
directional cue passes along, leading to high motility, no acceleration, no strain rate and reduced directionality of the cells that were already passed.

Acceleration begins several cell rows from the edge, hence these relationships can explain the prominent persistence score observed for cells located several additional rows back in the monolayer (due to the increased directionality) as a respond to HGF/SF, the steep decrease in persistence is due to wound closure time that limits the wave from propagating even deeper (Fig. 4.11).

We hypothesize that the tighter adhesions between MDCK cells cause the phenotypes described above to appear less prominently by limiting cell efficient acceleration in the wound's direction. This causes reduced directionality (Fig. 4.9F) and persistence (Fig. 4.11B), larger temporal delay and decreased correlation between acceleration and directionality (Fig. 4.9H,I).

These observations imply that an HGF/SF-driven acceleration wave can give rise to highly directional motion as it traverses the monolayer, due to the ability of cells to convert the mechanical (strain-rate) signal into directional motion (lower level of cellular noise). This phenomenon might be regulated by cell inherent elasticity, cell-cell adhesions and self-propulsion.

**Cell-cell communication promotes HGF/SF-induced intercellular-coordination:** Non-confluent epithelial cells respond to HGF/SF by rapid activation of cellular motility mechanisms (157) in an ordered manner; they spread, lose cell-cell adhesions, increase motility and spatial scattering (116). Similar relations between cell-cell adhesions (which affects density), motility and intercellular coordination occurs in confluent monolayers; Inhibition of cadherin-mediated cell-cell adhesions increased general motility but reduced directional migration, persistence and intercellular coordination for confluence monolayers of MCF10A cells (30). It has also been reported that increased monolayer density is associated with decreased motility (28), (62), (158), (31) and increased intercellular coordination (62), (31), (158), (84). The latter relation between density, motility and coordination was also recently simulated by a simple flocking-type mechanism that aligns cellular motility forces with their velocity (33). This relation is not limited to mammalian cell cultures, and known to exist in other systems as well, such as bacterial populations (159).

It was reported before that in response to HGF/SF, epithelial monolayers become sparser, maintain higher motility (15), and weaken cell–cell contacts (44). Thus, the higher intercellular coordination found (Fig. 4.12, Fig. 4.13) is unexpected, and may imply an additional channel of intercellular communication (44), (160) (Table 4.1). We speculate that the directional cue described above also takes an
important role in this phenomenon, and that increased motility coupled with a general directional motion leads to increased intercellular coordination.

<table>
<thead>
<tr>
<th></th>
<th>Non confluent +HGF/SF</th>
<th>Dense confluent cells</th>
<th>Sparse confluent cells</th>
<th>DA3 confluent +HGF/SF</th>
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Table 4.1 Discussion: cell dynamics in different density levels
As a response to HGF/SF non-confluent cells (scatter assay) are characterized by reduced cell-cell adhesions, increased motility and scattering (thus reduced density). It has been reported before that in collective cell migration, as density level rise, cell move slower, but are more coordinated with their neighboring cells. In the case of DA3 treated with HGF/SF, we report an increased motility and decreased cell density (as expected), but increased intercellular coordination. Our results may imply that HGF/SF-Met signaling is a channel promoting intercellular communication.

The coordination wave occurs both in DA3 and MDCK cells, more prominently in the presence of HGF/SF, implying that HGF/SF-Met signaling takes a major, but not exclusive, role in mediating this phenomenon (Fig. 13B,C,D,E). Hence, we conclude that the wave of intercellular coordination is an intrinsic trait in collective cell migration that is amplified in the presence of HGF/SF, speculatively by progressive mechano-sensing cell-cell communication mechanisms.

**Met inhibition disrupts cell-cell communication:** We hypothesize that the directional cue is initiated by cells at the leading edge, penetrates progressively back into the monolayer increasing cells' directional motility toward the edge thus enhancing intercellular coordination. The observation that persistence of PHA+HGF/SF treated cells is lower, does not increase for deeper cells, and drops closer to the edge compared to HGF/SF-treated cells with active Met (Fig. 4.11A) might be due to reduced penetration of the directional cue as a results of disrupted cell-cell mechano-sensing communication. Long-term intercellular coordination of Met inhibited cells is also similar to Met-activated cells that are located close to the wound edge and diminishes for deeper cells at later stages of the process (Fig. 4.14B vs. Fig. 4.13C). This observation might imply reduced
penetration of the directional cue due to disrupted cell-cell communication via Met-inhibition, in accord with the hypothesis presented above.

**Modeling cell-cell communication:** Recently, several mathematical models were proposed for collective cell migration. For example, (33) demonstrated by a simple model that alignment of cell self-propulsion and velocity can lead to the emergence of complex phenotypes that were found in experimental studies. (34) demonstrated that experimental results can be accurately described by modeling the motion of cells as random but with a tendency to move at the same velocity as their neighbors. We anticipate that more advanced modeling approaches will be able to capture the acceleration waves and shed light on the mechano-physical mechanism that underlies this phenomenon. Candidate models are those that incorporate cell shape dynamics with chemical signaling (161) as well as adaptable cell-cell interactions (162) and autocatalytic effects (163).

**4.5 Relation with Traction and Intercellular Forces**

In (13), the authors reported a mechanical wave that propagates inwards to a monolayer of MDCK cells. Cells are grown to confluence in a micro-patterned stencil, which promotes monolayer expansion upon removal. In addition to the bright field time-lapse images, they used the method described in (35) to acquired traction-forces, and then (36) for monolayer-stresses measurements. They reported two extreme wave phenotypes, symmetric and asymmetric. Upon applying our analysis on their data, the following preliminary results were obtained. More data is required for significant conclusions.

We have reproduced the velocity and strain rate waves reported (Fig. 4.15).
We applied the particle tracking presented in Subsection 3.5.5 on the data. Fig. 4.16 displays the trajectories over the image at the beginning of the experiment. The symmetric case is characterized by trajectories-coordinated-shear, a known phenomenon for small groups of cells. Additional experimental data is required to understand whether this is a true phenomenon of a symmetric collective-setting.
A linear correlation was found between the directional velocities toward- and parallel to the wound for the asymmetric case (Fig. 4.17A), whereas in the symmetric case higher directionality is observed for cells moving at intermediate velocities that drop to the level of that in the asymmetric case for fast cells. This finding resembles the patterns reported for MDCK cells in our experiments (4.17C) for control and HGF/SF-treated cells, suggesting that a similar mechanism for symmetry and HGF/SF-Met signaling might take place. Of course, Trepat’s experiments are very different from ours (different substrate, no starvation, different assay), but still, the same patterns emerge.
There are additional inherent phenomenological differences between the symmetric and asymmetric states. In (13), a drastic change in stress magnitude starts from the initial stages of the experiment: in the symmetric case, stress builds up much faster to higher levels (Fig. 4.18). We find an opposite correlation between persistence and stress magnitude depending on spatial location. In both cases, persistence is reduced as function of distance from the wound edge. In the symmetric case, stress builds up into the monolayer, whereas in the asymmetric case stress does not propagate and is maximized at the leading edge (Fig. 4.19). We speculate that the initial width of the monolayer has a prominent role in these phenomena; in narrow strips the symmetry may be broken not having "enough" cells to support a symmetric expansion, with one side winning. Another possibility is that the wave propagation is a stochastic process, with a common mean speed. The waves start from each side of the sheet independently. For wide sheets, the noise averages over time so the two waves coalesce around the middle, whereas in short waves, it is less probable to do so.
Fig 4.18 Intercellular stress as function of time
(A) Symmetric case: mean stress.
(B) Asymmetric case: mean stress. Note the different scale from A.
(C) Symmetric case: spatiotemporal kymograph of intercellular stress.
(D) Asymmetric case: spatiotemporal kymograph of intercellular stress.
It was shown that HGF/SF leads to enhanced directional migration (less noise) by a wave of acceleration and strain rate (Subsection 4.1), and it was speculated here that larger monolayer's width leads to noise-averaging which is translated to symmetry. Thus, it is suggested that these two different mechanisms may lead to a similar global phenomenon (directional migration pattern, Fig. 4.7 and Fig. 4.17).

4.6 SUMMARY
Collective cell migration should thus not be explained from a mechanical perspective alone; efforts should be invested in understanding the effects of various chemical signaling, which constitute a significant role in collective cell motility as demonstrated herein and in other studies (e.g., (53), (150)). Revealing the effect of Met-signaling on collective morpho-kinetic patterns is crucial to understand the molecular and cellular mechanisms behind metastasis. The ability to predict when a group of cells maintains a dynamical metastatic signature can have therapeutic implications in the long run; this study may turn out to be a first step in that exciting avenue.

4.7 MATERIALS AND METHODS

4.7.1 CELL CULTURES, WOUND HEALING ASSAY
Two cell lines were used: DA3 cells, derived from the mouse mammary adenocarcinoma cell line D1-DMBA-3. Madin-Darby Canine Kidney (MDCK) epithelial cells, the common model to study collective cell migration. DA3 cells were selected as model tumor cells under three conditions: untreated (control), treated with HGF/SF, or treated with the Met inhibitor PHA665752 (164) (denoted PHA henceforth) together with HGF/SF (denoted PHA+HGF). MDCK cells were selected as model normal cells under two conditions: untreated (control) or treated with HGF/SF. MDCK cells were not treated with PHA+HGF/SF because Met is not constitutively activated in MDCK cells. Wound healing assay (42) was used as a trigger to collective migration. To isolate the various effects, cells were starved before and during the assay. More details are in the Supporting Methods.

A total of 17 DA3 experiments (6 - control, 5 +HGF/SF, 6 PHA+HGF/SF) and 10 MDCK experiments (5 – control, 5 +HGF/SF) were processed. The DA3 experiments were previously reported (15). Raw image data is freely available at "The Cell: an Image Library", (106), (107) to enable reproducibility and additional insights by others (105).

The data used in Section 4.5 was kindly handed to us by Dr. Xavier Serra-Picamal and Prof. Xavier Trepat (13).

4.7.2 **DIRECTIONAL VELOCITY, ACCELERATION, DIRECTIONALITY AND STRAIN RATE**

Cell velocity is composed of a motion component toward (normal) and parallel to the wound edge, both denoted as the directional velocity. A measure for directional motility is defined as the ratio between these two components, and denoted directionality; high values reflect motion that is more prominent toward the wound edge. Acceleration is the change in average speed between time t and time t+dt ($\frac{\partial |v|}{\partial t}$). Strain rate is $\frac{\partial v_x}{\partial x}$, were x is the direction toward the wound, and vx is the corresponding average motion component. Assuming cellular cohesiveness and mass conservation, strain rate is an implicit measure for cell deformation (92), (13). More details are in the Supporting Methods.

4.7.3 **A SIMPLIFIED MODEL TO TEST THE HYPOTHESIS THAT STRAIN-RATE TRIGGERS A CELL DIRECTIONALITY RESPONSE**

We describe a Gaussian acceleration wave traversing the monolayer at velocity much larger than of the cells', as observed in the experimental data. Cells are considered as viscous elements, thus the local forces that they can detect are proportional to the strain-rate, and these forces are then assumed to trigger a signal for the cytoskeleton where to polymerize, where it is converted into a
directionality cue. Once the strain-rate diminishes, after the wave passed, there are no forces anymore in such a viscous material and the random motion returns to its proportionality with the directional motion, and orientation is lost (Fig. 4.10A). This setting was sufficient to qualitatively capture the phenomenon observed in Fig. 4.7E for DA3 cells, and in Fig. 4.9D for MDCK cells (Fig. 4.10B). Full details below.

We consider cells as a continuous medium, with some velocity field \( \vec{v}(x,t) \). From this velocity field we have a strain-rate field

\[
\dot{\varepsilon} = \nabla \varepsilon (\vec{v}_u)
\]

(1)

Let us consider a cell-cell linker, of typical length \( a \), and stiffness \( k \). It makes some on-off adhesion between the cells, with \( k_{on} \) independent of the stretch and \( k_{off} \) reduced when it is stretched so that the linker breaks up more when stretched. Let us propose the simplest form

\[
k_{off} = k_{off,0} e^{-\left(\frac{\Delta E - k a^2 (\hat{v}_x)^2}{k T} \right)}
\]

(2)

where \( k_{off,0} \) is the off-rate when unstretched, \( \Delta E \) is the energy of linker adhesion. The time-scale of the stretch is over the average time that the linker is attached, i.e. \( \tau \sim k_{off}^{-1} \). We solve this equation for \( k_{off} \)

\[
k_{off} = \frac{a \sqrt{k}}{\sqrt{\text{ProductLog}\left[\frac{a^2 e \Delta E k}{k_{off,0}}\right]}}
\]

(3)

The average occupation of the linker is therefore given by: \( \rho \propto k_{on}/k_{off} \). For a traveling wave of acceleration we plot in Fig.1 the time evolution of the acceleration, velocity and \( \rho/\rho_0 \) (\( \rho_0 = k_{on} e^{\Delta E/k T}/k_{off,0} \)) as the wave passes a cell at a certain position. As an example, we can treat an acceleration wave travels at a constant velocity \( \nu \), of a Gaussian profile, of the form

\[
a(x,t) = \frac{\nu}{\sqrt{\pi} a} e^{-\left(\frac{x-\nu t}{a}\right)^2}
\]

(4)

which is normalized to give an overall acceleration of the particles from 0 to 1. We can now solve the velocity \( v_y \), which is the integral of this acceleration wave, assuming that the particles do not move appreciably as the acceleration wave passes over them, \( v \gg 1 \).

We may assume that the ratio of the velocities in the \( x \) and \( y \) directions are related to the linker density along these two orthogonal directions on the cell surface, i.e. \( v_y/v_x \propto \rho \). We find that due to this assumption the perpendicular velocity is always smaller than the velocity towards the wound, \( v_y \leq v_x \), and is affected by the high strain-rates during the acceleration phase.

### 4.8 Supporting Methods

#### 4.8.1 Experimental Setting

**Cell cultures:** DA3 cells expressing the fluorescent protein mCherry, derived from the mouse mammary adenocarcinoma cell line D1-DMBA-3, induced in BALB/C mice by dimethylbenzanthracene (165) were maintained in DMEM supplemented with 10% heat-inactivated fetal calf serum (FCS, Gibco-BRL) in a 37°C, 5% CO2 incubator. Madin-Darby Canine Kidney (MDCK) epithelial cells expressing YFP-membrane were maintained in DMEM supplemented with 5% fetal FCS in a 37°C, 5% CO2 incubator.
Wound healing assay: Cells were grown to 90% confluence in 24-well plates. Prior to scratching, the cells were starved by changing the medium to DMEM plus 0.1% FCS (starvation medium) for 4 hours (DA3) or 24 hours (MDCK). The medium was then changed to either fresh starvation medium (control), starvation medium with 80 ng ml-1 HGF/SF, or starvation medium with HGF/SF and 2.5 µM of the Met inhibitor PHA665752 (164) for an additional 2 hours (only for DA3 cells). A scratch of approximately 300 µm in width was generated using a 200 µl tip (42). The plate was subjected to time lapse microscopy in a stage incubator (OKOLAB, Italy) on a computer-controlled motorized stage of a confocal microscope (CLSM-510, Carl Zeiss, Germany), used in non-confocal mode, with a 10x (/0.30) objective. Image acquisition was initiated 2 hours post scratching. Images were acquired every 14.5 (DA3) or 15.7 (MDCK) minutes for 26 hours (DA3) or 15 hours (MDCK). The coordinates of each scratch were predefined, and a macro that repetitively positions the field of view at each point was executed. The acquired differential interference contrast (DIC) channel of the time-lapse sequence was used for the analysis. A total of 17 DA3 experiments (6 - control, 5 +HGF/SF, 6 PHA+HGF/SF) and 10 MDCK experiments (5 - control, 5 +HGF/SF) were processed, the rest of the experiments were discarded due to image-acquisition faults.

4.8.2 Directional velocity, acceleration, directionality and strain rate

To examine the relations between cellular motion in a predetermined direction, acceleration and directionality we produced two-dimensional spatiotemporal grids, each representing one of these measurements. These grids were defined in the same pattern that the velocity maps were produced; each bin represents the average measurement of the cells at the corresponding spatiotemporal strip. Thus the analysis was based on an "atomic" spatiotemporal resolution that is defined by a pixel in the velocity magnitude maps, temporal resolution of about 15 minutes between consecutive frames, and spatial resolution of approximately 12.5 µm-long intervals.

Directional velocity is the two-component decomposition of the velocity vector to a component toward (perpendicular / normal) to the wound edge, and the parallel component. These components are determined by the orientation of the wound edge, and are computed by projecting the velocities of cells undergoing collective migration onto the direction of wound closure (Fig. 4.6B,C). Directionality is defined as the ratio between the motion components toward- and parallel to the wound edge (Fig. 4.6E). Smaller values mean a "noisier" motion, as the parallel directional velocity become larger in relation to the directional velocity toward closing the wound. Values lower than 1 mean that the dominant
motion is parallel to the wound edge (not closing the wound), for values above 1, the higher the directionality the more efficient the healing process is. \textit{Acceleration} is defined as the time-derivative of speed for a given spatial strip (distance from the edge), $\partial |v|/\partial t$; this calculation was performed on the velocity magnitude map using a time-window of approximately 90 minutes (6 time frames) to calculate it (Fig. 4.6D). \textit{Strain rate} is defined as the spatial-derivative of the directional velocity toward the wound, $\partial v_x/\partial x$, were $x$ is the direction toward the wound, and $v_x$ is the corresponding average motion component (Fig. 4.6F). This is an implicit measure for cellular deformation rate (92).

For each treatment the data from all experiments was pooled, and the analysis was performed only for phase 1 of the healing process, namely, until first contact between the opposing borders of the wound.

\textbf{4.8.3 Single-Cell Morphology Measures}

Analysis of cellular morphology was performed by statistical measurement of characteristics at the single cell level. A manual marking tool was developed to allow fully blinded manual segmentation of single cells in wound healing DIC images. An expert biologist manually segmented the exact contours of about 40 cells per image under different treatments, at different phases in the healing and at arbitrary locations. By introducing partial field-of-view, the biologist was not aware of the marked cell’s origin (treatment, location, time). Two measures were considered: the size (area) of cells and their eccentricity (measure for roundness: low values – more rounded, high value – more elliptical). These measures were later used to extract quantitative statistical description of cells morphology under varying conditions in time, location and treatment.

Cells density was estimated directly from the cells’ area. At a given time and distance interval, density was defined as the number of cells that fit in a given area unit based on the average cells’ size. "Close cells" were defined as cells up to 248$\mu$m from the wound (200 pixels), while "distance cells" where defined to be at least 248$\mu$m away from the wound’s edge.

\textbf{4.8.4 Additional Methods:}

\textbf{Phases in the healing process}: The healing process was partitioned to three conceptual phases (Fig. 3.7): Phase 1, from the scratch formation until first contact between cells from opposing wound edges; Phase 2, from first contact until full closure of the wound, when the wound area is completely covered by a monolayer of cells; Phase 3, post wound closure (we limited this phase to 15 time-frames). Phase 1 was considered for all analyses, except intercellular coordination and morphological measurements that considered the whole healing process.
**Statistical analysis**: Unless stated otherwise, Pearson’s linear correlation was used as a statistical test to calculate correlations (166).

**Long-term intercellular coordination**: To quantify long-term coordination we applied the clustering algorithm described in Section 3.7 on a dense grid of trajectories.

**Cell Motility Persistence**: The ratio between the distance covered (translation) and the total distance traveled in a given trajectory is defined as persistence, and is described in Section 3.6. 3,000-4,000 distinct trajectories per treatment were used for averaging persistence as function of distance from the edge.
Chapter 5

APPLICATIONS

Motility is one of the main parameters of cancer cell invasiveness and aggressiveness. In this chapter we apply our approach for high throughput phenotyping of tumor cells during collective migration. In one application we study the effects of glucose deprivation, glycolysis energy metabolism inhibition and activation of HGF/SF signaling on cells collective migration. In the second application we study cell individuality phenotype under different activating and inhibiting Met variants and HGF/SF signaling. Both applications serve as a general "proof of concept" as for our framework’s high throughput phenotyping capabilities.
5.1 The Interplay Between Glucose-Metabolism and Activation of HGF/SF on Cells Collective Migration

As a "proof of concept" for our high throughput phenotyping capabilities, the interplay between glucose metabolism and HGF/SF-activation was investigated by applying the spatiotemporal dynamics measurements. The effects of Met signaling were examined at different metabolic states on collective tumor cell motility during wound healing.

5.1.1 BACKGROUND

Glucose is essential in all eukaryotic organisms, especially mammalian cells, as an energy source to help sustain cell life and as a carbon source for most cell building blocks. Therefore, it is not surprising that glucose catabolism (glycolysis) is elevated in many cancers, particularly those with the most rapid growth rates as was discovered 80 years ago by Otto Warburg (167), (168). In accord with the Warburg effect, cancer cells exhibit increased activity and overexpression of intracellular glucose transporters (GLUT1 and 3) (169) and consume larger amounts of glucose than normal cells (170).

The analogue 2-Deoxy-glucose (2-DG) competitively inhibits cellular uptake and utilization of glucose. Upon entering the cell, it is phosphorylated and the product, 2-DG-6P undergoes no further metabolism, hence glycolysis is inhibited (171). Clinical trials have evaluated safety issues related to treating humans with 2-DG and found that doses up to 250 mg/ml are safe along with radiation therapy in patients with glioblastoma multiforme (172).

Involvement of Tyrosine kinase growth factor signaling pathways in cancerogenesis is well studied and targeted by several drugs (173), yet the functional relationship between these pathways and the alteration in cellular metabolic activities are poorly understood. Much less is known about the cellular proteins mediating the alterations in bioenergetics induced by Met signaling. HGF/SF increases both glycolysis and oxidative phosphorylation in breast cancer cell line DA3 (174), but HGF/SF-mediated activation decreases the mitochondrial membrane potential (data not published).

5.1.2 EXPERIMENTAL SETTING AND ANALYSIS

DA3 cells were pre-incubated with 5 mM glucose for 90 minutes, followed by 90 minutes in media containing the following constituents: 25 mM glucose (hyperglycemic); 5 mM glucose (normoglycemic), with or without 5 mM 2-DG; no glucose (hypoglycemic); with or without 5 mM 2-DG. Then, the monolayer was scratched by a 200 μl tip, and the medium was replaced to hyperglycemic (25 mM
glucose and fetal calf serum) for the remaining time of the experiment. Cells were imaged every 15 minutes for 24-72 hours with DIC microscopy and photographed by 510 Zeiss Meta confocal laser scanning microscope (CLSM).

Velocity magnitude maps were calculated for each experiment. The two first components of a PCA on the compact representation of these maps were used for visualization (Fig. 5.1). Discrimination between different treatments was performed by dividing the experiments to groups, and then applying SVM classification with "leave one out" validation, as described in Chapter 3.

5.1.3 PRELIMINARY RESULTS

The effect of HGF/SF was compared in different metabolic states. HGF/SF activated cells located far from the leading edge, in all the glucose-treatments, but in treatments lacking HGF/SF, these cells did not significantly start to migrate (Fig. 5.2A). Glucose deprivation of HGF/SF-treated cells induced enhanced migration compared to glucose-fed cells treated with HGF/SF (Fig. 5.2B). Finally, 2-DG inhibited collective cell migration in cultures treated with glucose but without HGF/SF (Fig. 5.2C).
5.1 The Interplay Between Glucose-Metabolism and Activation of HGF/SF on Cells Collective Migration

5.1.4 SUMMARY

To study the effects of hypoglycemia and HK inhibition on HGF/SF-induced cell motility, the wound healing experiment was performed in the presence of different glucose and 2-DG concentrations. Principal component analysis and SVM classification of the spatiotemporal representation of cells' speed demonstrated that HGF/SF-treated cells were characterized by high motility and possessed the...
"wave" phenotype regardless of other treatments (with or without glucose, and with or without 2-DG).

Based on these results we conclude that (1) HGF/SF-induced Met-signaling is more prominent than glucose in collective cell migration, (2) Glucose deprivation increases HGF/SF-induced motility, (3) Inhibition of cellular uptake and glucose-utilization further reduces these phenomena. These finding strengthen our hypothesis that HGF/SF signaling induces metabolism-plasticity of the cells altering from glycolysis to oxidative phosphorylation or beta oxidation thus increasing collective cell motility. However a profound inhibition of hexokinase activity by 2-DG significantly reduces it (174).

This quantification method provides an infrastructure for biologists to quantitatively answer questions about collective cell migration. Treatments are to be divided to several groups; the discrimination score between a pair of groups can serve as a measure for the different phenotypes (Fig. 5.2). A complementary approach would be to perform hierarchical clustering on the vector-representations and thus provide full structure on the phenotypic-relations between the different experimental conditions.

5.2 Screening Met-Variants for “Individuality” Phenotype

Previous studies in the Tsrfaty lab suggested that aberrant Met receptor activation by overexpression induces in some lines cell rounding and dissociation from substrate and neighboring cells, leading to cell migration and metastasis (unpublished). Constitutive activation of Met through point mutations at key residues of the tyrosine kinase domain may result in more motile and detached cells (unpublished).

Aggressive breast cancer cells that exhibit constitutive Met activation display extensive membrane blebbing and amoeboid motility that can be blocked by Met inhibition. Expressing high levels of Met in Human embryonic kidney cells HEK293T cells induces a constitutive phosphorylation of Met in a ligand-independent manner and prominent membrane blebbing, leading to amoeboid motility and invasion (123).

To further investigate HGF/SF-induced path finding amoeboid motility a model system has been generated of HEK293T cells exogenously overexpressing fluorescent-tagged (FT) wild type (WT) and mutated Met constructs within a monolayer of WT cells. The effects on this system of Met activation and its downstream signaling molecules were studied. A wound was generated within the cellular monolayer to induce migration and quantitative image analysis was used to evaluate cellular dynamics.
Understanding the cellular and molecular mechanisms enabling the cell to dissociate from the primary tumor and adopt a dynamic motile morphology that allow for migration within the normal tissue, is essential to combat the metastatic process and develop anti-metastatic therapies.

5.2.1 EXPERIMENTAL SETTING AND THE INDIVIDUALITY MEASURE

We investigate the effect of HGF/SF induced Met signaling and several Met-activating and repressing mutations on cells decision to move individually of their neighboring cells during collective cell migration. Wound healing assays were imaged with time-lapse confocal microscopy using HEK293T cell line expressing high levels of YFP-mutated Met to examine individual cell motility. A new measure was devised to quantify cell individuality that is based on combined processing of bright field and fluorescence live cell time-lapse confocal microscopy, and applied to study the relations between Met-activating or suppressing variants and HGF/SF.

Low concentrations of YFP-Met forms were transfected to HEK293T cells and spread in a monolayer to investigate individual-motion of cells (Fig. 5.3) in an experimental setting as that of (175). These experiments simulate the phenomenon of cells that acquire amoeboid-like motility while traversing in a tissue consisting of untreated cells. We suggest a novel fully-automated quantitative measure to examine the motility of transfected cells in relation to their vicinity of untreated cells (denoted individuality of cells, Fig 5.4A). It exploits the fluorescent channel to track single-transfected-cells and to define region of interests whereas the bright field channel to estimate motion of all cells in the monolayer. Spread of each transfected cell is recorded and compared to that of non-transfected cells in its vicinity throughout its trajectory to define trajectories relative speed, as a percentile related to its normal neighboring cells. Thus, the individuality measure of an experiment is defined by the trajectories relative speed of all its tracked cells.
5.2 Screening Met-Variants for “Individuality” Phenotype

5.2.2 Preliminary Results

The individuality measure applied on the HEK293T cells demonstrate that transfected cells maintain distinct motility relative to their surrounding cells: activating Met-variants (over-expressed WT, M1268T, D1246N, Y1248C,
L1213C/M1268T) are generally characterized by lower relative-motility derived from cells’ amoeboid-like motility; Met-suppressing TDN induces motility also characterized by lower relative-motility, whereas HGF/SF induces increased relative-motility (Fig. 5.4B). Statistical analysis between individuality measure on all pairs of HGF/SF and Met-variants conclude that the activation of Met-signaling by HGF/SF is the prominent component in individuality of cells (Fig. 5.5).

![Change in relative motility](image)

**Fig 5.5** HGF/SF-Met plays a major role in cells’ individuality. HGF/SF is more prominent than any Met-mutation in inducing enhanced cell individuality. Color represents change (%) in individuality.

## 5.3 Summary

We applied high throughput phenotyping to investigate the interplay between HGF/SF-Met and glucose-metabolism on collective migration, and the effect of various Met mutations and HGF/SF on cell individuality. In both cases Met activation by HGF/SF takes the prominent role in motility. Additional experiments are required to validate the biological results, but our computational framework can clearly serve for high throughput quantification.
Chapter 6

ONGOING WORK AND PRELIMINARY RESULTS

In previous chapters we have presented pioneering efforts to quantify and model in vitro collective cell migration. This field is in its infancy, so naturally, many new avenues open for future research. In the following we plant seeds that hopefully will sprout in the near future. Some ideas are enhancements for current quantification methods or suggestions for additional measures that currently are manually quantified or not quantified at all, such as measuring cell density or proliferation events. Some leads refer to new insight on general collective cell migration: intercellular coordination, broader understanding of the acceleration wave, transitions from single- to collective migration, genotype-phenotype mapping. Additional suggestions refer to new applications using similar tools presented herein, such as neural cell rosette or membrane cell blebbing quantification.
6.1 Multi-Cellular Segmentation for Live Imaging
As discussed in Subsection 3.2, many tools, algorithms and image-acquisition approaches exist to segment multi-cellular live images (70), (71), (72), (73), (93), (81), (80), (14), but these were designed to deal with single image segmentation thus ignoring specific domain information in live imaging of in vitro collective migration assays. These approaches may fail when handling varying imaging conditions and are prone to local algorithmic errors. Multi-cellular segmentation is the basic step in almost any analysis, hence its accuracy and robustness is crucial.

As far as we know, (8) was the first exploiting temporal information to improve segmentation robustness. Automatic parameters tuning have been suggested based on specific data (14), (93), (81), (80), but this requires an initial step of manual segmentation / cell detection, and offers no simple routine to perform this task.

We propose a new approach that utilizes annotated images from Borad's BBBC multi-cellular segmentation benchmark (16) to automatically adjust segmentation parameters without the need to annotate manually, and exploits temporal information to enhance robustness. The algorithm is composed of two main steps:

- Use several images from the time-lapse sequence and texture-descriptors to select the most similar images from the rich manually-annotated benchmark, and then use these images and their annotation to train a classifier that was specifically optimized to the given experiment;
- Exploit temporal information, the trait that the monolayer only expands in time, to further enhance segmentation robustness to noise and local error.

Additional enhancement can enter in the final localization, by e.g., using texture-based active contour methods (176).

6.2 Quantify Cell Density and Proliferation in Confluent Monolayers
Direct measurements of cell density and proliferation events, phenotypes have important implications to study the mechanisms that promote in vitro collective cell migration, are currently performed manually. Devising automatic measurements to enable objective quantification of these phenomena is thus highly important.

6.2.1 Mapping Image Texture to Cell Density
In most of the studies examining the effect of cell density on collective migration (e.g., (28), (62), (158), (31), (84)), density was determined by manual counting of single cells in the monolayer. The ability to estimate density at high confluence levels will extend these investigations to high throughput applications, as well as improve understanding of this process by using spatiotemporal density measurements.

The suggested approach is based on regression from high dimensional texture features to density estimation. Since texture contributes to discriminate between cells with different morphological characteristics (Section 3.4), this approach is considered promising.

6.2.2 Automatic Detection of Cell Division in Collective Cell Migration

The role of proliferation in collective cell migration is still vague. In the few studies that quantify proliferation, counting and localizing cell division were performed manually (44), or semi-automatically (62). Only a few attempts have been made to quantify mitosis (95). Automatic detection of such events will enable inclusion of cell proliferation measurements in future studies.

This problem is inherently a Computer Vision "detection" task. The idea proposed here is to use a "sliding window" and features extracted from texture and motion estimation. Sliding window is the standard method used to search an object in an image/video in detection tasks. A rectangular window with various scales and aspect ratios in a predefined time-interval is applied; each is scored based on SVM classification of the corresponding feature vectors. Manual labeling of cell division events is required as training data for enable this application. Alternatively, designated experiments can be performed using fluorescent nuclear markers, which help to detect division events using existing tools and can later serve as annotated data to train a classifier based by the bright-field channel.

6.3 An Open-Source Software for High-Throughput Analysis of Collective Cell Migration

There are very few public and freely available tools for automatic quantification of live in vitro collective migration (8), (9). We propose to develop open source software that will incorporate the measurements proposed through this dissertation. This software will have high throughput phenotyping ability, based on spatiotemporal characteristics of the cellular dynamics. One inherent feature that is missing in currently existing tools is the treatment-clustering and answering discriminative questions about an experiment. The framework described in Section 3.3, was (partially) validated in Section 5.1 will be combined
6.5 Model the Wave

with suitable user interface (102) to define an effective tool for application such as drug screening and molecular investigations.

6.4 Characterization of Intercellular Coordination

The direct method to cluster cells that migrate coherently as part of a group in the monolayer was presented in Section 3.7. This method was used to reveal the wave of intercellular coordination (Section 4.2). Its great advantage over other available approaches for quantification of intercellular coordination is the ability to explicitly grasp these groups of cells hence enabling investigations of the cellular and molecular mechanisms behind it.

The future step would be to investigate phenotypic and molecular characteristics of cells in the clusters. First, the phenotypic differences (velocity, directionality, persistence and morphology) between coordinately-moving and less cooperating cells will be examined in different lines and treatments. Next, the differences between clusters at different sizes will be studied. Both steps can be performed using the available experimental data. For deeper understanding of the underlying molecular mechanisms, additional experiment should be performed with various fluorescent molecular markers, such as of actin and myosin. Finally, downstream perturbations of the Met signaling pathway should be performed to understand the role of Met-signaling in this process.

6.5 Modeling the Wave

The mechanical waves reported above and by others are now simulated and modeled. A simple simulation demonstrates that an acceleration wave, traversing the monolayer at constant speed, can induce the phenotypes observed.

The general idea was to simulate a virtual particle in the experimental data, that is affected by the wave (by self propulsion to the edge direction) and by cell-cell interactions with its neighbors (in a spring-like relation). The main parameters are the wave propagation rate, its effect on migration, and the spring constant of the particle integration with its neighbors. Virtual particles are seeded in the monolayer (Fig. 6.1), in each frame a particles velocity is determined based on its current speed, the wave's effect, and the interaction with its neighbors (based on the real calculated local motion estimation). Particles that exceed beyond the monolayer limits (determined by the real segmentation) are discarded from the rest of the simulation.
The simulated cells were used to define velocity magnitude maps (Fig. 6.2A), and to extract measurements such as persistent migration (Fig. 6.2B). The velocity magnitude maps were visualized via first two components of PCA to demonstrate the transformation from phenotypes of untreated or cells treated with HGF/SF and Met inhibition to similar dynamics of HGF/SF treated cells (Fig. 6.2C).

Next, a possible mechanism is proposed to govern the acceleration wave, based on secretion of chemical signals by cells, beginning at the leading edge and propagating inwards the monolayer, activating cells from behind. To this end, we use a static grid of simulated cells; each bin in the grid has an "energy" level, which resembles the level of the chemical component that promotes cell acceleration. Cells being in an inactive state, and are activated by cells in front of them. Upon activation, signal secretion probability is dependent on the current "energy" level, and the signal propagates by diffusion to neighboring cells (Fig. 6.3A).

Preliminary results show that an energy wave emerges (Fig. 6.3B), similarly to the phenomenon observed experimentally (Fig. 6.3C). The propagation of the simulated signal (Fig. 6.4D) resembles the acceleration and strain-rate propagation (Fig. 6.3D). We are currently trying to understand the relations between the different parameters and to get better fit to the experimental results.
The next step would be to apply the static propagation model on to the dynamic experimental-based simulations.

Fig 6.2 Simulation results
(A) Left: the velocity map extracted from an experiment of untreated cells, based on manual tracking of cells. Center: simulation of treatment with HGF/SF from 700 virtual particles. Right: a representative velocity map of HGF/SF treated cells, based on manual tracking of cells.
(B) Persistent measurements of simulated experiments. Both simulations are characterized by increased persistence.
(C) Two-dimensional PCA on the experimental and simulated velocity magnitude maps.
6.6 **TRANSITION FROM SINGLE- TO COLLECTIVE-MOTILITY UNDER MET-SIGNALING**

Single cell migration has been studied extensively for many years now. Much effort is currently invested to investigate collective cell migration. It is shown here and by others, that single- and collective-migration can be characterized by different phenotypes. One example is the response of cells to HGF/SF. Surprisingly, very limited research was performed on the intermediate stage, migration of small groups of cells. (177) revealed a phase-transition from single-to group-motility that occur in Bacteria, and is based on the group's size. (178) proposed a framework to quantify migration of cell colonies. We intend to apply a similar approach on small cell colonies and quantify the effect of HGF/SF on motility measurements with respect to colony size. Initial microfluidic experiments were performed, and preliminary results were obtained (data not shown).

6.7 **GENOTYPE-PHENOTYPE MAPPING OF COLLECTIVE CELL MIGRATION**

To investigate the relations between metastatic potential, genomics and HGF/SF-Met signaling, we propose a genotype-phenotype mapping for several metastatic cell lines; gene-expression data have been acquired for 6 metastatic cells lines.
before and after exposure to HGF/SF. We are setting up an experimental framework to acquire time-lapse images of wound healing experiments with the same cell lines and conditions. Once the experimental data is ready, we suggest investigating the relations between cell line-dynamics-gene-expression data. Such a study was recently performed by Yuan et al., who combined information from tumor histological images and gene-expression data to report enhanced prognosis-prediction capabilities for breast cancer (179).

Our plan is to extract the phenotypes based on the framework presented in this dissertation, and then apply advanced machine learning techniques and statistical correlation methods to connect between the genetic data, collective motility dynamics, metastatic potential, and the relation to HGF/SF-Met.

The pairs of spatiotemporal dynamics features and genomic data is a natural input for the problem of learning to map between two vector spaces given pairs of matching vectors, one from each space. We shall use classical approaches, such as Canonical Correlation Analysis (CCA) (180), as well as modern tools (181), (182) to find new connections between genetic traits and different phenotypes in corresponding phenotypes, metastatic potential and HGF/SF, which might lead to early detection and better understanding of breast cancer in general, better prognosis-prediction capabilities, and aid in identifying new targets for personalized therapy.

### 6.8 Quantify Generic Neural Stem Cell Rosette

Pluripotent stem cells (PSCs) are defined by their ability to provide an unlimited source for generating the three germ layers. Since PSCs provide access to early cell fates previously inaccessible to researchers, they have an important role in development and disease investigation. The advent of induced PSCs (iPSCs) - pluripotent stem cells artificially derived from somatic cells by “forced” expression of pluripotent transcription factors – has brought a wave of experimental paradigms for systematic derivation of iPSCs from many types of diseases, and has transformed our understanding of disease modeling. This should ultimately lead to the development of robust therapeutic drug discovery strategies, which will eventually be customizable to individual patients.

Dr. Yechiel Elkabetz and his group from Tel Aviv University isolated a novel neural stem cell (NSC) from PSCs, termed rosette-NSC (R-NSC), and revealed a striking correlation between the capacity of rosettes to maintain their morphology *in vitro* and their ability to retain a broad differentiation potential. This encourages further deepening the knowledge on the cellular dynamics within the rosette structure, and how it relates to NSC function. However, features reflected by rosette structure, such as velocity and directional patterns of cellular
components within the rosette, cannot be simply measured by conventional stem cell assays.

Our approach for measuring collective cell migration is adjusted and applied to neural rosette phenotyping. The general idea is to extract high dimensional spatiotemporal representation of rosettes based on morphology and dynamics and to utilize it for clustering and discriminating between different variants of healthy and patient iPSC derived rosettes towards gaining first insights into neurodevelopment pathology. Preliminary results suggest that modification of our approach to this arena can be highly beneficial to this field (data not shown).

An example rosette is presented in Fig. 6.4. This project is performed as part of the M.Sc. studies of Omer Ziv from the Elkabetz’s laboratory.

![Fig6.4 Neural rosette](image)

**6.9 QUANTIFY DYNAMICS OF AMOEBOID BLEBBING CELLS**

To correlate between the alteration of signaling and amoeboid single cell motility, we develop a new approach for blebbing analysis. It is based on tracking the contour of a cell and producing a 2-dimensional spatiotemporal map to visualize and enable robust automatic quantification of dynamic blebbing characteristics such as bleb formation- and retraction-times, bleb growth/retraction rate and molecular activity localization in space and time (Fig. 6.5). The method is similar to those presented in (151), (183). Additional measurements can be obtained by quantification at the cell’s level, for example, careful examination of blebbing frequency and location can help answer questions such as whether there is a preference for blebbing at specific regions of a cell. The question whether bleb formation events are dependent or independent of each other can be resolved by examining the probability to get simultaneous two blebs dynamics. Preliminary
results indicate that this quantitative approach can be used to analyze blebbing parameters in computer simulated blebbing cells and in experimental data. Fig. 6.6 demonstrates blebbing formation and retraction dynamics of a simulated cell.

Fig 6.5 Blebs quantification
(A) Simulated blebbing cells (by Inbal Hect et al., unpublished).
(B) Spatiotemporal map of blebbing dynamics. X-coordinates are time, Y-coordinates is an arbitrary cyclic spatial representation of the cell, color represents estimated change in location of a specific point on the cells contour at a specific time. Red – is motion outwards from the cell, Blue – inwards motion. Large clusters of outward motion followed by inward motion are blebs.
(C) An example of a segmented bleb in the same spatiotemporal representation as in B.
(D) Bleb’s area as function of time. The most basic measurement that can be extracted.
6.9 Quantify Dynamics of Amoeboid Blebbing Cells

![Graph showing bleb area as a function of time for a single cell.](image)

**Fig 6.6** An example of a cell's blebbing dynamics. Bleb area as a function of time for a single cell.
Chapter 7

SUMMARY

In vitro collective cell migration is a model system for development, wound repair and cancer invasion. Despite numerous studies that investigate this mode of migration, only few attempts have been made to systematically quantify it. In this thesis we presented a comprehensive and robust tool box to measure dynamic spatiotemporal phenotypes ranging from cell velocity, persistent migration, intercellular coordination and morphology. Our toolbox surpassed current available tools, and was proven to be beneficial to study several specific research questions.

In this thesis we focus on HGF/SF-Met induced collective cell migration, as a specific signaling pathway of interest. We revealed that the activation of Met signaling by HGF/SF enhanced all aspects of migration in tumor and normal cells. We thoroughly investigated acceleration waves, a recently reported phenomenon that occurs during collective cell migration under certain conditions. We found that activation of Met by HGF/SF is a sufficient condition to induce acceleration waves, reported the relations between different characteristics of these waves and discovered a directional cue the follows it, and an additional wave of intercellular coordination.

Our tool box was proven to be effective for high throughput phenotyping. We exploited it to investigate the effects of HGF/SF signaling along with different metabolic conditions, or with various Met mutations. We found that Met activation by HGF/SF is the prominent factor in inducing collective migration in both cases. Finally, we proposed new measurements, several applications and open research questions as future work.
6.9 Quantify Dynamics of Amoeboid Blebbing Cells

AUTHOR CONTRIBUTIONS

Main articles from the Ph.D.:


The following paper is not related to the Ph.D. but was published during my studies: Zaritsky A, Barzilay O. Computer Science as a Community Involvement Activity. Proceedings of the 17th ACM SIGCSE conference on Innovation and Technology in Computer Science Education (ITiCSE2012), 2012.

Co-authored articles:

I contributed minorly to these articles, which correspond to projects carried out by collaborators.


Public datasets, benchmarks


Conference contribution:

- The 2nd Meeting of the Israeli Forum for Cytoskeleton and Motility, Be’er-Sheva, Israel. February 2013. **Talk title:** HGF/SF Induced Waves of Organized Motility during Collective Migration of Tumor and Normal Cells.


- The 46th Annual Scientific Meeting of ISM (Israel Society for Microscopy), Israel, 2012. **Poster title:** HGF/SF-Met-Induced Cell Motility: Cooperation versus Individuality. **Best poster award.**

- The Fourth Annual Meeting of the Israel Society for Cancer Research (ISCR), Israel, 2012. **Poster title:** Exploiting Bright Field Microscopy to Quantify Collective Cells Migration.

- Automated Imaging & High-Throughput Phenotyping, Cold Spring Harbor Laboratory, 2012. **Talk title:** Exploiting Bright Field Microscopy to Quantify Collective Cells Migration.


- The 45th Annual Scientific Meeting of ISM (Israel Society for Microscopy), Israel, 2011. **Talk title:** A Novel Approach to Quantify the Effect of HGF/SF on Cell Motility Patterns in DIC-Based Time-Lapse Microscopy Wound Healing Assays.
COLLABORATIONS

This thesis is a result of interdisciplinary collaboration. Many people have contributed to it, and without them I would have not been able to perform this research:

- Prof. Lior Wolf supervised my work.
- Prof. Ilan Tsarfaty and Prof. Eshel Ben-Jacob guided many aspects of my work.
- Dr. Doron Kaplan, Sari Natan, and Dr. Judith Horev performed the wound healing experiments related to HGF/SF-Met signaling.
- Dr. Doron Kaplan has integrated my code and implemented a user friendly interface for the Tsarfaty lab.
- Yaniv Goikhman performed the HEK293T "individuality" experiments.
- Dmitry Zabejinsky performed the wound healing experiments related to Met and Metabolism.
- Prof. Nir Gov supervised me on the relations between directionality, strain rate and the wave, and performed the simulation connecting these parameters.
- Avner Peled worked on the wave modeling.
- Dr. Inbal Hecht helped with productive discussions and helped in writing some of the papers.
- Prof. Xavier Trepat and Dr. Xavier Serra-Picamal supplied MDCK sheet expansion experiments with force measurements.
- Dr. Yechiel Elkabetz and Omer Ziv work on neural rosette quantification.
- Tammy Diamant and Dr. Adi Laser-Azogui performed single cell blebbing experiments, Naama Kisra worked on blebbing quantification.
REFERENCES


15. Zaritsky A, Natan S, Ben-Jacob E, & Tsarfaty I (2012) Emergence of HGF/SF-Induced Coordinated Cellular Motility. (Translated from English) Plos One 7(9) (in English).
16. Zaritsky A, Wolf L, Ben-Jacob E, & Tsarfaty I (2013) Benchmark for Multi-
Cellular Segmentation of Bright Field Microscopy Images. Submitted.
during Collective Migration of Tumor and Normal Cells. Submitted.
Suppresses the Malignancy Phenotype of Melanoma Brain Metastasis.
Submitted.
dimensional extracellular matrix. (Translated from eng) Cell Mol Life Sci
57(1):41-64 (in eng).
and escape mechanisms. (Translated from eng) Nat Rev Cancer 3(5):362-
374 (in eng).
22. Eccles SA, Box C, & Court W (2005) Cell migration/invasion assays and
morphogenesis and cancer. (Translated from eng) Int J Dev Biol 48(5-
6):441-449 (in eng).
Modular Mechanical Properties. (Translated from English) Science
322(5907):1502-1505 (in English).
English) Journal of Cell Science 122(18):3209-3213 (in English).
in infiltrating lobular carcinoma. An analysis of "classical" and variant
of invasion, bleomycin sensitivity, and clinical course in squamous cell
carcinoma of the oral cavity. (Translated from eng) Cancer 51(12):2175-
2180 (in eng).
Epithelium. (Translated from English) Biophysical Journal 98(9):1790-
1800 (in English).
cell migration using an siRNA screening approach. (Translated from
English) Nature Cell Biology 10(9):1027-1038 (in English).
regulates cadherin-dependent collective migration through myosin-II
contractility. (Translated from English) Journal of Cell Biology 199(3):545-
563 (in English).
migration driven by cooperative substrate deformation patterns.


58. Lee P & Wolgemuth CW (2011) Crawling Cells Can Close Wounds without Purse Strings or Signaling. (Translated from English) Plos Computational Biology 7(3) (in English).


শিক্ষক𫚃: רימונד ובברלי סאקלר

ביה הנشرح למדעי המחשב

שיטות חישוביות להיבחר ממדי❧ תנועה קבוצית של תאים

עיבוד לשים קבלת תואר דוקטור לפילוסופיה

נאות

אסף זריצקי

עיבוד ועיטשת המדריך

יפורפ' ליאור 롤ף

הוגש לסנט של אוניברסיטת תל אביב

מאי 2013
A movement collective of cells plays a central role in many biological processes. Understanding the molecular and cellular mechanisms of such movement is very limited despite decades of extensive research. Various computational methods, algorithms, and image processing software have been developed for individual cell dynamics, but the treatment of collective movement has been neglected.

Enormous amounts of information collected in experiments of this type require high data processing capacity in order to enable applications such as molecular and cellular mechanisms of cancer, drug screening, and personalized medicine. By simulating cells in real-time, this work presents a set of quantitative measures for collective cell movement that transcend the existing methods. The tool set includes cell segmentation—multivariate, tracking and collective motion, movement in groups, individual cell movement measures, and various presentations of time-space and more.

Validation of the measures was achieved by comparing with leading methods in the field. The study is based on the inspiration of collective cell movement in normal and cancerous cells through the receptor Met ligand of Hepatocyte Growth Factor / Scatter Factor (HGF/SF), which induces various biological responses such as growth and culture, migration and morphological changes in cells and tissues in normal and cancerous cells. It is found that the activation of HGF/SF-Met is sufficient to enable a directional signal through a conduction layer below the epithelial cell layer.

The use of the data analysis methods developed for the study of collective cell movement can be used for various applications such as personalized medicine and drug screening. The results of the study were examined between the early stages of creating a scar and mutations activated and repressed of Met in the culture of normal cells. Therefore, a method was developed that measures the movement of a single cell in relation to its close environment. It is found that the activation of HGF/SF-Met is a major factor in the functioning of the personalized medicine. The studies described above confirm that the method developed by us can be used for diagnosis, drug screening, and personalized medicine methods.
תקציר

פרק פעיל

לתוך התפתחות, קבוצות גדולות של תאים עוברים מרחקים עצומים על מנת להגיע ליעד הбиולוגי הסופי שלהם. בתיקון רקמות, יריעות של תאים נעות בתיאום כדי לרפא רקמה שנפגעה. בסרטן, קבוצות של תאים פולשות למטריצה הבインタ-תאית (ECM), חוצות רקמה בריאה ביעילות גבוהה العراقילים. הבנה המנגנונים התאתיים של אופן תנועה כזה מוגבל מאוד. מחקרים באמצעות תמנונים ניסויים במבחנה (in vitro) עשויים להיות שימושיים ביותרологии, הבנת תופעות מבית התאים, והבנה של המנגנונים התאתיים שנ_reactionים במערכת ניסויים שונות. שיטה בסיסית לכימות כוללת רכישת תמונות בזמנים שונים (או באופן רציף) במקביל, ומדידת קצב הריפוי באמצעות מדד ידני או אוטומטי של פיצויים. עיבוד הנתונים הוא צוואר הנקמה במחקרים אלה. שיטות מחישוביות, מדדים, ותוכנות לעיבוד תמונות מיקרוסקופיות, הוצגו ומעטים. למקרה הנוכחי, שיטה אוטומטית של דינמיקה תאית מאופיינת באובייקטיביות, ורמות כדי גילוי תכונות שלא נראות בעין האנושית. קיימות שיטות מחישוביות, מדדים, ותוכנות לעיבוד תמונות מיקרוסקופיות, אך הטיפול בגרסת התנועה הקבוצתית הוזנח. }

ברקע

 движения קבוצתית של תאים משחקת תפקיד מרכזי בתהליכים ביולוגיים רבים. בשアイ ציעום, נגרם ב(radius תאיATOM) תאי רקמות, יריעות של תאים נעות בתיאום כדי לרפא רקמה שנפגעה. בסרטן, קבוצות של תאים פולשות למטריצה הבinta-תאית (ECM), חוצות רקמה בריאה ביעילות גבוהה العراقילים. הבנה המנגנונים התאתיים של אופן תנועה כזה מוגבל מאוד. מחקרים באמצעות תמנונים ניסויים במבחנה (in vitro) עשויים להיות שימושיים ביותרולוגיה, הבנת תופעות מבית התאים, והבנה של המנגנונים התאתיים שנreaction התאים בשאיש מ 이것이, שיטה בסיסית לכימות כוללת רכישת תמונות בזמנים שונים (או באופן רציף) במקביל, ומדידת קצב הריפוי באמצעות מדד ידני או אוטומטי של פיצויים. עיבוד הנתונים הוא צוואר הנקמה במחקרים אלה. שיטות מחישוביות, מדדים, ותוכנות לעיבוד תמונות מיקרוסקופיות, הוצגו ומעטים. למקרה הנוכחי, שיטה אוטומטית של דינמיקה תאית מאופיינת באובייקטיביות, ורמות כדי גילוי תכונות שלא נראות בעין האנושית. קיימות שיטות מחישוביות, מדדים, ותוכנות לעיבוד תמונות מיקרוסקופיות, אך הטיפול בגרסת התנועה הקבוצתית הוזנח.
The meaning is that the genotype represents the outcomes of algorithms because using fewer of the least accurate trajectories provides more information about the dynamic nature of motility than using the actual trajectories. The algorithm is wrapped in a graphical user interface, and today it is available through a GUI interface and code.

The reality is an illustration of a complete experiment through a vector descriptor in a high-dimensional space containing varying properties. Changes as small as possible through the use of a dynamic representation. This approach was proposed as a method to determine the collective movement of cells in a dataset.

We have developed a method to refine trajectories based on the division of vector motion:

- In the second stage of the method, we determined the signal-to-noise ratio (SNR) and the local speed of the trajectory at each point in the image, which together with the movement vector and the rate of strain presents the basis for advanced processing.

- The segmentation of the image into a set of vector motion fields allows visualization of the dynamic nature of all atoms for the purpose of checking the quality of each measurement independently because it can be used to evaluate.

- The quality of the measurements is measured in terms of the ability to diagnose diseases and develop treatments for related or similar conditions.

- The idea is a representation of a complete experiment through a vector descriptor in a high-dimensional space containing varying properties. Changes as small as possible through the use of a dynamic representation. This approach was proposed as a method to determine the collective movement of cells in a dataset.

- We developed an algorithm for this purpose, which excels beyond its competitors.

- In the next paper, we show the benefits of using related or similar conditions and the dynamic nature of all atoms for the purpose of checking the quality of each measurement independently because it can be used to evaluate.
HGF/SF-Met

A novel approach to understanding Hepatocyte Growth Factor / Scatter Factor (HGF/SF) expression in cancer cells.

HGF/SF-Met is a receptor tyrosine kinase that is overexpressed in various types of cancer, including liver cancer. Its expression is regulated by various factors, including Met receptor dimerization, which is essential for the activation of HGF/SF-Met.

In this study, we aimed to develop a new method for analyzing the expression of HGF/SF-Met in cancer cells. We used a combination of computational and experimental approaches to study the expression of HGF/SF-Met in liver cancer cells.

Our results showed that HGF/SF-Met expression is significantly higher in cancer cells compared to normal liver cells. We also found that the expression of HGF/SF-Met is correlated with the aggressiveness of the cancer cells.

In conclusion, our study provides new insights into the expression of HGF/SF-Met in liver cancer cells, which could lead to the development of new therapeutic strategies for treating liver cancer.
מכניים בתאים
tאית מוגברת שגורמת לתנועה
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rק שהיא התאים המתואמים הללו לא מטופלים מאופיינים בירידה בתנועה
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ספציפי תחת הפעלת מנגנון האיתות של
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החווים יותר מהגלים הללו ינועו באופן יותר עקבי מתאים
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בשכבה חד
היחס בין רכיב התנועה בכיוון ובמקביל לפצץ קבוע
המאפיינת את

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ברגע שהגל ממשיך לתאים פנימיים יותר בשכבה חד
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In this article, the authors discuss the high productivity phenotype in the third part of the work. They also present additional sets for high productivity phenotype analysis. The goal was to conduct a test of the implementation for the already defined metrics for high productivity tasks.

In the first implementation, they investigated the relationship between the metabolism of sugars and the operation of the \( \text{HGF/SF-Met} \) mechanism for collective cell movement through the use of dynamic space-time volume visualization and classification in the high-dimensional space of the experiments according to different treatments. The division of the treatments into groups was done based on the biological question asked at that time.

It was found that remote cells participated in collective grouping when treated with \( \text{HGF/SF-Met} \) at one of the different concentrations of sugars, but there were no statistically significant consequences of the treated remote cells. The decrease of sugars in the \( \text{HGF/SF-Met} \) group increased the motility compared to the \( \text{HGF/SF-Met} \) group, and at the various sugar concentrations. At cells that were treated with a block of sugar flux without \( \text{HGF/SF-Met} \), there was a decrease in motility compared to the cells treated at various sugar concentrations.

In summary, it was found that the operation of the \( \text{HGF/SF-Met} \) significantly affects the phenotype in a much more prominent way than any of the nutrition treatments that were examined.

In the second implementation, they investigated the early stages of fiber production through \( \text{Met} \) mutations and combinations in the cultivation of normal cells. In order to do so, they developed a test that measures the individual cell movement in relation to its surroundings. It was found that the operation of \( \text{HGF/SF-Met} \) is the main factor in the individual movement compared to different \( \text{Met} \) versions.

The implementations mentioned above are expected to be useful in diagnostics, drug testing and developing personalized medical techniques. Further experiments are planned in order to verify the reported results in this section.

Projects are ongoing, future results and preliminary results in the last part of the work are presented. Some ideas for a new generation of cell collective movement tests: improving the segmentation of the cells using the data at the moment and better use of the tagged data, ideas for algorithms to count cell density in a single layer and cell division events.

Research is involved in understanding more in-depth the mutual movement corresponding to the data reported and the analysis of the transition from the movement of individual cells to characteristics of collective movement.

An additional project at the beginning of which is mapping of information on gene expression, for the phenotype collective movement for different types of cancer cells. Other application is the use of the tools developed for analysis of fiber traits of bone cells.

A last project is a count of hearts, morphological change in a cell associated with increased motility and guiding properties.