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**Decoding cell migration – the impact of analytical
choices on biological interpretation**

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ABSTRACT

Cell migration is a fundamental biological process underlying development, tissue repair, immune cell trafficking, and pathological conditions such as tumor invasion and metastasis. Because migratory behavior critically influences metastatic potential, quantitative analysis of cell motility has become central to cancer research. However, migration is a complex and dynamic process, and its experimental investigation is complicated by methodological diversity, limited standardization, and the wide range of available descriptors, often leading to inconsistent or non-comparable results across studies.

This dissertation aimed to systematically investigate how methodological and analytical choices influence the quantitative and biological interpretation of cell migration. To this end, we combined experimental, computational, and statistical approaches to dissect sources of variability in migration measurements across different analytical frameworks.

Large-scale videomicroscopic datasets comprising thousands of individually tracked cells were analyzed to evaluate the behavior and robustness of commonly used migration descriptors, which were grouped into displacement-, path-, and directionality-based categories. We further examined how tracking strategies, intrinsic cell-specific properties, and analytical refinements – including noise filtering and controlled simulation-based perturbations – shape migration readouts and parameter sensitivity. To place single-cell findings into a broader experimental context, we performed a comparative analysis of widely used *in vitro* migration assays, including single-cell tracking, gap-closure assays, and transwell migration, using a standardized panel of human melanoma cell lines and the multikinase inhibitor nintedanib as a model compound.

Across all analyses, migration descriptors differed markedly in sensitivity and, in some cases, even in the direction of response to identical biological perturbations. Certain parameters yielded discordant or opposing outcomes under the same treatment, demonstrating that the apparent effect of a perturbation depends strongly on the chosen analytical framework. Displacement-based descriptors proved the most robust across tracking strategies, whereas path-based metrics were highly sensitive but susceptible to tracking-related noise. Directionality-related measures showed pronounced cell line-dependent variability, reflecting biological diversity in migratory control. In addition, intrinsic cell-line-specific properties, especially morphology, were linked to

characteristic migratory behavior and tracking reliability, indicating that shape-dependent motility is accompanied by shape-dependent detectability in trajectory-based analyses.

Comparative analysis across migration assay formats revealed that identical biological perturbations produced assay-specific and sometimes non-concordant inhibitory patterns, underscoring that migration readouts are not directly interchangeable across experimental contexts. Even when the same cell lines and treatment conditions were applied, the apparent magnitude and direction of inhibition depended strongly on the assay format, reflecting the distinct biological constraints and readout principles captured by each system.

In conclusion, this work demonstrates that cell migration cannot be reliably characterized by a single assay or descriptor. Instead, meaningful interpretation requires careful consideration of methodological context, analytical choices, and assay-specific biases. By providing an integrative framework for comparing migration parameters and experimental formats, this study highlights the importance of methodological awareness in achieving biologically reliable and reproducible conclusions in migration research.

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1. LIST OF ABBREVIATIONS

<i>Abbreviation</i>	<i>Definition</i>
AR	Aspect ratio
ATA	Average turning angle
AUC	Area under the curve
AV	Average velocity
CI	Circularity
CLSV	Cell-line specific velocity
CN	Cell number
COL	Colonization
COLS	Colony size
CONV	Convexity
COV	Coverage
CS	Cell size
CV	Coefficient of variation
D	Displacement
DMEM	Dulbecco's Modified Eagle Medium
DR	Directionality ratio
ECM	Extracellular matrix
FBS	Fetal Bovine Serum
IC50	Half maximal inhibitory concentration
Max D	Maximal displacement
MF	Multiplication factor (to modify speed)
ML	Machine learning
MSD	Mean squared displacement
P	Probability of changing direction (to modify direction)
PBS	Phosphate-buffered saline

RND	Roundness
SD	Standard deviation
SEM	Standard error of the mean
SOL	Solidity
SRB	Sulforhodamine B
TCA	Trichloroacetic acid
TTD	Total traveled distance
TVZC	Turning vector zero crossing
V	Velocity
Z-E (assay)	Zone-exclusion (assay)

2. INTRODUCTION

2.1. Cell migration as a fundamental cellular behavior

Cell migration refers to the ability of cells to relocate in a directed or biased manner within space and time (Lauffenburger & Horwitz, 1996). In practical terms, migration allows cells to move from an initial location to a well-defined destination in response to specific cues, enabling tissue organization, adaptation to environmental conditions, and the execution of specialized physiological functions (Te Boekhorst et al., 2016). At the cellular level, this relocation does not occur as a single event, but as the outcome of multiple interconnected processes that together transform intracellular biochemical activity into productive physical movement (Friedl & Wolf, 2003; Ridley et al., 2003)

2.2. Cellular and molecular mechanisms of migration

Cell migration emerges from the coordinated operation of cytoskeletal force generation, adhesion-mediated force transmission, and polarity-driven directional organization (Ridley et al., 2003; Friedl et al., 2012). Rather than representing a single event, migration is produced by a conserved mechanistic cycle in which cells establish front-rear polarity, extend protrusions at the leading edge, form and remodel adhesive contacts, translocate the cell body, and retract the trailing edge (Friedl & Wolf, 2003; SenGupta et al., 2021). This cyclic organization is summarized in Figure 1. The efficiency of this cycle depends on the tight coupling between biochemical signaling and mechanical feedback, which together shape the observable motility phenotype, including migration speed, persistence, and directionality (Schaks et al., 2019; Shellard & Mayor, 2020).

2.2.1. Cytoskeletal dynamics and actin remodeling

The actin cytoskeleton forms the structural and mechanical basis of essentially all modes of eukaryotic cell migration. At the leading edge, protrusions are driven by rapid actin polymerization and network remodeling. Two major classes of actin-regulatory complexes dominate this process. The Arp2/3 complex promotes branched actin networks that drive lamellipodia formation, generating broad sheet-like protrusions that push the plasma membrane forward (Schaks et al., 2019). In contrast, formin proteins promote the elongation of unbranched actin filaments, thereby

supporting the formation of filopodia, slender spike-like protrusions that probe and sense the surrounding microenvironment. These protrusive activities are spatially controlled by the small Rho-family GTPases Rac and Cdc42, which regulate when and where lamellipodia and filopodia are formed. By coordinating actin polymerization with external cues, these regulators determine the direction and exploratory behavior of the leading edge (Pollard & Borisy, 2003).

At the trailing edge, actomyosin contractility driven by myosin II and modulated by RhoA-ROCK signaling retracts the rear of the cell and pulls the cell body toward the newly formed front. Thus, forward movement emerges from a coordinated cycle in which front-driven protrusion extends the leading edge while rear contraction translocates the cell body, resulting in net forward displacement (Figure 1). Productive migration requires a balance between these processes; disruptions in this balance reduce net motility (Pollard & Borisy, 2003).

Actin filaments undergo rapid turnover (nucleation, elongation, branching, severing) mediated by accessory proteins such as cofilin, coronin, cortactin, and fascin. Their activities fine-tune filament organization and mechanical properties, enabling cells to switch between stable and exploratory protrusions. In pathological contexts, dysregulation of actin remodeling supports invasive phenotypes and migratory plasticity (Panková et al., 2010; George et al., 2023).

2.2.2. *Adhesion turnover and integrin signaling*

For migration to produce net displacement, intracellular force generation must be transmitted to the extracellular environment. This mechanical coupling is mediated by cell-matrix adhesions, which are primarily formed by integrins, heterodimeric transmembrane receptors that link the actin cytoskeleton to extracellular matrix components such as fibronectin, collagen, and vitronectin (Sun et al., 2016; Figure 1).

As the leading edge advances, integrins engage available ligands and initiate nascent adhesions by recruiting structural and regulatory proteins, including talin, vinculin, paxillin, and focal adhesion kinase. With increasing contractile tension, these contacts mature into larger, force-bearing focal adhesions that stabilize protrusions and support forward translocation (Geiger & Yamada, 2011). Efficient migration requires continuous adhesion turnover: new adhesions form at the front, while older adhesions disassemble at the rear through mechanisms such as proteolysis or endocytosis to permit tail retraction. Consequently, migration efficiency reflects a dynamic

balance, consistent with the biphasic relationship between adhesion strength and motility, where insufficient adhesion yields poor traction but overly stable adhesion impairs detachment and slows movement (Elosegui-Artola et al., 2018).

Beyond their mechanical role, adhesions act as signaling hubs. Force-dependent adhesion maturation activates Src/FAK-linked signaling and engages Rho-family GTPases, enabling mechanotransductive feedback that allows cells to sense ligand density, stiffness, and spatial organization of the matrix and adapt their protrusive and contractile behavior accordingly (Plotnikov et al., 2012).

In physiological three-dimensional tissues, these processes unfold within structurally heterogeneous extracellular matrices, where fiber architecture, porosity, and viscoelasticity impose additional constraints on migration. In contrast, in two-dimensional *in vitro* environments, including the single-cell and gap-closure used in this dissertation, cells migrate on a planar substrate with defined ligand density and stiffness. While simplified, these systems capture core aspects of adhesion-dependent traction and remain highly informative for dissecting fundamental motility mechanisms. At the same time, planarity can introduce context-specific behaviors such as contact guidance, whereby cytoskeletal organization becomes biased by subtle microtopographical features or surface chemistry (Yamada & Sixt, 2019; Kim et al., 2012).

2.2.3. Polarity establishment and directional sensing

A cell can generate force and traction without producing effective migration if movement is not organized in a persistent direction. Directional migration therefore, depends on establishing and maintaining a polarized front-rear axis. Cells achieve this by detecting spatial cues, whether chemical, mechanical, or topographical, and translating them into stable asymmetry in signaling and cytoskeletal organization (Shellard & Mayor, 2020). This polarity is typically characterized by enrichment of pro-protrusive signaling at the leading edge and contractile signaling at the rear, reinforcing persistent movement (Ridley et al., 2003; SenGupta et al., 2021).

Directional sensing enables a broad range of guided behaviors, including chemotaxis along soluble gradients, haptotaxis along matrix-bound ligand gradients, durotaxis toward stiffer substrates, and topotaxis in response to geometrical features of the environment (Shellard & Mayor,

2020). Importantly, the stability of polarity and the ability to suppress competing protrusions are key determinants of whether cells move persistently or follow more tortuous trajectories.

2.2.4. *Mechanochemical integration: the motor-clutch view*

Although cytoskeletal dynamics, adhesion turnover, and polarity signaling can be discussed as separate modules, migration is generated by their continuous mechanochemical coupling. The motor-clutch model provides a widely used conceptual framework for understanding how intracellular forces are converted into productive movement through regulated engagement with the extracellular matrix (Chan & Odde, 2008; Bangasser et al., 2017).

In this model, actin polymerization and myosin II-based contractility act as the motor, driving retrograde actin flow. Integrin-based adhesions act as transient clutches that intermittently engage the actin network with the substrate. When clutches engage, retrograde flow slows, and traction forces build; when they disengage, the cytoskeleton slips, releasing tension and resetting the cycle. Migration efficiency is therefore determined by the dynamics of clutch engagement, which are modulated by matrix stiffness and ligand availability (Elosegui-Artola et al., 2018; Yamada & Sixt, 2019; Roca-Cusachs et al., 2013).

Beyond actin, other cytoskeletal systems shape this integrated response. Microtubules contribute to polarization and adhesion turnover, while intermediate filaments provide structural integrity and help stabilize directional movement (Etienne-Manneville, 2013). Together, these interacting systems define a regulatory space in which cells can adjust how rapidly they move and how persistently they maintain direction, depending on internal state and external conditions (Dogterom & Koenderink, 2019).

Importantly, this mechanochemical framework predominantly reflects adhesion-dependent, mesenchymal-like migration typical of fibroblast-type cells and many two-dimensional in vitro systems. However, alternative migration modes exist that rely on distinct mechanistic principles. Amoeboid migration, for example, is characterized by reduced dependence on integrin-mediated adhesion and increased reliance on cortical actomyosin contractility, enabling rapid movement in confined environments (Friedl & Wolf, 2003; Panková et al., 2010). In contrast, epithelial-derived systems, such as epidermal keratinocytes, often exhibit collective migration behavior, where coordinated cell-cell interactions and tissue-level polarity play a dominant role (Mayor & Etienne-

Manneville, 2016). These differences highlight that the relative contribution of adhesion, contractility, and polarity can vary substantially across migration modes and experimental models.

2.3. Modular control of migration – speed and directional persistence

A key consequence of mechanochemical integration is that migration can be decomposed into at least two partially independent output dimensions: how fast a cell moves (speed) and how consistently it maintains its direction (directional persistence). Conceptually, these dimensions reflect independent control over propulsion and directional guidance, analogous to modulating acceleration and steering rather than executing a single pre-defined migration program. Accordingly, migration speed and directional persistence are governed by overlapping but non-identical signaling networks and mechanical processes. This conceptual separation is illustrated in Figure 1.

At the mechanistic level, protrusive actin dynamics, actin turnover, actomyosin contractility, and the formation and disassembly of adhesions primarily regulate movement intensity and therefore shape speed. In contrast, persistence depends more strongly on the stability of polarity, spatial restriction of signaling to the leading edge, and suppression of competing protrusions, which together maintain a coherent front-rear axis over time (Schaks et al., 2019; Shellard & Mayor, 2020). As a result, external cues and pharmacological perturbations can selectively affect one dimension while leaving the other relatively preserved.

This conceptual separation has direct consequences for quantitative migration analysis. Path-based descriptors primarily capture changes in movement intensity, whereas displacement- and directionality-based parameters reflect the spatial organization and persistence of trajectories. As a result, cells may exhibit increased instantaneous velocity without achieving greater net displacement when trajectories become more tortuous, while slower-moving cells can traverse larger distances by maintaining high directional persistence. Consequently, no single descriptor can fully represent migratory behavior, and biologically meaningful interpretation requires simultaneous consideration of both dimensions and their potential decoupling.

A limited number of conserved signaling networks provide major entry points into this modular control of migration and can be selectively perturbed by pharmacological interventions. Experimental and pharmacological studies have demonstrated that migration speed and directional

persistence are not obligatorily co-regulated, but can be differentially modulated by targeting distinct signaling nodes. This provides direct evidence that these two dimensions constitute partially independent regulatory outputs rather than inseparable properties of a single migratory program. Concrete examples of pro- and anti-migratory interventions are discussed in Section 2.6.

Among these pathways, PI3K signaling plays a central role in directional sensing and chemotactic polarity. Local PI3K activity promotes asymmetric PIP3 accumulation at the leading edge, reinforcing Rac and Cdc42 signaling and stabilizing persistent protrusions; accordingly, PI3K inhibition preferentially impairs gradient sensing and directional persistence, while baseline motility speed may remain relatively preserved under non-directed conditions as depicted in Figure 1 (Sawyer et al., 2003; Huang et al., 2005; Weiger et al., 2013).

In contrast, the MAPK/RAF/MEK cascade integrates growth-factor signaling with cytoskeletal regulation and migratory plasticity. Depending on cellular context and mode of intervention, MAPK modulation can affect migration speed, directional persistence, or both, frequently resulting in complex and multidimensional motility phenotypes (Molnár et al., 2018; Rittler et al., 2020; Figure 1).

Finally, integrin-mediated adhesion signaling provides the mechanical coupling between intracellular force generation and the extracellular matrix. Changes in integrin expression, activation state, or downstream signaling predominantly influence traction generation and migration efficiency, thereby exerting a stronger effect on movement intensity and speed (Figure 1), particularly in mesenchymal migration modes (Geiger & Yamada, 2011; Laszlo et al., 2015). This mechanical output is tightly coupled to actomyosin contractility (regulated by RhoA/ROCK pathway); accordingly, pharmacological inhibition of ROCK reduces contractile force generation and typically leads to decreased speed (Vicente-Manzanares et al., 2005).

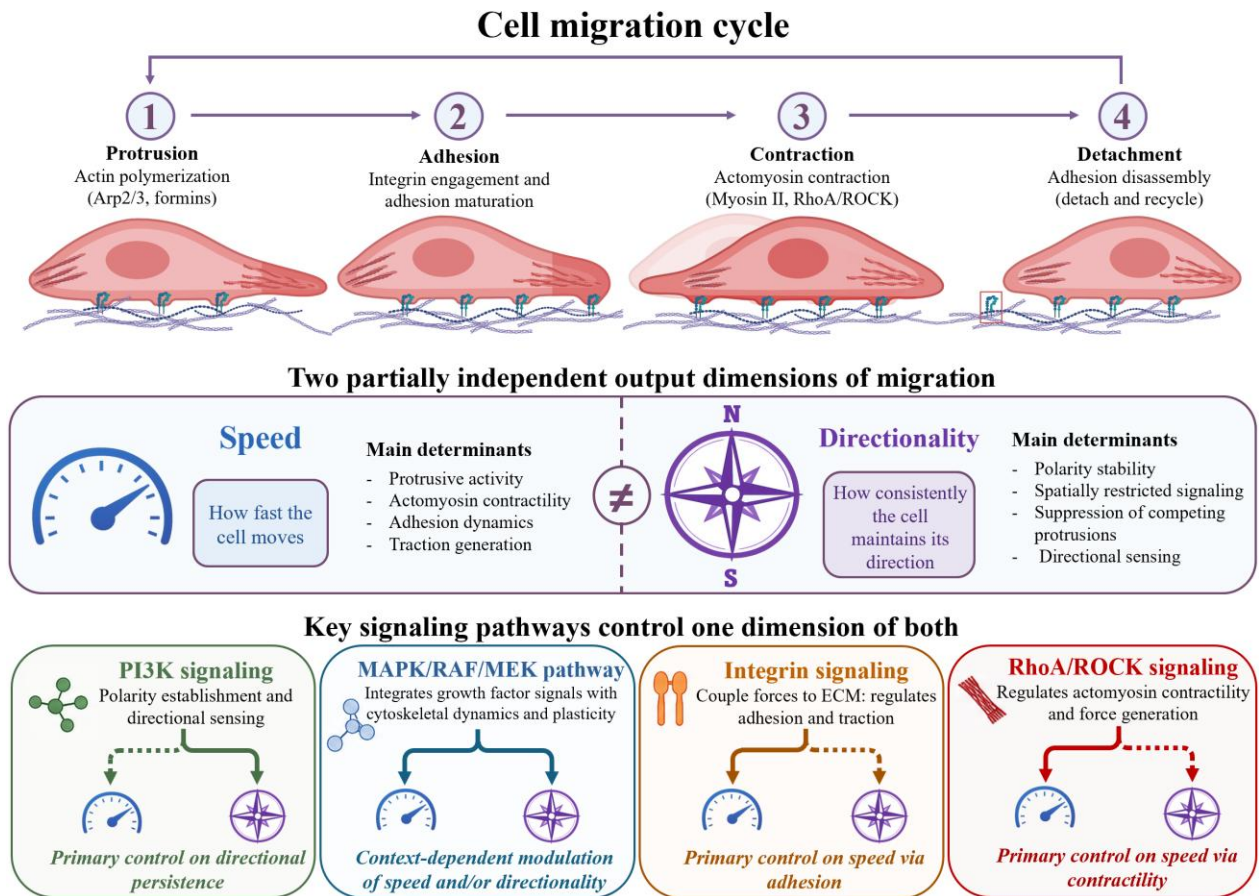


Figure 1. Conceptual framework of cell migration as a two-dimensional output defined by partially independent regulatory modules.

Taken together, these considerations highlight that pharmacological modulation of cell migration frequently gives rise to multidimensional motility phenotypes whose apparent outcome depends on both the targeted signaling pathway and the quantitative descriptor used for evaluation. This principle is particularly relevant in cancer research, where many therapies alter cytoskeletal organization, adhesion dynamics, or polarity control, thereby reshaping migratory behavior in ways that may not be captured by a single metric. Accordingly, migration assays provide sensitive and informative readouts of pathway-specific perturbations, revealing mechanistic effects that extend beyond proliferation or viability endpoints (Molnár et al., 2019; Schelch et al., 2018; Diazzzi et al., 2022).

2.4. The biological significance of cell migration

In multicellular organisms, cell migration underlies a wide range of biological processes, including embryonic morphogenesis, tissue homeostasis and repair, immune surveillance, inflammation, and tumor invasion and metastasis (Friedl & Wolf, 2003; Friedl & Gilmour, 2009; Shellard & Mayor, 2020; Szabó & Mayor, 2018).

During embryogenesis and organogenesis, migrating cells form the structural and functional foundations of developing tissues, contributing to vasculogenesis, neural tube closure, limb formation, and neural crest dispersal (Szabó & Mayor, 2018; Olson & Nechiporuk, 2018). In the developing nervous system, precisely coordinated neuronal migration ensures the correct positioning of post-mitotic neurons and the establishment of functional connectivity patterns. Disturbances in these migratory programs can lead to severe developmental abnormalities (Cooper, 2013).

In adult tissues, migration remains essential for maintaining homeostasis and regeneration. Epithelial cells continually replace damaged or senescent neighbors to preserve barrier integrity in the skin and mucosal surfaces (Dekoninck & Blanpain, 2019). Following injury, effective wound repair depends on the rapid and coordinated migration of keratinocytes and fibroblasts into the damaged area, coupled with extracellular matrix remodeling and angiogenesis. The efficiency of these processes strongly influences tissue regeneration and scar formation (Krndija et al., 2019). Within the immune system, highly regulated migratory programs orchestrate leukocyte trafficking between tissues and lymphoid organs, enabling immune surveillance, timely inflammatory responses, and host defense (Luster et al., 2005; Liu et al., 2007; Nourshargh & Alon, 2014).

When migration becomes dysregulated, it contributes to pathological conditions such as chronic inflammation, autoimmune diseases (Nourshargh & Alon, 2014), and cancer progression.

2.5. Cell migration in cancer progression and metastasis

Cell migration is an essential requirement for metastatic dissemination, as tumor cells must acquire the ability to traverse the surrounding tissue, breach the basement membrane, intravasate into the circulation, and eventually colonize distant organs (Majidpoor & Mortezaee, 2022). Since metastasis accounts for the majority of cancer-related mortality (Bergers & Fendt, 2021), understanding how cancer cells regulate, adapt, and reprogram their migratory behavior is

essential. Tumor cells display pronounced migratory plasticity, allowing them to transition between mesenchymal and amoeboid modes or to migrate as cohesive multicellular groups depending on environmental conditions (Panková et al., 2010; George et al., 2023; Bravo-Cordero et al., 2012). Because many anticancer treatments alter cytoskeletal organization, adhesion dynamics, or signaling pathways controlling motility, assessing migration is a central objective of drug-testing workflows (Molnár et al., 2019; Schelch et al., 2018; Diazzi et al., 2022).

2.5.1. Local invasion, intravasation, extravasation, colonization

Local invasion represents the first step of metastatic dissemination. To infiltrate the surrounding stroma, tumor cells remodel their cytoskeleton, reorganize integrin-mediated adhesions, and adapt their mode of migration to the physical properties of the extracellular matrix.

Individual-cell invasion may proceed via mesenchymal motility, characterized by strong adhesions, elongated morphology, and proteolytic ECM degradation, or via amoeboid migration, which relies on weak adhesion, high cortical contractility, and the ability to squeeze through matrix gaps with minimal proteolysis (Panková et al., 2010; George et al., 2023).

In contrast, collective invasion involves multicellular groups that maintain intercellular junctions while migrating as coordinated units. These collectives often move more efficiently through tissue barriers and benefit from emergent properties such as mechanical cooperation, shared polarity cues, and division of labor among subpopulations (Bravo-Cordero et al., 2012).

Following local invasion, cells must intravasate into blood or lymphatic vessels, a process facilitated by cytoskeletal contractility, disruption of endothelial junctions, and interactions with immune or stromal cells. Once in circulation, cells must survive hemodynamic stress and extravasate at distant sites by adhering to and transmigrating through the endothelium. The final step, colonization, requires cells to adapt to new microenvironments and reinitiate proliferation (Valastyan & Weinberg, 2011).

2.5.2. Migratory plasticity in tumors: mesenchymal, amoeboid, hybrid states

Tumor cell motility is not fixed but dynamically reprogrammable. Cancer cells frequently undergo phenotype switching events such as epithelial-mesenchymal transition (EMT) or

mesenchymal-amoeboid transitions (MAT), producing diverse and highly adaptable migration modes (Brabletz et al., 2018; Pandya et al., 2017; Zhang et al., 2025).

Importantly, many tumors adopt hybrid migration states, combining features of amoeboid and mesenchymal motility. These hybrid modes confer a high degree of flexibility, enabling cells to rapidly switch between strategies when encountering physical constraints, stromal resistance, or drug-induced perturbations. This plasticity explains why migration outcomes are highly treatment- and context-dependent, and why *in vitro* assays may reveal divergent effects even with the same compound or cell line (Graziani et al., 2022; Te Boekhorst et al., 2016).

2.5.3. Collective invasion and leader-follower organization

Collective invasion is increasingly recognized as a major contributor to early metastatic spread. Within migrating collectives, leader cells occupy the invasive front, generating protrusions, sensing environmental cues, degrading ECM, and establishing directional polarity. Follower cells maintain group cohesion, transmit mechanical forces, and contribute to coordinated movement by amplifying or stabilizing guidance cues originating from leader cells (Barriga & Mayor, 2019).

Leader-follower specialization arises from differential cytoskeletal organization, integrin expression, matrix-degrading enzyme activity, and responsiveness to chemotactic or haptotactic signals. The emergent properties of collectives, such as force transmission, cooperative ECM remodeling, and distributed sensing, often make collective invasion more efficient than single-cell migration, particularly in dense or heterogeneous tissues (Barriga & Mayor, 2019).

Because collective and single-cell migration modes rely on partially distinct regulatory pathways, pharmacological perturbations can selectively alter migration efficiency, coordination, or invasion strategy without uniformly affecting cell viability or proliferation. This functional separability makes migration-based readouts particularly informative for dissecting pathway-specific drug effects *in vitro*.

2.6. **Pharmacological modulation of migration**

As outlined in Section 2.3, cell migration is governed by at least two partially independent regulatory dimensions – movement speed and directional persistence – which can be differentially

modulated by external cues and pharmacological perturbations. Consequently, pro- and anti-migratory compounds do not exert uniform effects on motility but rather target distinct signaling modules controlling cytoskeletal dynamics, polarity stabilization, and adhesion-mediated force transmission. This functional modularity provides a mechanistic framework for interpreting treatment-induced changes in migration and underscores why different assays and quantitative descriptors may yield divergent outcomes.

2.6.1. Pro-migratory stimuli used as positive controls

Growth factors are commonly used as positive controls in migration assays, as they activate conserved signaling pathways that promote cytoskeletal reorganization and motility. In particular, fibroblast growth factor 2 (FGF2) and epidermal growth factor (EGF) stimulate migration predominantly through MAPK-dependent signaling, enhancing protrusive activity and adhesion turnover and thereby increasing movement intensity and migration speed.

Depending on cellular context and baseline migration mode, growth factor signaling may also stabilize polarity and increase directional persistence, resulting in a combined pro-migratory effect. In malignant pleural mesothelioma cells, FGF2 and EGF induce EMT-like phenotypic transitions associated with increased migration (Schelch et al., 2018), while in melanoma, long-term BRAF inhibition promotes adaptive EGFR upregulation and enhanced motility (Molnár et al., 2019).

2.6.2. Anti-migratory strategies and targets

In contrast to growth factor stimulation, several clinically relevant compounds exert anti-migratory effects by targeting signaling pathways that regulate cytoskeletal organization, adhesion dynamics, or polarity control. Importantly, such interventions may selectively impair specific dimensions of migration, leading to complex phenotypic outcomes that are not adequately captured by a single quantitative descriptor.

Nintedanib (BIBF1120) is a potent multi-kinase inhibitor targeting VEGFR, FGFR, and PDGFR signaling axes (Wind et al., 2019). While its anti-fibrotic and anti-angiogenic properties are well established, particularly in the context of therapy resistance, its effects on intrinsic tumor cell migration have been less extensively characterized. In BRAF-mutant melanoma models, nintedanib suppresses extracellular matrix remodeling and delays tumor relapse by inhibiting a

pro-fibrotic, mesenchymal-like state driven by the miR-143/145 cluster (Diazzi et al., 2022). Additional studies demonstrated that combining nintedanib with immune checkpoint blockade enhances antitumor immunity through suppression of cancer-associated fibroblasts and increased CD8⁺ T cell infiltration, highlighting its capacity to modulate the tumor microenvironment (Kato et al., 2021). Novel delivery strategies further revealed its ability to reduce vasculogenic mimicry and clonogenic capacity in resistant melanoma cells, while in uveal melanoma nintedanib treatment was shown to reduce metabolic activity and induce oxidative stress (Pawlik et al., 2024).

Beyond melanoma, nintedanib has consistently been reported to inhibit migration and invasion in multiple tumor types, including pancreatic cancer, glioblastoma, lung cancer, and mesothelioma (Awasthi et al., 2015; Laszlo et al., 2018; Andreucci et al., 2023; Gadhave et al., 2025). Mechanistically, inhibition of receptor tyrosine kinase signaling interferes with growth factor–dependent cytoskeletal regulation and integrin-mediated adhesion dynamics, thereby reducing traction generation and migration efficiency. Such effects are expected to primarily impact effective displacement and movement persistence, although context-dependent effects on migration speed may also occur.

Collectively, these observations emphasize that anti-migratory compounds do not simply “slow down” cells but can reshape migratory behavior in a multidimensional manner by selectively targeting the regulatory modules underlying speed, directional persistence, or both. This principle forms the conceptual basis for the experimental analyses presented later in this work, where migration inhibition is evaluated across multiple assay formats and quantitative descriptors.

2.7. *In vitro* methods to analyze migration: assay landscape

In vitro migration assays provide controlled and experimentally tractable platforms for quantifying cell movement and assessing how biological or pharmacological perturbations alter migratory behavior (Kramer et al., 2013; Pijuan et al., 2019). Because different assays capture distinct mechanistic aspects of motility, ranging from pure single-cell migration to collective sheet movement or chemotactic invasion, the choice of assay strongly shapes the observed outcomes (Justus et al., 2014). Moreover, migration analysis involves multiple methodological steps – from data acquisition to parameter calculation and interpretation – that further influence the final readout (Figure 2). Understanding the strengths, limitations, and biological focus of each system is

therefore essential when comparing migration datasets or interpreting treatment effects (Masuzzo et al., 2016).

2.7.1. 2D single-cell time-lapse and track-based analysis

Single-cell migration assays constitute one of the most direct experimental approaches for quantifying intrinsic motility. In contrast to collective migration formats, single-cell assays isolate the autonomous behavior of individual cells, enabling the measurement of movement that is unconfounded by proliferative expansion, cell-cell interactions, or matrix remodeling by neighboring cells (Svensson et al., 2018). When cells are plated at low density, each cell migrates freely in a multi-directional manner, allowing researchers to capture pure migratory behavior in a simplified, yet mechanistically informative, environment. Although this reductionist setting lacks the architectural and biochemical complexity of tissues, it provides a uniquely clean framework in which cytoskeletal dynamics, polarity cues, and adhesion turnover can be examined without higher-order collective effects.

The practical implementation of single-cell migration analysis relies on time-lapse microscopy. Cells are imaged at regular intervals, producing a sequence of frames from which trajectories must be extracted. This multi-step workflow comprises image preprocessing, segmentation, tracking, and the computation of quantitative descriptors (Masuzzo et al., 2016; Piccinini et al., 2016; Chenouard et al., 2014). As illustrated in Figure 2, each of these steps introduces methodological variability, and the final migration readout is shaped as much by computational decisions as by underlying biology.

2.7.1.1. Pre-processing and segmentation

Accurate segmentation is the foundation of reliable cell tracking. Preprocessing typically involves illumination correction, background subtraction, denoising, and contrast enhancement to stabilize the appearance of cells across frames (Terry et al., 2009). Nevertheless, segmentation remains challenging, particularly for human cells, which display extreme morphological heterogeneity. Even within a single cell line, cells may vary substantially in size, circularity, solidity, or aspect ratio depending on their cell-cycle stage, spreading state, or polarity program.

Tumor cells amplify these complexities, exhibiting dynamic shape fluctuations and protrusive activity that frequently alter their apparent morphology in successive frames (Meijering, 2012).

These traits make automated segmentation difficult to standardize. Low contrast, fluctuating edge intensities, overlapping protrusions, or transient cell-cell contacts often lead to incomplete masks, merged objects, or artificial fragmentation (George et al., 2023). Moreover, the magnitude of true biological movement between frames can be small, and when displacement is similar to the segmentation error margin, centroid estimates can shift even when the cell is essentially stationary. Such jitter introduces downstream distortions in all trajectory-dependent metrics (Chenouard et al., 2014; Masuzzo et al., 2016). For this reason, segmentation quality is a major determinant of analytical precision in single-cell migration assays, directly influencing the reliability of downstream feature extraction (Figure 2).

2.7.1.2. Tracking strategies

Tracking links cell positions through time to generate trajectories (Meijering et al., 2012). Three general strategies are used.

Manual tracking relies on human annotation and is highly accurate when carefully performed. Operators can resolve ambiguities in identity, making it the gold standard for precision. Its major limitations are throughput and operator bias, as users tend to preferentially select well-behaved, clearly segmented cells (Masuzzo et al., 2016).

Semi-automatic tracking combines algorithmic linking with manual correction. This approach balances scalability and accuracy, yet remains susceptible to segmentation errors and morphological variability (Piccinini et al., 2016). Centroid jitter, shape-dependent fluctuations, or brief occlusions can still lead to path distortion.

Fully automated tracking uses algorithmic linking without human intervention. Machine learning and probabilistic frameworks have improved performance, yet no universally reliable free or open-source solution exists for irregularly shaped human cells (Chenouard et al., 2014; Maška et al., 2014; Stringer et al., 2021). Automated methods are particularly vulnerable to identity swaps, trajectory fragmentation, and jitter in cases of small displacements, low contrast, or rapid shape changes. Commercial high-end systems mitigate some of these issues but remain costly and not widely accessible.

Because tracking precision directly affects path-based and directional descriptors, the choice of tracking method fundamentally shapes the quantitative output (Figure 2).

2.7.1.3. Quantitative descriptors of single-cell migration

Once trajectories are obtained, numerical metrics are computed to characterize motility. Numerous descriptors exist (Masuzzo et al., 2016; Hu et al., 2023), but they can be organized into three mechanistically distinct groups. This taxonomy, although conceptually recognized in past publications, is rarely made explicit and has not been formalized in the literature in the structured way adopted in this dissertation. As summarized in Figure 2, different parameter classes capture distinct aspects of migration and may therefore lead to different interpretations of the same underlying behavior.

Displacement-based parameters (e.g., net displacement, maximal displacement, mean squared displacement) quantify the spatial extent of movement relative to the point of origin.

Path-based parameters (e.g., total traveled distance, instantaneous and mean velocity) describe dynamic movement along the trajectory, integrating moment-to-moment changes in position.

Directionality-based parameters (e.g., turning angles, directionality ratio, persistence metrics) capture how consistently a cell maintains its orientation over time. Directionality reflects polarity regulation and cytoskeletal organization, and they can provide mechanistic insight into whether treatments affect protrusion orientation or the stability of polarity cues (Gorelik & Gautreau, 2014).

Because many descriptors are mathematically related, descriptor selection should be hypothesis-driven rather than exhaustive. For instance, a cell with high velocity but low displacement must be following a tortuous path, whereas high displacement with low instantaneous speed suggests strong directionality.

2.7.1.4. Morphological descriptors and their relevance

An additional strength of single-cell assays is that migration data can be enriched with morphological information. From either segmentation masks or manual contouring, cell area,

circularity, aspect ratio, solidity, convexity, and other shape descriptors can be extracted (Masuzzo et al., 2016; George et al., 2023). These parameters are biologically meaningful because morphology is tightly linked to cytoskeletal organization and migration mode. Elongated or polarized cells tend to exhibit more persistent, mesenchymal-like movement, whereas rounded, low-solidity cells often adopt amoeboid-like, less productive motility. Morphological features also influence segmentation robustness: elongated cells with sharp protrusions provide clearer edges, whereas round, dim, low-contrast cells frequently cause tracking uncertainty. Thus, shape descriptors contribute not only to biological interpretation but also to the reliability of the computational pipeline.

2.7.2. *Wound healing assays*

Wound healing assays evaluate the coordinated migration of cell populations into an initially cell-free area. Although widely used, these assays do not measure migration in isolation, but rather a composite of collective motility, cell-cell rearrangements, proliferation, cell size, and tissue-scale mechanics (Hohmann et al., 2022; Kramer et al., 2013; Pijuan et al., 2019). They are therefore highly informative for studying sheet-like epithelial movement and wound closure, but their readouts must be interpreted in the context of these overlapping processes.

The conventional **scratch assay** is generated by mechanically disrupting a confluent monolayer, typically using a pipette tip or blade (Liang et al., 2007; Jonkman et al., 2014). The scratch creates an irregular cell-free region whose width and shape can vary substantially across fields and experiments. Because the scratch physically injures cells, cellular debris and damage-associated molecular patterns (DAMPs) released from lysed cells may stimulate or inhibit migration and can activate wound-specific signaling programs that are independent of intrinsic basal motility (Jonkman et al., 2014; Pijuan et al., 2019). Irregular wound geometry and uncontrolled mechanical stress together result in considerable inter-experimental variability and can obscure subtle treatment-induced differences. These factors represent assay-specific sources of variability that directly influence the final migration readout (Figure 2). To improve reproducibility, standardized multi-pin scratch tools and automated wound-generation platforms have been introduced to produce more uniform and high-throughput scratches compared with manual pipette-based methods (Chen et al., 2023). Nevertheless, independent of the level of automation, mechanical scratching inevitably causes cell lysis and surface damage, leaving behind

cellular debris and exposed or redistributed adhesion molecules that can locally facilitate migration and bias the interpretation of intrinsic motility (Pijuan et al., 2019; Jonkman et al., 2014).

Insert-based **zone-exclusion** assays were developed to mitigate several of these limitations. In this format, removable silicone or agarose inserts are placed on the culture surface before seeding, preventing cell attachment in defined circular or rectangular regions. Once the monolayer has formed, the insert is removed, leaving a highly reproducible, debris-free gap with well-defined geometry and without mechanical damage to the cells (Shiode et al., 2017; Veres-Székely et al., 2022). Cells at the margin then migrate inward in a coordinated fashion, driven by collective cues rather than injury-induced signals. The uniform initial geometry and absence of mechanically induced cellular disruption substantially improve reproducibility and reduce assay noise, making zone-exclusion assays more suitable than scratch assays for quantitative comparison of collective migration across treatments or cell lines (Figure 2).

Despite these differences in implementation, both scratch and zone-exclusion assays are typically quantified using similar image-analysis strategies. Time-lapse or endpoint images are acquired at defined intervals, and the cell-free area or wound region is segmented either manually, semi-automatically, or fully automatically. Manual delineation (for example, in ImageJ or similar tools) remains common and can be highly accurate but is labor-intensive and operator-dependent (Liang et al., 2007). Semi-automated and automated pipelines use intensity thresholding, edge detection, or machine-learning-based segmentation to distinguish the cell-free region from the cell-covered area, and then track its evolution over time (Cortesi et al., 2017; Masuzzo et al., 2016; Garcia-Fossa et al., 2020; Figure 2). As in single-cell assays, segmentation quality strongly depends on image contrast, uniform illumination, and the visibility of cell boundaries, and artefacts or debris at the wound edge can lead to over- or underestimation of the gap area.

From segmented images, a variety of quantitative metrics can be derived. The simplest are wound width, wound area, and percentage wound closure, computed by comparing the cell-free region at each time point to its initial size (Liang et al., 2007; Pijuan et al., 2019). Closure kinetics are often summarized by the slope of wound area versus time (an angular coefficient or closure rate), which condenses the dynamics into a single number describing average closure speed. High-content, instrument-specific platforms such as the IncuCyte system additionally provide metrics such as wound confluence and relative wound density, the latter defined as the cell density within

the wound area relative to the surrounding monolayer, which partly normalizes for changes in global cell density due to proliferation or treatment effects (Topman et al., 2012; Johnston et al., 2015). Other analytical frameworks normalize closure rates to geometric features, such as initial gap width or the number of advancing cell fronts, to derive standardized “cell front velocity” measures that improve comparability across experiments with different technical settings.

In contrast, explicit normalization of wound closure to cell size is rarely applied in the wound-healing literature. Most studies implicitly assume comparable cell dimensions between conditions or focus on normalizing for gap width and cell density rather than for individual cell area. As a consequence, differences in mean cell size can directly influence apparent closure rates, since larger cells occupy a greater portion of the cell-free area per unit of displacement. This is particularly relevant when treatments or cell lines exhibit pronounced morphological differences. In such contexts, incorporating cell-size information or normalizing closure dynamics to cell area would provide a more accurate representation of true migratory capacity, but this practice has not yet become standard in routine wound-healing assay analysis.

2.7.3. Transwell/chemotaxis under confinement (semi-3D)

Transwell assays quantify directed cell migration across a porous membrane toward a chemotactic or chemokinetic stimulus, such as serum, growth factors, or extracellular matrix components (Justus et al., 2014; Zaki et al., 2019; Ou et al., 2019; Figure 2). During migration, cells actively traverse vertical pores, requiring deformation of their cytoskeleton and nucleus and the generation of contractile forces to squeeze through constrictions that may be smaller than the nuclear diameter (Denais et al., 2016; Conboy et al., 2024). This confined movement has led to the assay sometimes being described as three-dimensional (Goh et al., 2020). However, it does not represent true 3D migration, as cells do not move within a surrounding extracellular matrix unless matrix components (e.g. Matrigel) are introduced (Tomasova et al., 2019).

As a consequence, transwell migration is strongly influenced not only by intrinsic motility but also by cell size, shape, stiffness, and deformability. These biophysical properties critically determine whether a cell can successfully pass through a pore and therefore have a disproportionate impact on the measured outcome. Pharmacological treatments that alter cytoskeletal organization or cell morphology may thus modify transwell readouts independently of their effects on true

migratory capacity. For example, treatments that reduce cell size or increase cellular plasticity can facilitate pore transit, whereas agents that induce cell spreading or nuclear stiffening may suppress transmigration even when basal motility remains unchanged (Justus et al., 2014).

Unlike single-cell or gap-closure assays, transwell experiments typically provide endpoint rather than kinetic information, as illustrated in Figure 2. After a defined incubation period, cells that have migrated to the lower surface of the membrane are fixed, stained, and quantified. Several quantification strategies are commonly used. The most widespread approach involves manual or semi-automated counting of migrated cells in representative microscopic fields (Justus et al., 2014). Alternatively, colorimetric or fluorometric assays measure total signal intensity from stained cells on the lower membrane surface, providing a bulk estimate of migration (Zaki et al., 2019). More recently, automated image-analysis pipelines have been employed to segment and count transmigrated cells across the entire membrane area, improving throughput and reducing observer bias (Ou et al., 2019).

Regardless of the quantification method, transwell results are most often expressed as absolute cell counts, normalized counts relative to control conditions, or migration indices calculated as ratios of migrated cells to the total number of seeded cells (Figure 2). In invasion-style adaptations using ECM-coated membranes, invasion indices are similarly derived by comparing treated and control conditions (Justus et al., 2014). Notably, comparison of cell size, nuclear size, or deformability is rarely performed, despite the well-established influence of these parameters on pore traversal efficiency. Consequently, differences in transwell readouts may reflect alterations in cell mechanics rather than changes in intrinsic motility or chemotactic responsiveness.

Taken together, transwell assays are highly sensitive tools for detecting migration under confinement and chemotactic guidance, but they capture a composite phenotype that integrates motility, morphology, and mechanical adaptability. Interpretation of transwell data, therefore, benefits from complementary measurements of cell size and shape, ideally obtained from single-cell migration assays, to disentangle true migratory effects from morphology-driven changes in pore passage efficiency. This underscores that migration outcomes are inherently assay-dependent and must be interpreted within the methodological framework outlined in Figure 2.

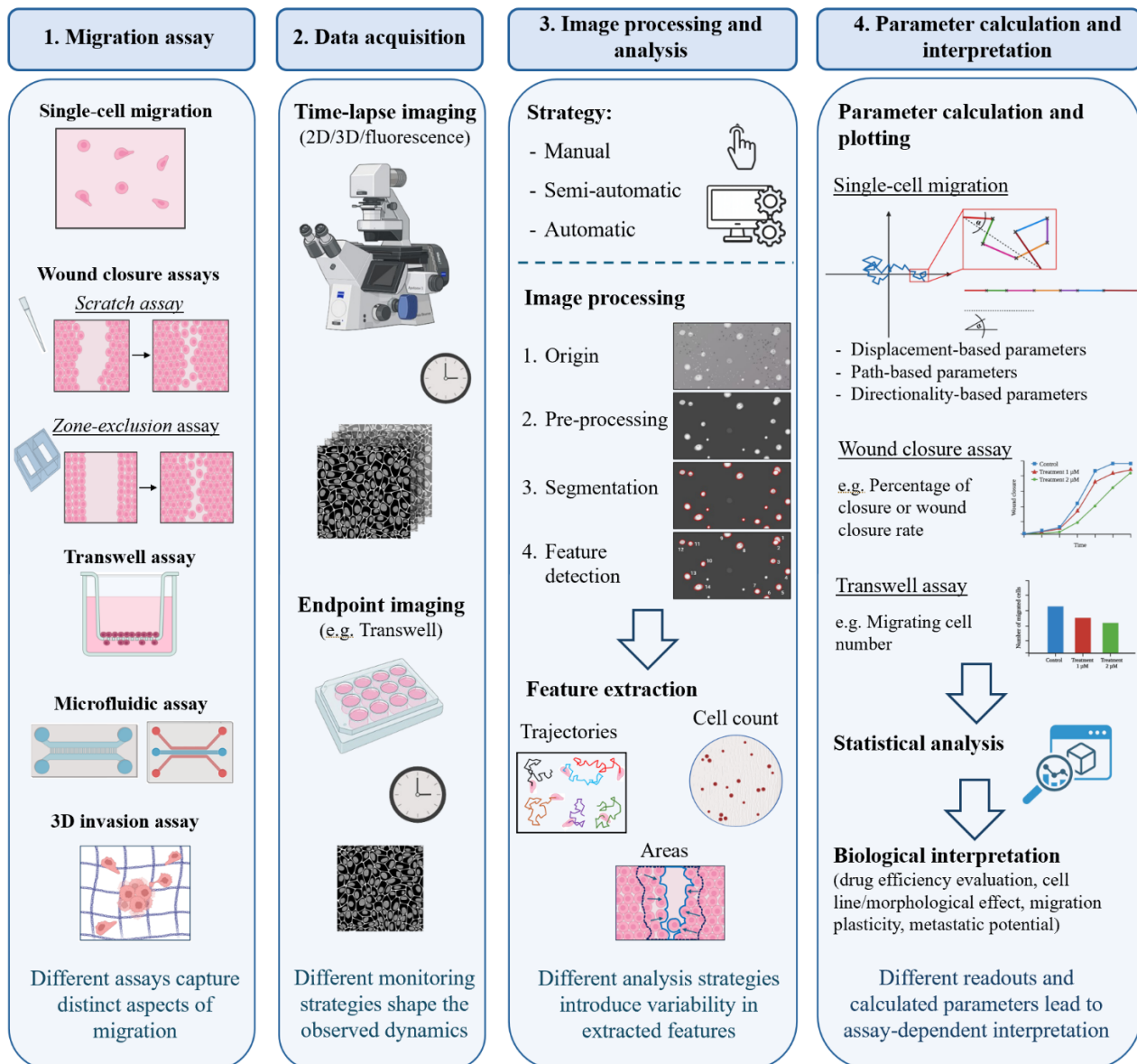


Figure 2. Analytical workflow of migration assays: from imaging to biological interpretation

2.7.4. *Advanced systems (microfluidics, 3D matrices)*

Several advanced experimental platforms enable the investigation of cell migration in settings that capture additional layers of biological complexity and may more closely approximate physiological environments (Figure 2). Microfluidic systems allow the generation of stable and quantifiable chemical gradients, supporting controlled studies of chemotaxis and gradient sensing (Shih et al., 2019; Wong et al., 2021). Three-dimensional collagen or hydrogel matrices recreate key structural and mechanical features of extracellular matrices, enabling the analysis of migration modes that are not accessible in two-dimensional systems, including matrix remodeling, confined

movement, and nuclear deformation (Doyle et al., 2009; Figure 2). In addition, engineered substrates with defined topographical or mechanical properties permit systematic interrogation of durotaxis, topotaxis, and stiffness-dependent motility.

While these approaches provide a richer environmental context for studying migration, they also involve increased experimental and analytical complexity, as well as challenges in standardization and quantitative comparison across conditions. For this reason, the present dissertation focuses on three widely used and experimentally tractable platforms (single-cell tracking, gap-closure assays, and transwell migration), which together represent the principal methodological classes employed in cancer cell motility research and allow systematic cross-assay comparison under controlled conditions.

2.8. Data-driven approaches and machine learning in biological research

Machine learning (ML) and deep learning approaches have become increasingly prominent across biomedical research, driven by the growing availability of large, high-dimensional datasets and advances in computational power. Unlike classical rule-based or threshold-driven analyses, ML methods learn patterns directly from data, enabling the identification of complex, non-linear relationships that are difficult to capture using predefined metrics. As a result, ML is now widely applied in areas such as image analysis, phenotype classification, drug response prediction, and systems-level modeling of cellular behavior (Moen et al., 2019; Caicedo et al., 2019; Greener et al., 2022; Kumar & Srivastava, 2024).

At a conceptual level, ML algorithms can be broadly divided into supervised and unsupervised approaches. Supervised learning relies on labeled data and is commonly used for classification or regression tasks, where the objective is to predict discrete classes (e.g., treated vs. control) or continuous outcomes based on input features. Classifiers such as support vector machines, random forests, and neural networks are particularly well-suited for evaluating whether multidimensional feature sets can reliably distinguish between experimental conditions (Morales & Escalante, 2022). In contrast, unsupervised methods aim to uncover intrinsic structure in unlabeled data, such as clustering or dimensionality reduction, revealing latent phenotypic states or trajectories.

The application of machine learning in biological research has expanded rapidly in recent years, particularly in the analysis of microscopy-based imaging data. In cell imaging studies, ML and

deep learning approaches have shown clear advantages over classical algorithms in tasks such as segmentation and tracking, especially when cells exhibit complex, heterogeneous, or dynamically changing morphologies and when image quality is compromised by noise or uneven illumination (Moen et al., 2019; Jan et al., 2024; Krikid et al., 2024; Annasamudram et al., 2025).

Beyond image processing, machine learning is increasingly used as a data-driven analytical framework to integrate multiple quantitative features into higher-order phenotypic representations. Supervised classification approaches, in particular, allow the combination of motility-related and morphology-related descriptors to distinguish experimental conditions, cellular states, or treatment responses that may not be separable using individual parameters alone (Kesapragada et al., 2024; Way et al., 2021). In this sense, ML does not replace mechanistic analysis, but rather complements it by revealing multivariate patterns that emerge from complex datasets.

In migration research, ML-based approaches have begun to appear as tools for classifying migratory phenotypes and evaluating assay performance. Previous studies demonstrated that combinations of trajectory-derived features, including speed, persistence, and turning behavior, can discriminate migration modes more effectively than single descriptors (Masuzzo et al., 2016; Gorelik & Gautreau, 2014), and more recent work has extended these concepts using machine-learning-based frameworks to analyze and model migratory behavior (Olalla et al., 2024). Some studies further showed that ML classifiers can separate treatment conditions or genetic perturbations based on migration features, even when conventional statistical analyses yield ambiguous or weak effects (Kimmel et al., 2021; Garcia-Moreno et al., 2024).

In the context of this dissertation, machine learning was applied not as a replacement for quantitative migration analysis but as an integrative layer to assess how different migration assays and parameter sets capture treatment-induced phenotypic changes. Because distinct migration assays probe fundamentally different biological processes and generate non-equivalent quantitative readouts, direct comparison of their outputs is inherently challenging. By quantifying classification performance across assays and feature combinations, ML provides an objective measure of assay sensitivity and discriminatory power. In this way, ML helps to bridge heterogeneous migration readouts, linking quantitative descriptors, cell morphology, and experimental design, while preserving the need for biologically grounded interpretation.

3. OBJECTIVES

Cell migration can be analyzed through a wide range of experimental and computational approaches, each capturing distinct aspects of motility and reflecting specific biological mechanisms. This methodological diversity is both an advantage and a challenge: while it enables the investigation of migration from multiple perspectives, it also introduces substantial variability in data acquisition and interpretation. Beyond assay design, the analytical framework, particularly the selection and computation of migration parameters, has a profound influence on the observed outcomes. This is especially evident in single-cell tracking experiments, where numerous quantitative descriptors can be calculated, each sensitive to different aspects of cell behavior such as velocity, persistence, or morphological plasticity.

The overarching aim of this dissertation was to obtain a comprehensive understanding of how methodological and analytical choices influence the quantitative and biological interpretation of cell migration. Thus, the following research were performed:

- 1) Evaluate the sensitivity, repeatability, and robustness of common migration descriptors using large-scale videomicroscopic datasets analyzed through multiple tracking strategies (manual, semi-automatic, and noise-filtered semi-automatic).
- 2) Dissect the determinants of parameter variability through a two-dimensional random-walk simulation framework, systematically testing how controlled alterations in speed and directionality affect quantitative metrics.
- 3) Investigate the relationship between intrinsic cell morphology and motility, by correlating cell line-specific morphological and behavioral descriptors to reveal how cell-specific features shape both migratory behavior and tracking performance.
- 4) Compare the behavior and interpretational scope of different migration assays, including single-cell, scratch/zone-exclusion, and transwell assays, under pharmacological modulation with nintedanib.
- 5) Integrate findings across analytical levels to formulate methodological recommendations for the standardized and biologically meaningful evaluation of cell migration.

Rather than seeking a single optimal assay or descriptor, this work aims to establish an integrative framework illustrating how diverse experimental and computational perspectives collectively contribute to a more nuanced, context-aware understanding of cell motility in cancer research.

4. MATERIALS AND METHODS

4.1. Reference dataset – previously acquired videomicroscopic images

To establish a robust analytical framework for quantifying single-cell migration, we performed a systematic evaluation of how commonly used migration parameters behave under different biological and methodological conditions. For this purpose, we analyzed previously acquired large-scale videomicroscopic datasets containing thousands of individually tracked trajectories across multiple cancer cell lines. These recordings provided a comprehensive **reference dataset** that enabled us to assess the sensitivity, robustness, and inter-parameter relationships of single-cell migration descriptors, as well as their dependence on cell line-specific morphological features. The computational analyses described for this **reference dataset** are implemented in the `single-cell_experiment.m` MATLAB script, available in the accompanying GitHub repository (https://github.com/banyaigreti/Decoding_cell_migration-Banyai_G_L), except for the determination of cell line-specific features, which was performed in ImageJ.

All datasets were obtained from time-lapse recordings performed between 2009 and 2018 at Semmelweis University (Budapest, Hungary). Images were captured using an inverted phase-contrast microscope equipped with a motorized stage and temperature-controlled incubation system. These measurements were conducted for specific research purposes and preliminary experiments and included partially unpublished data. Each experiment monitored solitary cells maintained in low-density cultures under standardized conditions to minimize cell-cell interactions and ensure migration behavior independent of collective effects.

The datasets covered multiple tumor-derived cell lines subjected to distinct pharmacological treatments, allowing for a comprehensive evaluation of migration parameters across heterogeneous experimental contexts. The cell lines and treatments included in these datasets are summarized in Tables 1 and 2, together with references to the corresponding publications where detailed methodological and treatment-related information is provided.

Category	Experimental perturbation (with references)
Treating agent	BEZ235 (Rittler et al., 2020), dabrafenib (Hegedűs et al., 2020; Molnár et al., 2018), EGF (T. Garay, Molnár, et al., 2015), FGF (T. Garay, Molnár, et al., 2015), selumetinib (Hegedűs et al., 2020; Molnár et al., 2018; Rittler et al., 2020; Schelch et al., 2018), sorafenib (Molnár et al., 2018), vemurafenib (Hegedűs et al., 2020), zoledronic acid (T. Garay, Kenessey, et al., 2015), nintedanib (Laszlo et al., 2018; Schelch et al., 2018), Zn , ELA-32 , ML-221 , DTIC (T. Garay, Kenessey, et al., 2015), Temsirolimus (Lötsch et al., 2013), MK-2206 (Lötsch et al., 2013), SB-431542 (Hoda et al., 2012), MASP1 , RQ , SA , apelin (Berta et al., 2014), activin (Hoda et al., 2012), BI 853520 (Laszlo et al., 2019)
Coating agent	laminin (Laszlo et al., 2015)
Transfection	ITGA7 (Laszlo et al., 2015), siRNA (Hoda et al., 2012), cldn4 , PMCA4b (Hegedus et al., 2017), siMVP (Lötsch et al., 2013)

Table 1. Investigated treatments with references

Tumor	Cell lines (with references)
Breast	MDAMB231 (T. Garay et al., 2016; Molnár et al., 2018), H7/MVP (Lötsch et al., 2013), H7/vc (Lötsch et al., 2013)
Colorectal	HT25 (Tátrai et al., 2017), HT29 (T. Garay et al., 2016; Rittler et al., 2020; Tátrai et al., 2017)
Lung	A549 , Calu-6 , CRL5885 (T. Garay et al., 2016; Molnár et al., 2018; Rittler et al., 2020), CRL5922 (T. Garay et al., 2016; Molnár et al., 2018), EKV1 (T. Garay et al., 2013), H146 (T. Garay et al., 2013), H1650 (T. Garay et al., 2013), H1975 (T. Garay et al., 2013), HCC-15 (T. Garay et al., 2013), HTB-182 (T. Garay et al., 2013), LC42 (T. Garay et al., 2013), LCLC103H (T. Garay et al., 2013), SELS (T. Garay et al., 2013), SW900 (T. Garay et al., 2013), VMC47 , VMC50
Lymphatic endothelial	(TERT)-transduced LEC (Berta et al., 2014)

Malignant pleural effusion	PF49 (Hegedűs et al., 2020)
Melanoma	A2058 (T. Garay et al., 2013; T. Garay, Kenessey, et al., 2015; T. Garay, Molnár, et al., 2015; T. Garay et al., 2016; Hegedus et al., 2017; Rittler et al., 2020; Tátrai et al., 2017), A375 (T. Garay et al., 2013; T. Garay, Kenessey, et al., 2015; T. Garay, Molnár, et al., 2015; T. Garay et al., 2016; Hegedus et al., 2017; Hegedűs et al., 2020; Molnár et al., 2018; Rittler et al., 2020), HT168 (T. Garay et al., 2013), HT199 (T. Garay et al., 2013; T. Garay, Kenessey, et al., 2015; T. Garay, Molnár, et al., 2015), LOX (T. Garay, Kenessey, et al., 2015), M1 (T. Garay et al., 2013; T. Garay, Kenessey, et al., 2015; Tátrai et al., 2017), M24met (T. Garay et al., 2013; T. Garay, Kenessey, et al., 2015; T. Garay, Molnár, et al., 2015), Mel Pt-4 pre (Molnár et al., 2019), Mel Pt-4 post (Molnár et al., 2019), Mel Pt-3 pre (Molnár et al., 2019), Mel Pt-3 post (Molnár et al., 2019), Mel Pt-1 pre (Molnár et al., 2019), Mel Pt-1 post (Molnár et al., 2019), MEWO (T. Garay et al., 2013; T. Garay, Kenessey, et al., 2015; T. Garay, Molnár, et al., 2015; Hegedus et al., 2017), SKMEL28 (T. Garay et al., 2016; Rittler et al., 2020), VM-15 (T. Garay, Kenessey, et al., 2015; T. Garay, Molnár, et al., 2015; Hegedus et al., 2017), WM239 (T. Garay, Kenessey, et al., 2015; Rittler et al., 2020), WM35 (T. Garay et al., 2013; T. Garay, Kenessey, et al., 2015; T. Garay et al., 2016; Rittler et al., 2020), WM3629 (T. Garay et al., 2016; Molnár et al., 2018), WM3670 (T. Garay, Kenessey, et al., 2015; T. Garay et al., 2016; Molnár et al., 2018), WM983A (T. Garay et al., 2013), WM983B (T. Garay et al., 2013)

Mesothelioma	I2 (T. Garay et al., 2013; Hoda et al., 2012; Laszlo et al., 2015, 2018; Schelch et al., 2018), M38K (T. Garay et al., 2013; Hoda et al., 2012; Laszlo et al., 2015, 2018; Molnár et al., 2018), Meso49 (Laszlo et al., 2015), Meso71 (Schelch et al., 2018), Meso84 (Schelch et al., 2018), Meso92, Meso100, Meso103 (Schelch et al., 2018), Meso110 (Schelch et al., 2018), Met5a (Hoda et al., 2012; Laszlo et al., 2015, 2018), NP2 (Laszlo et al., 2018), p31 (T. Garay et al., 2013; Hoda et al., 2012; Laszlo et al., 2015; Schelch et al., 2018), p31res (T. Garay et al., 2013; Hoda et al., 2012; Schelch et al., 2018), SPC111 (T. Garay et al., 2013; Hoda et al., 2012; Laszlo et al., 2015, 2018; Schelch et al., 2018), SPC212 (T. Garay et al., 2013; Hoda et al., 2012; Laszlo et al., 2015, 2018; Schelch et al., 2018), VMC12 (Laszlo et al., 2015; Schelch et al., 2018), VMC14 (Schelch et al., 2018), VMC20 (Hoda et al., 2012; Laszlo et al., 2015; Schelch et al., 2018), VMC23 (T. Garay et al., 2013; Laszlo et al., 2015), VMC28 (Schelch et al., 2018), VMC31 (Schelch et al., 2018), VMC33 (T. Garay et al., 2013; Laszlo et al., 2015), VMC40 (Laszlo et al., 2015; Schelch et al., 2018), VMC45 (Schelch et al., 2018), VMC48, VMC6 (Hoda et al., 2012; Laszlo et al., 2015; Schelch et al., 2018), CRL5820 (T. Garay et al., 2013; Hoda et al., 2012; Laszlo et al., 2015; Schelch et al., 2018), CRL5915 (T. Garay et al., 2013; Laszlo et al., 2015)
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Table 2. Analyzed cell lines with references

4.2. Primary experimental dataset – experiment design, cell culture, and treatment procedures

To broaden the analytical perspective gained from 2D single-cell migration studies and to evaluate how different assay formats capture distinct facets of cell motility, we complemented single-cell analyses with additional *in vitro* migration assays. Specifically, we incorporated collective migration approaches (scratch and zone-exclusion gap-closure assays) as well as a semi-3D transwell assay, enabling a systematic comparison across fundamentally different migratory contexts. For this purpose, we designed and executed a dedicated experimental series exclusively for this comparative framework; throughout the thesis, this dataset is referred to as the **primary experimental dataset**. These experiments were performed using five human melanoma cell lines

and the multikinase inhibitor nintedanib, allowing us to assess assay-specific reproducibility, sensitivity, and the consistency of treatment-induced migration inhibition across formats.

4.2.1. Cell lines and culture conditions

The A2058 melanoma cell line is available through ATCC, while the WM983B cell line, derived from a metastatic site, is accessible from the Wistar Institute, Philadelphia, PA. Mel Pt-3 pre and Mel Pt-3 post cell lines were derived from the same patient, before and during vemurafenib treatment, respectively. The Mel Pt-4 post cell line was similarly isolated from a patient undergoing vemurafenib therapy at the time of cell collection. The three Mel Pt cell lines were established and kindly provided by Professor Peter Hersey from the Oncology and Immunology Unit, Calvary Mater Newcastle Hospital, and the Kolling Institute, Royal North Shore Hospital, University of Sydney, NSW, Australia (Lai et al., 2012).

All cell lines were maintained in DMEM (4.5 g/L glucose, with L-glutamine and sodium pyruvate; Capricorn Scientific), supplemented with 10% fetal bovine serum (FBS; EuroClone) and 1% penicillin–streptomycin–amphotericin (Lonza). Cultures were incubated at 37 °C in a humidified atmosphere containing 5% CO₂, unless otherwise indicated.

4.2.2. Drug treatment and viability assessment

Nintedanib (Sigma-Aldrich, St. Louis, MO, USA), a multi-kinase inhibitor with anti-migratory potential, was used as the reference compound. To determine the non-cytotoxic concentration range suitable for migration assays, a Sulforhodamine B (SRB) assay was performed. Briefly, 3,000 cells per well were seeded in 96-well plates and treated with serial dilutions of nintedanib (1–8 µM) in complete medium for 72 hours.

After incubation, cells were fixed with 10% trichloroacetic acid (TCA) and stained with SRB dye for 15 minutes. Unbound dye was removed with 1% acetic acid, and protein-bound SRB was solubilized in 10 mM Tris-HCl buffer (pH 8.0). Absorbance was measured at 570 nm, and IC₅₀ values were calculated by nonlinear regression using GraphPad Prism. Across all melanoma cell lines, IC₅₀ values were higher than 1 µM and 2 µM nintedanib. Accordingly, these concentrations were chosen as sub-cytotoxic doses, ensuring that any observed effects predominantly reflect migration-specific responses rather than treatment-induced loss of viability.

4.3. Single-cell tracking

As described in Sections 4.1 and 4.2, two distinct sets of time-lapse recordings were used for single-cell migration analysis. In both cases, quantitative characterization of cell movement required the extraction of individual cell trajectories from video data by assigning time-resolved x-y coordinates to tracked cells across successive frames.

The **reference dataset** (Section 4.1) consisted of previously acquired video microscopic recordings analyzed to evaluate migration parameters. In total, 1,287 recordings were analyzed, representing more than 6,000 manually and over 8,000 semi-automatically tracked individual cells across 293 unique cell-line $\times$ treatment combinations. Cells were seeded at low density to ensure sparse distribution, allow unrestricted, multidirectional migration, and minimize cell-cell contact. Exact seeding densities differed between experiments, reflecting cell line-specific characteristics such as size, proliferation rate, and intrinsic migratory capacity. Imaging was performed under standard cell culture conditions using phase-contrast microscopy, and most recordings spanned approximately 72 hours with a 5- or 10-minute video rate. **Manual** and **semi-automatic tracking** were performed for a minimum of 24 hours after treatment initiation. In addition, semi-automatic tracking results were subjected to **noise filtering** to evaluate how algorithm-related artefacts affect quantitative migration parameters and to refine the reliability of parameter estimation.

The **primary experimental dataset** (Section 4.2) comprised experimentally generated recordings of melanoma cell lines treated with nintedanib, used to compare migration assay performance. For these experiments, cells were seeded at a density of 1,000 cells per well in 96-well plates. Following 24 hours of attachment, treatment with 1 μ M or 2 μ M nintedanib was applied, while control wells received only complete DMEM supplemented with 10% FBS. Plates were maintained on an inverted phase-contrast microscope equipped with a motorized stage and on-stage incubation chamber providing stable physiological conditions (37 °C, 5% CO₂). Images were captured at 10-minute intervals for 72 hours. Three independent experiments were conducted on separate plates, each with three replicate wells per condition and two distinct fields of view per well. In these recordings, **semi-automatic tracking** was used exclusively.

4.3.1. *Cell tracking methods*

4.3.1.1. *Manual tracking*

Manual tracking was performed exclusively on the **reference dataset** (Section 4.1). All manual tracking was performed by a single observer using an in-house developed tracking tool. Similar to the image acquisition, the manual tracking had been completed beforehand and was provided to us for analysis. The observer positioned the marker at the approximate geometric center of each cell in the initial frame and subsequently repositioned it only when the cell exhibited clear translational movement beyond its immediate vicinity. This approach ensured that minor morphological fluctuations did not artificially contribute to displacement. When no visible movement occurred between two selected frames, the marker position remained unchanged.

Because manual tracking is inherently time-consuming, trajectories were extracted at reduced temporal sampling. For datasets acquired at a 5-minute imaging interval, cells were tracked on every third frame, yielding an effective temporal resolution of 15 minutes, whereas for datasets recorded at 10-minute intervals, tracking every second frame resulted in a 20-minute effective resolution. These manually curated trajectories served as a reliable ground truth for subsequent comparison with semi-automatic tracking results and for the calibration of the noise filtering thresholds.

4.3.1.2. *Semi-automatic tracking: Image pre-processing, tracking, and noise-filtering*

The same semi-automatic tracking procedure, including the standardized image pre-processing steps, was applied identically to both the **reference and primary experimental datasets** (Sections 4.1 and 4.2).

Before tracking, all image sequences were pre-processed in ImageJ to minimize tracking errors and background noise. Each frame was smoothed using a Gaussian blur filter with a radius of 1.4 pixels, followed by background subtraction using the rolling ball algorithm (radius = 12 pixels). The resulting images were then inverted and contrast-enhanced using a 0.06 saturation threshold to optimize cell boundary detection.

Semi-automatic tracking was subsequently performed on the pre-processed recordings using *CellTracker* (Piccinini et al., 2016). The software identifies individual cells in each frame based on their intensity and size, and links their centroids across consecutive images to reconstruct complete trajectories. Two initialization parameters are required: the average cell diameter and the average

cell velocity (in pixels). These parameters were empirically optimized for each dataset based on preliminary ImageJ measurements. The cell diameter was set to correspond to the approximate pixel size of single cells, while the average velocity parameter was slightly adjusted upward or downward according to observed cell motility. Such fine-tuning enhanced trajectory stability and reduced the likelihood that the tracking window would drift off the cell. However, these modifications exerted negligible influence on the resulting coordinate data, since all incomplete or misaligned trajectories were subsequently excluded during manual validation. Individual cells were manually selected in the first frame and automatically tracked for at least 24 hours. For reference recordings, the tracking duration matched that of the corresponding manual analyses. As mentioned earlier, tracking continuity was visually verified, and trajectories in which the tracking box lost the cell were excluded from further processing. Finally, the time-resolved x-y coordinates of each successfully tracked cell were exported for subsequent quantitative analysis.

For the **reference datasets** (Section 4.1), additional post-processing steps were performed to ensure full comparability with manually tracked results. First, trajectories were downsampled to match the effective frame interval used during manual tracking, that is, every third or second image, corresponding to a temporal resolution of 15 or 20 minutes, respectively.

Subsequently, a **noise-filtering** procedure was applied to the semi-automatic trajectories. The Euclidean distance between successive coordinates was calculated, and steps smaller than a predefined displacement threshold were reset to the previous position. The optimal threshold ($\sigma = 24 \mu\text{m}$, corresponding to 11 pixels) was empirically determined by comparing the outputs of semi-automatic and manual tracking. The threshold was defined as the point where the difference between manual and semi-automatic measurements fell below 10% for both displacement- and path-based parameters.

4.3.2. Processing and quantitative analysis of tracked cell trajectories

Prior to quantitative analysis, all trajectory data extracted from single-cell tracking were curated to ensure completeness and reliability. Any cell whose x-y coordinate series was incomplete – i.e., if the tracking was lost or the cell moved out of the field of view before the end of the observation period – was excluded from further analysis. Only trajectories with continuous positional data throughout the full tracking interval were retained.

The validated coordinate datasets were then used for quantitative computation of cell migration parameters. Each trajectory was represented as a two-dimensional positional vector:

$$p_i = (x_i, y_i) \quad (1)$$

4.3.2.1. Calculation of migration parameters

Using the sets of coordinates derived from **reference and primary experimental datasets** (Section 4.1 and 4.2), eight commonly used migration parameters, as well as the less frequently applied turning vector zero-crossing (TVZC), were computed for each tracked cell in MATLAB (Table 3).

Migration parameter		Equation
Displacement-based	Mean squared displacement	$MSD_i = \left(\frac{1}{N-i}\right) \sum_{k=1}^{N-i} d^2(p_k, p_{k+i})$
	Displacement	$D_i = d(p_1, p_i)$
	Maximal displacement	$\text{Max } D_i = \max_{k=1}^i D_k$
Path-based	Total traveled distance	$TTD_i = \sum_{k=1}^{i-1} d(p_k, p_{k+1})$
	Velocity	$V_i = \frac{d(p_i, p_{i+1})}{\Delta t_i}$
	Average velocity	$AV = \left(\frac{1}{N-1}\right) \sum_{i=1}^{N-1} V_i$
Directionality-based	Directionality ratio	$DR = \frac{D_N}{TTD_N}$
	Average turning angle	$ATA = \left(\frac{1}{N-1}\right) \sum_{i=1}^{N-1} \alpha_i$, where $\alpha_i = \tan^{-1} \frac{(y_{i+1} - y_i)}{(x_{i+1} - x_i)}$
	Turning vector zero crossing	$TVZC = \sum_{i=1}^{N-1} \delta(\alpha_i \cdot \alpha_{i+1} < 0)$

Table 3. Investigated migration parameters with their equation

In the equations, p denotes the positional vector (as defined in Eq. (1)), and i ranges from 1 to $N-1$, where N corresponds to the total number of frames. Notably, the parameters can be grouped according to the geometric basis of their calculation.

Displacement-based parameters (MSD, D, and Max D) quantify the extent of cell translocation from its original position. The mean squared displacement (MSD) represents the average squared distance from the starting point as a function of time, reflecting the overall diffusivity of movement. The displacement (D) describes the straight-line distance between the cell's initial and current positions, while maximal displacement (Max D) denotes the greatest such distance reached during the observation period.

Path-based parameters (TTD, V, and AV) describe migration dynamics along the entire trajectory. The total traveled distance (TTD) is the cumulative sum of all stepwise displacements, thus reflecting total motility activity. The instantaneous velocity (V) corresponds to the stepwise distance per unit time, while the average velocity (AV) represents its mean over the entire recording.

Directionality-based parameters (DR, ATA, and TVZC) capture the persistence and turning behavior of migration. The directionality ratio (DR) is defined as the quotient of net displacement and total travelled distance, providing a quantitative measure of trajectory linearity. Values approaching 1 indicate increasingly straight and persistent migration paths. This metric is also referred to in the literature as the confinement ratio, persistence ratio, meandering index, or straightness index, reflecting its widespread use for quantifying directional consistency across different analytical frameworks (Masuzzo et al., 2016). The average turning angle (ATA) quantifies the mean angular deviation between consecutive displacement vectors, reflecting local fluctuations in direction. Finally, the turning vector zero crossing (TVZC) represents the number of times the turning vector changes sign, corresponding to the frequency of left/right directional switches during the observation period.

4.3.2.2. *Quantitative analysis of temporal migration dynamics and statistical evaluation*

The first five parameters listed in Table 3 (MSD, D, Max D, TTD, and V) describe time-dependent trajectories of cell migration. Therefore, the area under the curve (AUC) was calculated individually for each cell using the MATLAB built-in `trapz` function. For **both datasets**, this

approach was applied to better capture the cumulative migratory behavior over time compared to single time-point evaluations, thereby providing a robust and integrative descriptor for subsequent quantitative analyses. As emphasized earlier before parameter calculation, all trajectories were screened to ensure completeness, and any cell whose tracking was lost before the end of the observation period was excluded from analysis. This filtering step was essential to avoid underestimation of AUC values arising from prematurely terminated trajectories.

For the **reference datasets** (Section 4.1), the parameter values or AUC values (in case of time-dependent parameters) obtained under treatment conditions were also normalized to the respective untreated controls for each cell line, expressed as **normalized migration parameters**.

To assess **divergent parameter responses** resulting from a treatment, the normalized values for all treatment conditions were first transformed using the $\log_2$ function. Subsequently, displacement-related parameters (MSD, D, Max D) were averaged to derive a displacement-based score, while path-related parameters (TTD, V, AV) were averaged to yield a path-based score. This aggregation was appropriate because all original metrics had been normalized to their respective controls – thereby eliminating absolute magnitude differences – and the parameters within each group were conceptually and mathematically related, reflecting similar aspects of migratory behavior. Finally, the frequency of discordant cases was quantified; these were defined as instances where the displacement-based score increased while the path-based score decreased, or vice versa. The number of such cases was expressed as a percentage of all treatment and cell-line combinations by calculating: $\text{discordant cases} / \text{total cases} \times 100$.

To evaluate parameter **sensitivity**, all video-microscopic datasets were analyzed using the three tracking modes (manual, semi-automatic, and noise-filtered semi-automatic). For each cell line and treatment combination, AUC ratios between the tracking methods were computed and averaged to quantify relative sensitivity. This analysis yielded three comparison groups: semi-automatic vs. manual, noise-filtered semi-automatic vs. manual, and semi-automatic vs. noise-filtered semi-automatic. Statistical comparisons were performed using one-way analysis of variance (ANOVA) followed by Tukey's post-hoc test.

The **repeatability** of each migration parameter was characterized by the coefficient of variation (CV), defined as the standard deviation divided by the mean, expressed as a percentage. Mean CV

values were then calculated across tracking methods. Statistical comparisons were performed using the same analytical framework as in the sensitivity analysis.

For the **primary experimental datasets** (Section 4.2), the inhibitory effect of nintedanib (**Inhibition%**) on cell migration was quantified using the MSD-based inhibition index, calculated as:

$$Inhibition\% = 100 * \left(1 - \frac{MSD\ AUC_{treated}}{Mean\ MSD\ AUC_{control}}\right) \quad (2)$$

Kruskal-Wallis analysis followed by Dunn's multiple-comparison test was used to evaluate statistical significance ($p < 0.05$).

4.3.3. Determination of cell line-specific features

As previously described, experiments were performed under low seeding density conditions, which minimize confluency-related effects on both cell morphology and motility. Multiple cells were sampled from multiple, randomly chosen fields of view across **each dataset**. Averaging across these independent replicates provided robust and statistically reliable morphological profiles for all investigated cell lines, and the high cell count effectively compensated for cell cycle-dependent variability.

4.3.3.1. *Semi-automated image processing method and correlation calculation*

For the **reference datasets** (Section 4.1), cell line-specific parameters were determined using a semi-automated image analysis workflow in *ImageJ*. For each cell line, three independent untreated control recordings were processed to extract representative morphological and spatial metrics.

Initial preprocessing included application of a Median filter (radius = 3 pixels) to reduce high-frequency noise, followed by background subtraction using the rolling ball algorithm (radius = 8 pixels). The smoothing step was repeated once more with a Median filter (radius = 3 pixels) to ensure uniform background correction. After these steps, automated thresholding was applied to generate binary mask images. A minimum size threshold of 120 pixels was applied using the particle analysis function to exclude debris and subcellular fragments. Any holes within identified regions were filled using the built-in Fill Holes command in ImageJ. These processed binary

regions, corresponding to individual cells or colonies, were then used to derive cell line-specific features.

The following parameters were quantified using ImageJ's Particle Analysis function (except convexity):

- Cell size (CS): defined as the mean area (in pixels²) of at least ten detected individual cells, with cell individuality verified through manual inspection.
- Circularity (CI): calculated as $4\pi \times \text{area}/\text{perimeter}^2$, where the perimeter denotes the length of the cell boundary (for at least ten individual cells - verified manually). CI yields values close to 1 for near-circular shapes.
- Aspect ratio (AR): calculated as the ratio of the major to minor axis of the best-fit ellipse, reflecting cell elongation (for at least ten individual cells - verified manually).
- Solidity (SOL): representing boundary regularity, defined as the ratio of the cell area to its convex hull area (at least ten individual cells - verified manually).
- Convexity (CONV): expressing the smoothness of the cell contour by dividing the convex hull perimeter by the perimeter (for at least ten individual cells - verified manually). CONV was extracted using the ImageJ Shape Filter plugin.
- Cell number (CN): determined by dividing the total covered area (sum of all detected particles – not only the manually verified individual cell area) by the average CS.
- Coverage (COV): the percentage of the total image area occupied by cells (covered area / whole field of view area).
- Colonization (COL): was calculated as the ratio of the CN to the number of distinct non-connected areas (detected particle number), describing spatial clustering.
- Colony size (COLS): calculated as the area of the largest connected region divided by the mean CS, reflecting colony formation potential.
- Cell line-specific velocity (CLSV): derived as the mean velocity from manually tracked control cells, included to capture the characteristic motility of each line (this parameter was not obtained from ImageJ).

To investigate relationships between these cell line-specific features and migration descriptors, Pearson correlation coefficients were computed using MATLAB's built-in `corrplot` function, enabling visualization of linear associations among parameters.

4.3.3.2. Manual evaluation of cell-line specific features

For the **primary experimental datasets** (Section 4.2), cell line-specific characteristics were determined manually using ImageJ. In contrast to the semi-automated image processing applied to reference data, these measurements were based on direct manual outlining and evaluation of individual cells from the live-cell recordings. For each melanoma cell line, at least ten cells were outlined manually in six representative fields of view.

From the manually traced contours, ImageJ's Particle Analysis function was utilized to automatically compute the following characteristics:

- Cell size (CS): same as defined in Section 4.3.3.1.
- Circularity (CI): computed as previously defined in Section 4.3.3.1.
- Aspect ratio (AR): computed as previously defined in Section 4.3.3.1.
- Roundness (RND): computed as $4 \times \text{area} / (\pi \times \text{major axis}^2)$, quantifying overall circular symmetry.
- Solidity (SOL): computed as previously defined in Section 4.3.3.1.

Within the same image sequences, cells were also manually counted at baseline (0 h), 24 h, and 48 h. This allowed us to evaluate cell morphology and proliferation under nintedanib treatment. Manual inspection additionally captured treatment-induced morphological variability that may not be reliably detected by automated segmentation. For the Mel Pt-4 post cell line, an additional measurement was performed at the 4-hour time point to evaluate early morphological changes, as this line reached excessive confluency at later stages, rendering shape measurements unreliable beyond 24 hours.

The **proliferation rate** was also calculated, as the ratio of the cell number at each later time point to that at baseline, enabling normalized comparison of growth dynamics across cell lines and treatment conditions.

4.4. Random walk simulation of cell migration

To extend our analysis beyond the experimentally acquired data described in Section 4.1, and to systematically evaluate how all nine quantitative migration parameters respond to controlled changes in motility, we implemented a computational random-walk model in MATLAB (Codling et al., 2008). Virtual cell trajectories were generated under predefined combinations of speed and directionality, mimicking distinct hypothetical “treatment” scenarios. The simulation framework enabled systematic assessment of the sensitivity and robustness of the migration descriptors under controlled, noise-free perturbations. In this way, it provided a complementary reference for interpreting the variability and treatment responses observed in the experimental single-cell tracking data. The complete MATLAB implementation of the simulation framework (simulation.m) is publicly available in the accompanying GitHub repository: https://github.com/banyaigreti/Decoding_cell_migration-Banyai_G_L.

4.4.1. *Mathematical model of cell displacement and directionality*

The effect of simulated treatments on migration speed was introduced through a multiplication factor (MF) that scaled displacement at each time step. Cell positions were iteratively updated according to the following equations:

$$\begin{aligned}\Delta x_i &= MF \times v_i \times \sin(\alpha_i) \\ \Delta y_i &= MF \times v_i \times \cos(\alpha_i)\end{aligned}\tag{3}$$

where Δx_i and Δy_i are the coordinate displacements at time point i , MF is the multiplication factor representing the influence on migration speed, v_i is the baseline instantaneous speed, randomly sampled between 0 and 7.5, and α_i denotes the turning angle at time point i .

The baseline speed distribution (v_i in 0-7.5 range) was chosen such that the migration parameters of the control simulations ($MF = 1$, $P = 0.5$) fell within the lower third of those observed experimentally, ensuring that the simulated control trajectories reflected a realistic low-to-moderate motility phenotype.

The simulation consisted of 96 time points (i), matching the temporal resolution of the experimental imaging (15-minute intervals over 24 hours).

The directionality of motion was determined by the probability P of changing direction – e.g., if P is greater than R , the cell will change its direction – as described in the following equation:

$$\alpha_i = \begin{cases} U(-5^\circ, 5^\circ) & \text{if } P > R \\ U(-180^\circ, 180^\circ) & \text{if } P < R \end{cases} \quad (4)$$

where $U(a,b)$ denotes a random uniform distribution between a and b , and R is a random number between 0 and 1.

4.4.2. Simulation design and treatment scenarios

Under control conditions, MF was set to 1, such that the instantaneous speed of each step followed the baseline random distribution (uniformly between 0 and 7.5) and was not altered by any multiplicative treatment effect. Directionality was set to $P = 0.5$, allowing equal probability of continuing straight or turning.

Four **combined “treatment”** conditions were initially defined to represent characteristic alterations in cell motility:

- (i) Increased speed and increased directionality,
- (ii) Increased speed and decreased directionality,
- (iii) Decreased speed and increased directionality, and
- (iv) Decreased speed and decreased directionality.

In addition to combined perturbations, four **single-parameter treatments** were simulated:

- (v) Increased speed with control directionality ($P = 0.5$)
- (vi) Decreased speed with control directionality ($P = 0.5$)
- (vii) Control speed ($MF = 1$) with increased directionality
- (viii) Control speed ($MF = 1$) with decreased directionality

Speed-inhibiting conditions were simulated by decreasing MF from 1 to 0.05, whereas speed-enhancing conditions increased MF from 1 to 2 (Table 4). When directionality decreased simultaneously (erratic trajectories), MF had to be extended up to 4 for displacement-based parameters to diverge significantly from control (Table 4); therefore, both $MF = 1-2$ and $MF = 1-4$ ranges are illustrated in this condition.

Directionality was increased by raising P up to 0.95 (5% turning probability), and decreased by lowering P to 0, allowing unrestricted turning (Table 4).

	Control	Decrease	Increase
Speed (<i>MF</i>)	1	0.05 – 1	1 – 2 or 4
Directionality (<i>P</i>)	0.5	0.05 – 0.5	0.5 – 0.95

Table 4. Parameter ranges used in random walk simulations. *MF* modulates migration speed, and *P* defines directional persistence.

The *MF* and *P* ranges (Table 4) were divided into 200 equally spaced intervals. For each *MF*-*P* combination, 20 independent simulations were performed, each with 10 cells simulated, yielding $200 \times 20 \times 10$ trajectories per treatment condition. Control conditions were simulated 100 times. In total, 360,100 simulated trajectories were generated:

- 100 control simulations
- $5 \times 200 \times 20 \times 10$ for the combined *MF*-*P* treatment (when *P* decreased, *MF* was measured between 1-2 and 1-4 as well)
- $4 \times 200 \times 20 \times 10$ for the single-parameter treatment

4.4.3. Calculation of migration parameters and statistical analysis for random walk simulations

All nine quantitative migration descriptors – mean squared displacement (MSD), displacement (*D*), maximal displacement (Max *D*), total traveled distance (TTD), velocity (*V*), average velocity (AV), directionality ratio (DR), average turning angle (ATA), and turning vector zero-crossing (TVZC) – were computed from the simulated trajectories using the same workflow and equations as described for the experimental single-cell data (Table 3, Section 4.3.2.1).

For the five time-dependent parameters (MSD, *D*, Max *D*, TTD, and *V*), the AUC was also calculated to capture their cumulative temporal behavior. The remaining parameters (AV, DR, ATA, and TVZC) were computed directly from the trajectory coordinates (as described in 4.3.2.2 as well).

Statistical evaluation was performed using one-way ANOVA. For each *MF*-*P* parameter combination, the migration parameters of the ten simulated cells within each replicate were averaged, yielding one value per replicate. Each of the 20 replicates was then compared to the control condition (*MF* = 1, *P* = 0.5). For every *MF*-*P* combination, we recorded how many of the

20 replicates showed a statistically significant difference from the control. These results were visualized using a color scale, where color intensity reflected the number of significantly deviating replicates (ranging from 0 to 20).

4.5. Gap closure assays

Gap closure assays were performed as part of the **primary experimental datasets** described in Section 4.2, using five human melanoma cell lines treated with the multi-kinase inhibitor nintedanib. These assays were conducted to enable cross-comparison between distinct *in vitro* migration assays.

Two assay formats were employed to evaluate collective cell migration: a conventional wound-healing (**scratch**) **assay** and a zone-exclusion (**Z-E**) **assay**. The scratch assay involved mechanically creating a gap in a confluent monolayer, whereas the Z-E assay utilized a silicone-based insert to produce a precisely defined cell-free area. Both assays were conducted under similar culture and imaging conditions to ensure comparability of closure kinetics across experimental formats.

4.5.1. Scratch wound-healing assay

For the scratch assay, 30,000 cells per well were seeded into 96-well plates to establish a confluent monolayer. On the following day, linear scratches were introduced using a sterile pipette tip. Detached cells were removed by two washes with phosphate-buffered saline (PBS), and the remaining adherent cells were exposed to 1 μ M or 2 μ M nintedanib in 150 μ L of DMEM supplemented with 10% FBS. Plates were maintained on an inverted phase-contrast microscope equipped with a motorized stage and an on-stage incubation chamber ensuring stable conditions (37 $^{\circ}$ C, 5% CO₂). Time-lapse images were captured every 4 hours for a total duration of 72 hours. Four independent experiments were performed on separate plates, each containing three replicate wells per condition, and two non-overlapping fields of view per scratch (per well) were recorded.

4.5.2. Zone-exclusion (Z-E) assay

In the Z-E assay, two-well silicone inserts (Ibidi GmbH) were placed into 24-well plates, and 15,000 cells were seeded into each chamber in 70 μ L of complete DMEM (10% FBS). After

overnight incubation, the inserts were gently removed to create a sharply defined gap between two cell populations. The wells were washed twice with PBS, and treatment with 1 μ M or 2 μ M nintedanib in 150 μ L of DMEM + 10% FBS was initiated. Plates were maintained under identical environmental conditions as in the scratch assay (37 °C, 5% CO₂). Time-lapse imaging was carried out every 4 hours for 72 hours. Four independent experiments were performed, each with one insert per condition per plate and three fields of view recorded per well.

4.5.3. Image processing and quantitative analysis

Gap closure was evaluated manually using *ImageJ*. For each time point, the cell-free area was delineated by hand, and its surface area was measured until complete closure was achieved.

The **relative gap area** (or relative scratch/Z-E area) was computed as the ratio of the area at time t to the initial area (relative gap area = A_t / A_0), such that values <1 indicate progressive gap closure.

For statistical comparison, the area under the curve (AUC) was calculated from the relative area-time plots using trapezoidal numerical integration (`trapz`, MATLAB), providing a single metric summarizing closure kinetics.

To account for variability in the initial gap area and differences in cell size, the **gap closure relative to cell size** was calculated as the difference between the initial and the time-point-specific gap area, normalized to the average single-cell area: Gap closure relative to cell size = $(A_0 - A_t) / A_{\text{cell}}$, where A_{cell} represents the average single-cell area obtained from independent single-cell videomicroscopy measurements (cell size (CS) parameters, Section 4.3.3.2). This correction ensured that variations in mean cell size between cell lines did not bias closure rate comparisons.

Mean gap closure velocity was determined as the change in gap area between consecutive time points divided by the corresponding time interval ($\Delta t = 4$ h) and subsequently averaged across the observation period (Gap closure velocity = $(A_t - A_{t+1}) / \Delta t$).

4.5.4. Quantification of nintedanib-induced inhibition

To quantify the inhibitory effect of nintedanib on collective cell migration, the percentage inhibition (**Inhibition%**) was calculated from the AUC of the relative gap area curves as:

$$Inhibition\% = 100 * \left(1 - \frac{Mean\ AUC_{control}}{AUC_{treated}}\right) \quad (5)$$

Here, the mean operator applies only to the control group, where all replicate AUC values were averaged to establish a robust baseline. Each treated sample was then normalized to this control mean, ensuring consistent reference scaling and appropriate variance representation.

Kruskal-Wallis analysis followed by Dunn's multiple-comparison test was used to evaluate statistical significance ($p < 0.05$).

4.6. Transwell assay

Transwell migration assays were performed as part of the **primary experimental datasets** described in Section 4.2, using the same five human melanoma cell lines treated with the multi-kinase inhibitor nintedanib. This assay model was also included in the cross-assay comparison.

4.6.1. *Experimental setup, treatment, and imaging*

Cell migration through porous membranes was assessed using hanging cell culture inserts with 8 μ m pore size (Sigma-Aldrich). Inserts were placed into 24-well plates, and 30,000 cells were seeded into the upper chamber in 100 μ L of serum-free DMEM. The lower chamber contained 750 μ L of DMEM supplemented with 1% fetal bovine serum (FBS) to establish a chemoattractant gradient. A low serum concentration was selected to maintain a stable directional stimulus while minimizing diffusion into the upper chamber, which would otherwise dissipate the gradient and diminish directed migration. For treatment conditions, both the upper and lower compartments received nintedanib at final concentrations of 1 μ M or 2 μ M. For the A2058, WM983B, Mel Pt-3 pre, and Mel Pt-3 post cell lines, incubation continued for 24 hours. For Mel Pt-4 post cells, the assay duration was reduced to 4 hours to avoid excessive migration and near-complete membrane coverage that precluded accurate quantification.

Following incubation, non-migrated cells remaining on the upper membrane surface were carefully removed using sterile cotton swabs. Inserts were washed twice with PBS, and migrated cells on the lower surface were fixed with 10% TCA for 10 minutes in the 24-well plate. Cells were subsequently stained with SRB solution for 15 minutes, and excess dye was removed by washing with 1% acetic acid. Three representative images from randomly selected fields of view were

captured per insert using an inverted phase-contrast microscope equipped with a 10× objective lens. At least three independent experiments were conducted on separate plates, with one insert per condition in each experiment.

4.6.2. Image analysis, migration quantification, and statistical evaluation

Image analysis was carried out using ImageJ software. Each image was first split into RGB channels, and the green channel was selected for further analysis, as SRB-stained cells appear dark in this channel, providing high contrast for segmentation. A fixed threshold value of 0.63 was applied to enhance contrast and isolate stained cell regions. To suppress background noise, a minimum filter (radius = 2-3 pixels) was applied, and particles smaller than this threshold were excluded to eliminate debris and artifacts. Subsequently, particle analysis was performed to quantify the number of migrated cells based on the remaining segmented objects. For the WM983B cell line, due to insufficient image contrast, cell counts were determined manually. To validate the automated workflow, a representative subset of images from each cell line was analyzed manually as well. Automated and manual results showed strong concordance, with an average deviation of approximately 5% (range: -18% to +11%), confirming the reliability of the semi-automated quantification procedure.

Migratory capacity was expressed as the **migration rate**, defined as the number of migrated cells divided by the incubation time (migration rate = number of cells / t). This normalization enabled direct comparison between conditions with different incubation durations.

Relative migration was calculated as the ratio between the number of migrated treated and control cells (relative migration = treated cell number / control cell number).

To quantify the inhibitory effect of nintedanib, the percentage inhibition (**Inhibition%**) was determined as follows:

$$Inhibition\% = 100 * \left(1 - \frac{Relative\ migration_{treated}}{Mean\ relative\ migration_{control}}\right) \quad (6)$$

Kruskal-Wallis analysis followed by Dunn's multiple-comparison test was used to evaluate statistical significance ($p < 0.05$).

4.7. Machine learning pipeline

Direct comparison of migration assays is inherently challenging, as each platform captures distinct biological processes and produces outputs in different scales and measurement units. To establish an objective and quantitative benchmark of assay sensitivity and reliability, a machine learning classification framework was developed. The underlying rationale was that assays with higher sensitivity to pharmacological perturbation should allow more accurate discrimination between control and nintedanib-treated samples. All analyses were performed in Python. Data handling and preprocessing were implemented using the pandas library (The pandas development team, 2020), while machine learning algorithms were built with scikit-learn (Pedregosa et al., 2011). The Python implementation of the machine learning workflow is publicly available in the accompanying GitHub repository (ML_single-cell_analysis.ipynb and ML_scratch_Z-E_assay_analysis.ipynb): https://github.com/banyaigreti/Decoding_cell_migration-Banyai_G_L.

4.7.1. *Feature generation and dataset preparation*

4.7.1.1. *Single-cell tracking data*

From the standardized single-cell trajectories (x-y coordinates, origin-shifted to (0,0)), a comprehensive set of quantitative migration descriptors was computed and used as input features for the machine learning model. These parameters were grouped according to their biological and mathematical relevance as follows (parameters whose computational definitions are provided in Table 3 are indicated accordingly):

1) Path-based parameters

- Distance between two consecutive points (Δd) – the Euclidean distance between each pair of successive coordinates; the mean and standard deviation of all Δd values were computed for downstream analyses.
- Total traveled distance (TTD) – see definition in Table 3.
- Average velocity (AV) – see definition in Table 3.

2) Displacement-based parameters

- Displacement (D) – see definition in Table 3.
- Maximal displacement (Max D) – see definition in Table 3.
- Mean squared displacement (MSD) – see definition in Table 3.

3) Directionality-based parameters

- Directionality ratio (DR) – see definition in Table 3.
- Average turning angle between consecutive steps (ATA) – average angular change between successive displacement vectors, see Table 3.
- Standard deviation of turning angle between consecutive steps – the variability (SD) of angular changes between successive displacement vectors.
- Turning vector zero crossing between consecutive steps (TVZC) – the number of sign changes in the turning vector between successive displacement steps; see Table 3.
- Average turning angle relative to the initial orientation axis – the mean angular deviation of each displacement vector relative to the trajectory's initial direction.
- Standard deviation of turning angle relative to the initial orientation axis – variability (SD) of angular deviations relative to the initial trajectory direction.
- Turning vector zero crossing relative to the initial point – the number of times the turning vector changes sign when computed relative to the initial displacement direction.

4) Morphology-normalized migration parameters – to account for cell-intrinsic variability, each migration descriptor listed above was further normalized to the cell's morphological profile, generating an extended feature set. Normalization factors derived from cell-specific parameters (from Section 4.3.3.2): Cell size (CS); Circularity (CI); Aspect ratio (AR); Roundness (RND); Solidity (SOL)

Thus, for each cell, both the raw migration features and the morphology-normalized counterparts were included, yielding a total of $14 (n_{\text{migration parameters}}) + 5 (n_{\text{cell specific parameters}}) \times 14$, resulting all together 84 quantitative features per trajectory.

4.7.1.2. Gap closure assays data

For both the scratch and zone-exclusion (Z-E) assays, the primary input variables consisted of the measured cell-free gap areas (μm^2) obtained at 4-hour intervals from 0 to 72 hours, until complete closure was achieved.

From these time-series data, the following numerical features were derived and used as input for the machine learning model:

- 1) Gap area at each time point (A_t) – measured cell-free area from 0 to 72 hours at 4-hour intervals.
- 2) Global derived parameters of gap area
 - Mean gap area – the average cell-free area across all time points.
 - Standard deviation of gap area – the temporal variability of the gap size.
 - Kurtosis of gap area – quantifies whether gap closure is concentrated within a narrow time window (high kurtosis, leptokurtic) or distributed more evenly (low kurtosis, platykurtic).
 - Skewness of gap area – measures temporal asymmetry of closure; positive skew indicates slow early closure followed by acceleration, whereas negative skew reflects rapid initial closure followed by plateauing.
- 3) Relative gap closure
 - Mean relative closure – the mean normalized reduction in gap area between consecutive time points.
 - Standard deviation of relative closure – variability in closure rates over time.
 - Kurtosis of relative closure – determines whether closure events cluster within a short phase or occur uniformly.
 - Skewness of relative closure – indicates acceleration (positive skew) or deceleration (negative skew) of closure dynamics.
- 4) Closure time – includes the time points (in hours) at which complete closure is achieved.

In total, 28 quantitative parameters were generated per assay condition (19 time points + 9 derived features), providing a multidimensional representation of closure dynamics for input into the machine-learning pipeline.

4.7.2. Model training and validation

For both datasets (single-cell tracking data, gap closure data – Section 4.7.1), the same supervised classification protocol was applied. The goal was to distinguish control versus nintedanib-treated samples based on the extracted features.

A Random Forest classifier was selected for its robustness and ability to handle heterogeneous input features. Each model consisted of 500 decision trees ($n_{\text{estimators}} = 500$). To ensure reproducibility and minimize sampling bias, a 10×5-fold cross-validation strategy (Browne, 2000) was employed. At the beginning of each run, the dataset was randomly partitioned into five subsets. In each fold, four subsets (80%) were used for model training, and the remaining subset (20%) served as the independent test set. This process was repeated five times per run, rotating the test subset each time, and the entire 5-fold procedure was reiterated ten times with different random splits. After each fold, the classification accuracy was recorded, and the final performance metric was expressed as the mean accuracy across all 50 test iterations ($10 \text{ runs} \times 5 \text{ folds}$).

Figure 3 provides a schematic overview of the entire machine-learning pipeline, including feature extraction, data processing, model training, and performance evaluation.

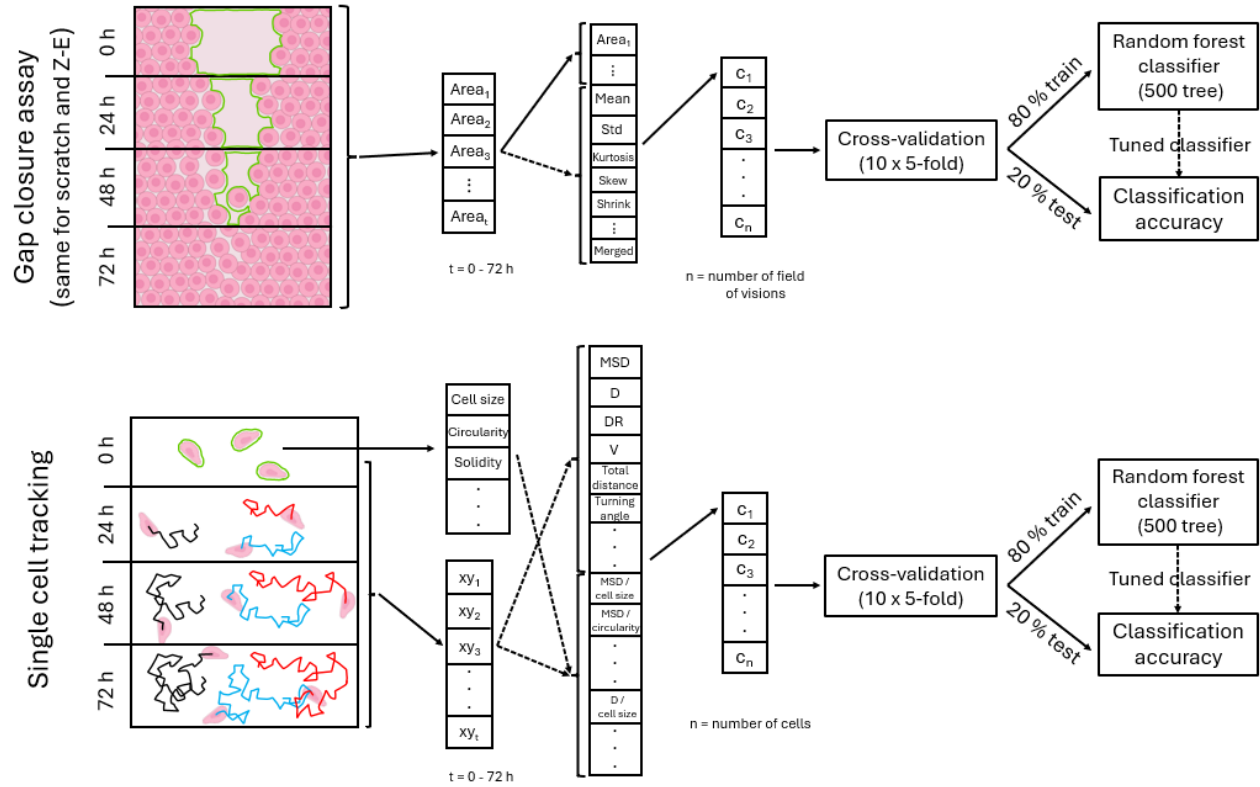


Figure 3. Overview of the machine learning workflow. Features were extracted from scratch, zone-exclusion (Z-E), and single-cell assays. For illustration, scratch and Z-E processing steps are shown together because their feature-extraction pipelines were identical, although datasets were analyzed separately. All datasets were evaluated using a 10×5-fold cross-validated random forest classifier, and classification accuracy is reported for each assay individually.

5. RESULTS

5.1. Quantitative analysis of single-cell migration parameters by videomicroscopy

To gain a deeper understanding of how quantitative migration descriptors capture distinct aspects of cell motility, we performed a systematic evaluation using the **reference dataset** described in Section 4.1. This extensive videomicroscopy collection encompassed multiple cell lines from diverse tumor origins and comprised 293 individual cell-line $\times$ treatment or control combinations. Our objectives were to determine how migration parameters respond to pharmacological perturbations and to assess how parameter outputs depend on the applied tracking strategy. Specifically, we compared manual and semi-automatic tracking approaches and, to account for potential algorithm-derived noise in the semi-automatic pipeline, additionally evaluated parameter behavior after noise filtering. We further examined parameter repeatability and correlation structure across methods, as well as the influence of intrinsic cell line-specific characteristics, including morphology, size, colonization behavior, and proliferation capacity, on the resulting quantitative readouts.

5.1.1. *Migration descriptors respond differentially to pharmacological treatment*

5.1.1.1. *Global assessment of concordance and discordance across migration metrics*

To evaluate whether displacement- and path-based migration metrics capture treatment-induced changes uniformly, we compared the two parameter groups directly. As described in Methods (Section 4.3.2.2), displacement-based descriptors (MSD, D, Max D) and path-based descriptors (TTD, V, AV) were aggregated into composite scores using $\log_2$ -transformed, normalized values, enabling direct comparison of their directional changes. The proportion of discordant cases, instances where the two aggregated scores changed in opposite directions, was quantified as a percentage (discordant cases / total $\times$ 100). These relationships are visualized in scatter plots and corresponding bar charts summarizing the frequency of discordant responses for each tracking method (Figure 4).

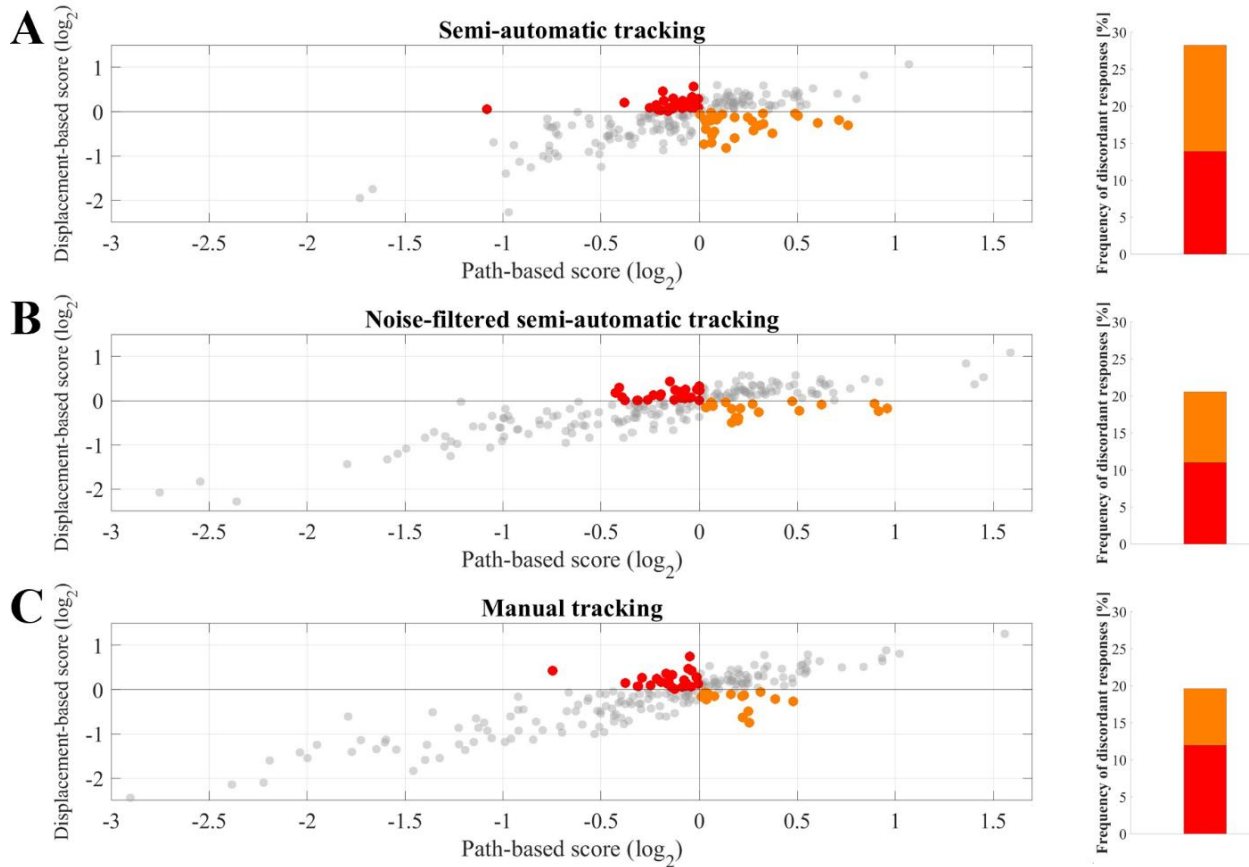


Figure 4. Divergent treatment responses across migration parameters and tracking methods. A–C) Left: Scatter plots of $\log_2$ -transformed displacement-based versus path-based scores. Gray dots indicate concordant changes; red and orange dots represent discordant responses (red: displacement increased while path decreased; orange: path increased while displacement decreased). Vertical and horizontal lines denote no change ($\log_2 = 0$). Right: Frequency of discordant responses for A) semi-automatic, B) noise-filtered semi-automatic, and C) manual tracking. Displacement-based score = average of MSD, D, Max D; Path-based score = average of TTD, V, AV.

Across the dataset, the scatter plots revealed well-defined subsets in which displacement- and path-based metrics responded divergently to treatment (Figure 4). Approximately 20-30% of all cases showed discordance, meaning that one parameter group increased while the other decreased. Importantly, this frequency was comparable across tracking methods, although semi-automatic tracking produced a slightly higher proportion of discordant outcomes. Overall, the prevalence of discordance suggests that this phenomenon primarily reflects genuine biological behavior intrinsic to cell motility rather than technical artifacts arising from tracking methodology.

These discordant patterns are of considerable biological and potential clinical relevance. For instance, a treatment that reduces instantaneous migration speed (reflected by decreased path-based scores) might superficially appear anti-migratory. However, if the same treatment simultaneously increases net displacement, cells may be able to traverse larger effective distances, potentially facilitating disseminative or metastatic behavior despite moving more slowly.

5.1.1.2. Divergent kinematic responses underlying discordant migration metrics

Such discordance can be explained by the fact that pharmacological agents often modulate both velocity and directional persistence in distinct ways. Displacement-based parameters (MSD, D, Max D) are strongly dependent on directionality, whereas path-based parameters (TTD, V, AV) are predominantly determined by instantaneous speed. Thus, if velocity decreases while directionality increases, displacement-based parameters may rise while path-based measures fall (Figure 5A). Conversely, when velocity increases, but directionality becomes erratic, path-based metrics will increase while displacement-based metrics decline (Figure 5B).

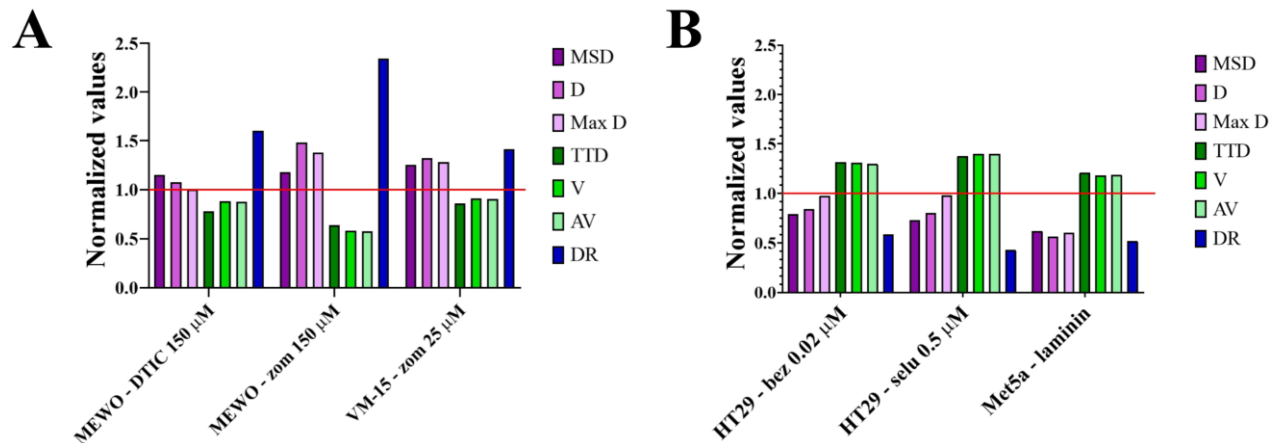


Figure 5. Divergent responses of displacement- and path-based migration parameters under opposing treatment effects. A) Representative cases in which velocity decreased while directionality increased. B) Representative cases in which velocity increased while directionality decreased. Normalized values (relative to control = red line at 1) are shown for displacement-based (MSD, D, MaxD), path-based (TTD, V, AV), and directionality-based (DR) metrics. Data from manual tracking are shown for clarity; cell line $\times$ treatment combinations are listed in Methods.

Taken together, our analyses indicate that pharmacological treatments modulate migration along four distinguishable kinematic modes:

- (i) Concordant increase – simultaneous rise in both speed and directional persistence, resulting in consistent increases across all parameters.
- (ii) Discordant (with orange color) – speed increases while directional persistence decreases, generating increased path length but reduced displacement.
- (iii) Discordant (with red color) – speed decreases while directional persistence increases, yielding reduced path length but increased displacement.
- (iv) Concordant decrease – simultaneous reduction in speed and directionality, leading to consistent decreases in all metrics.

This phenomenon reinforces the established understanding that velocity and directionality are mechanistically distinct and independently regulated components of cell motility (Shellard and Mayor, 2020; Schaks et al., 2019). These findings raised the question of whether discordant responses arise purely stochastically or if deterministic features, such as the cell line's morphology or its inherent motility, shape the likelihood of observing discordance.

5.1.1.3. Determinants of discordance: stochastic vs. cell-line-dependent factors

To address this, we analyzed the distribution of discordant events across all cell line $\times$ treatment combinations. No specific line or treatment consistently exhibited discordant behavior, indicating that discordance is not an intrinsic property of any particular biological system but rather a probabilistic outcome of how treatment-induced changes interact with cell-specific motility patterns.

We then performed two complementary statistical approaches to test whether discordance probability associates with intrinsic cell line specific features (ten parameters total; Methods 4.3.3.1):

- **Correlation analysis** between the displacement/path-score ratio and all cell line-specific features.
- **Group comparison (t-test)** between concordant vs. discordant cases for each intrinsic parameter.

Both analyses consistently showed no significant associations, indicating that discordance is primarily driven by treatment-induced modulation of the migratory machinery rather than by stable cellular traits.

The only consistent difference emerged for cell line–specific velocity (CLSV), which differed between concordant and discordant cases only when using semi-automatic tracking. Specifically, concordant responses were more frequent in inherently fast-moving cell lines. Because semi-automatic tracking exhibited a slightly higher rate of discordance, these results suggest that technical factors related to tracking noise may interact with biological velocity.

5.1.2. Random-walk simulations confirm migration parameter sensitivity to different treatment conditions

Building on the experimental findings, we next sought to dissect mechanistically how distinct combinations of motility changes give rise to divergent behaviors among migration parameters. To this end, we employed a controlled *in silico* framework based on random-walk simulations, in which cell speed (modulated by the multiplication factor, MF) and directional persistence (modulated by the turning-probability parameter, P) could be independently or jointly altered.

Simulation baseline settings were calibrated such that control trajectories (MF = 1, P = 0.5) produced migration parameter values falling within the lower third of the experimentally observed control distributions. This ensured that the simulated control phenotype corresponded to a realistic low-to-moderate motility regime, consistent with the behavior observed across many cell lines. To illustrate this calibration, Figure 6 compares the simulated control trajectories (shown in red) with all experimentally measured control trajectories (black-gray range), for all nine migration parameters.

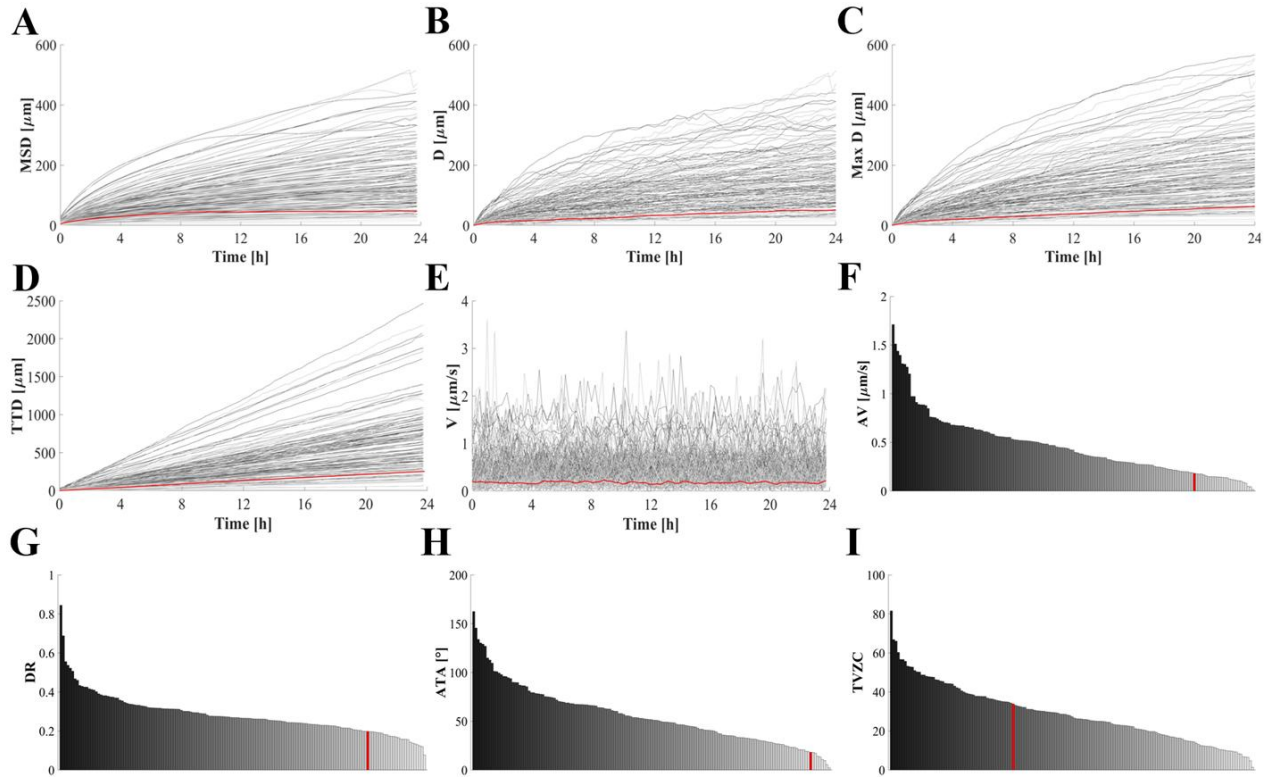


Figure 6. Migration parameters in control conditions: experimental measurements versus simulated random-walk trajectories. A–E) Time-dependent parameters: A) Mean squared displacement (MSD), B) displacement (D), C) maximal displacement (Max D), D) total traveled distance (TTD), E) velocity (V). F–I) Single-value parameters: F) average velocity (AV), G) directionality ratio (DR), H) average turning angle (ATA), I) turning vector zero-crossing (TVZC). Experimental control trajectories are shown in black-gray, while simulated random-walk controls (MF = 1, P = 0.5) are overlaid in red.

Because our experimental data demonstrated that treatments may modulate speed and directionality independently, we defined four characteristic motility-response patterns for systematic evaluation:

- (i) Increased speed and increased directionality,
- (ii) Increased speed and decreased directionality,
- (iii) Decreased speed and increased directionality, and
- (iv) Decreased speed and decreased directionality.

These four regimes represent the fundamental kinematic modes through which pharmacological interventions can reshape cell migration.

5.1.2.1. Simulated cell trajectories under different motility conditions

To obtain an intuitive overview of how various MF-P combinations translate into migration patterns, we first visualized representative x-y trajectories generated for each of the four principal response types. These synthetic paths clearly illustrate how distinct combinations of speed and directional persistence shape the geometry, curvature, and net displacement of migration.

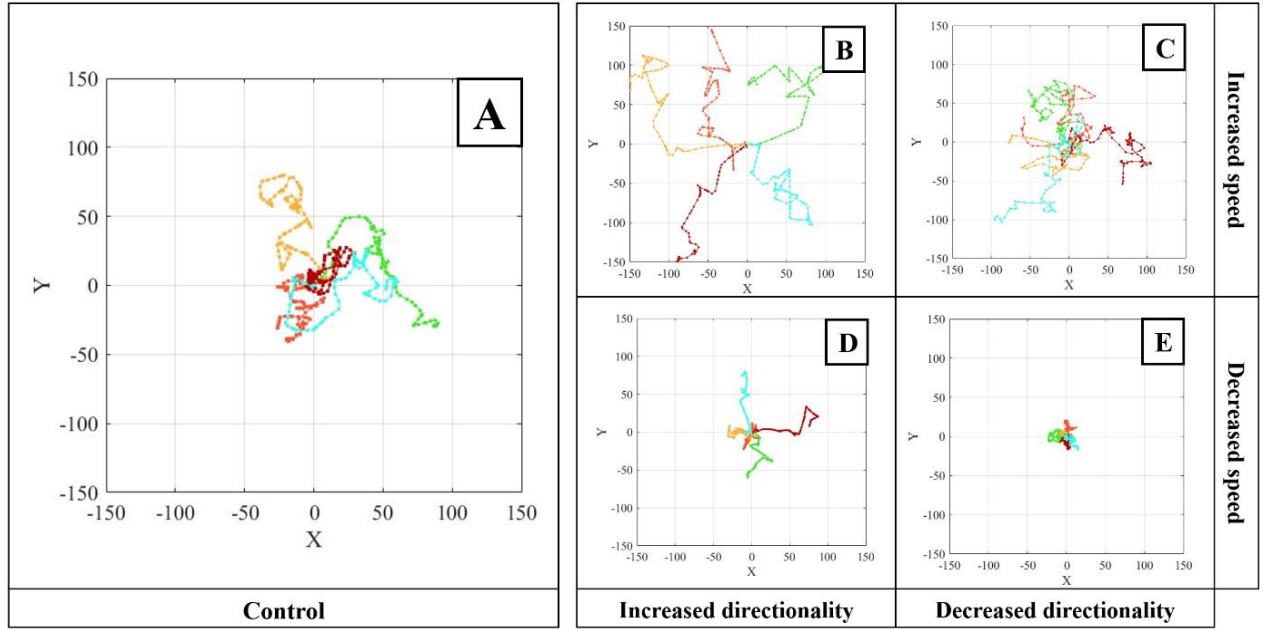


Figure 7. Cells were initialized at (0,0), and trajectories were generated for 96 time steps. A) Control (MF = 1.0, P = 0.5). B) Increased speed and increased directionality (MF = 1.5, P = 0.75). C) Increased speed and decreased directionality (MF = 2.5, P = 0.25). D) Decreased speed and increased directionality (MF = 0.5, P = 0.75). E) Decreased speed and decreased directionality (MF = 0.5, P = 0.25).

Under control conditions (Figure 7A), cells turned with 50% probability at each step, producing moderately curved trajectories and achieving net displacements of roughly one-third of the field of view. When both speed and directionality increased (Figure 7B), trajectories became markedly straighter and net displacement increased substantially, reflecting concordant enhancement of motility capacity. When speed increased, but directionality decreased (Figure 7C), cells moved faster but turned more frequently, generating tortuous paths. This yielded only modest net displacement despite elevated instantaneous speed, a characteristic scenario that can generate discordant responses (increased path-based values but decreased displacement-based values). In the opposite configuration, where speed decreased, but directionality increased (Figure 7D), straighter trajectories partially compensated for reduced step size. Some cells thus reached

distances comparable to controls, potentially producing the second type of discordant response (increased displacement despite lower path-based parameters). Finally, simultaneous reduction of speed and directionality (Figure 7E) produced highly erratic low-displacement trajectories, with both motility attributes impaired.

5.1.2.2. Sensitivity of migration parameters to simulated changes in speed and directionality

To quantify the responsiveness of each migration descriptor to defined perturbations in speed and directionality, we evaluated the deviation of simulated trajectories relative to the control condition across the full MF–P parameter grid. For every MF–P combination, twenty independent simulations were performed, and statistical significance was determined using one-way ANOVA. Figure 8 summarizes these outcomes: each marker represents one MF–P pair, and the color scale indicates the number of replicates (0-20) that differed significantly from the control (MF = 1, P = 0.5).

In addition to the four combined perturbation patterns (cases (i)-(iv)), we also simulated scenarios in which only one parameter was altered while the other was held constant.

- (v) Increased speed with control directionality (P = 0.5)
- (vi) Decreased speed with control directionality (P = 0.5)
- (vii) Control speed (MF = 1) with increased directionality
- (viii) Control speed (MF = 1) with decreased directionality

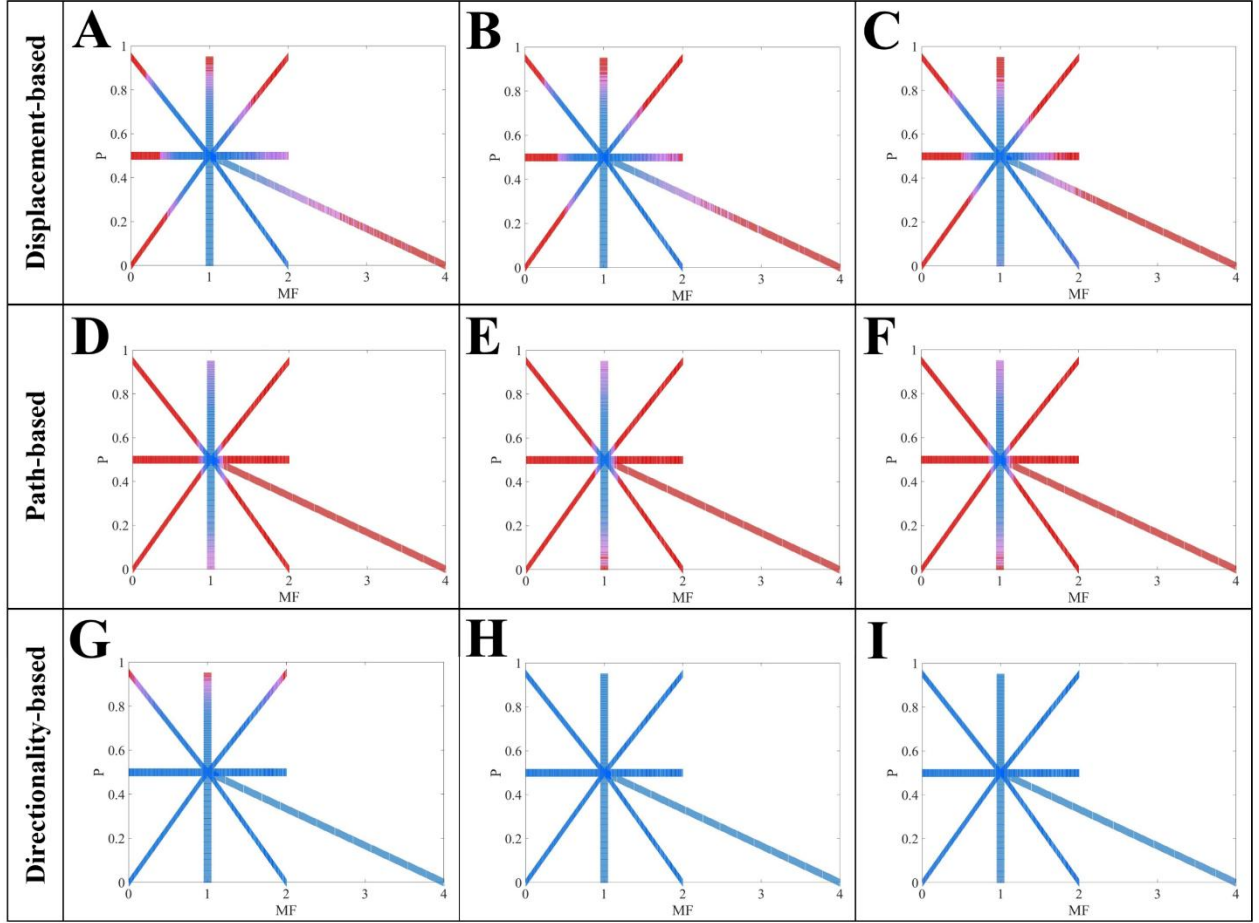


Figure 8. Sensitivity of migration parameters to simulated changes in speed (MF) and directionality (P). Each marker represents a specific MF–P parameter combination in the simulation grid. Marker color indicates the number of significant deviations (out of 20 replicates) relative to the control condition (MF = 1, P = 0.5), determined by one-way ANOVA (blue = 0/20, red = 20/20). Panels show: A) mean squared displacement (MSD); B) displacement (D); C) maximal displacement (Max D); D) total traveled distance (TTD); E) velocity (V); F) average velocity (AV); G) directionality ratio (DR); H) average turning angle (ATA); I) turning vector zero crossing (TVZC).

Displacement-based parameters showed clear and characteristic sensitivity patterns across the simulation landscape. Under conditions where directionality was reduced while speed increased, displacement-based parameters remained largely insensitive unless MF values were substantially elevated. Specifically, the MF range had to be extended from 2 up to 4 to achieve consistent significant deviations across all 20 replicates (Figure 8A-C; MF in the range 0-2 was also plotted in the figure for visualization). This underscores the compensatory interplay between

straightness and speed: highly erratic trajectories require disproportionately high velocity to achieve larger net displacement.

Among the displacement-based metrics, MSD was the least sensitive, requiring simultaneous, marked changes in both MF and P to diverge from control. In contrast, Max D exhibited the highest sensitivity, yielding significant differences under more moderate perturbations.

All three displacement-based parameters responded earlier and more strongly when MF and P were altered in the same direction (both increased or both decreased). When the two parameters changed in opposite directions, greater perturbation magnitudes were required to achieve deviation from the control, reflecting the opposing effects of speed and directional persistence on net displacement.

When only directionality was altered ($MF = 1$), displacement-based parameters responded predominantly to increased directionality, which promotes straighter trajectories and thus enhances displacement. Reduced directionality without a change in speed had minimal impact, confirming that velocity remains the primary determinant of displacement-related measures.

Path-based parameters (TTD, V, AV) demonstrated overall higher sensitivity than displacement-based metrics. Even relatively small deviations in MF or P produced consistent significant differences, and path-based measures responded reliably regardless of whether speed and directionality changed in the same or opposite directions. When only directionality was modified at constant speed ($MF = 1$), path-based parameters showed minimal deviation from the control, highlighting their strong dependence on speed rather than directional persistence.

Among **directionality-related parameters**, the directionality ratio (DR) displayed selective sensitivity: significant deviations were observed primarily under increased directionality, and only at relatively high parameter changes. Reduced directional persistence alone did not produce robust differences. Turning-related parameters (ATA, TVZC) showed no consistent significant differences across the tested conditions, although sporadic deviations occurred. Their overall insensitivity suggests limited responsiveness to moderate fluctuations in speed or directionality within the simulated framework.

5.1.3. *Sensitivity of migration parameters to tracking methods*

To determine how strongly different migration descriptors depend on the tracking approach, all videomicroscopy recordings were analyzed using both manual and semi-automatic tracking. To further dissect method-specific effects, the semi-automatic trajectories were additionally processed with a noise-filtering algorithm that removed sub-threshold movements. For each migration parameter, raw treatment and control values were compared, as well as normalized treatment responses relative to the corresponding controls. Time-dependent descriptors were evaluated via the area under the curve (AUC), whereas single-value parameters (AV, DR, ATA, TVZC) were directly compared. Statistical differences across methods were assessed by one-way ANOVA followed by Tukey's multiple comparisons test.

5.1.3.1. *Comparison of manual and semi-automatic tracking*

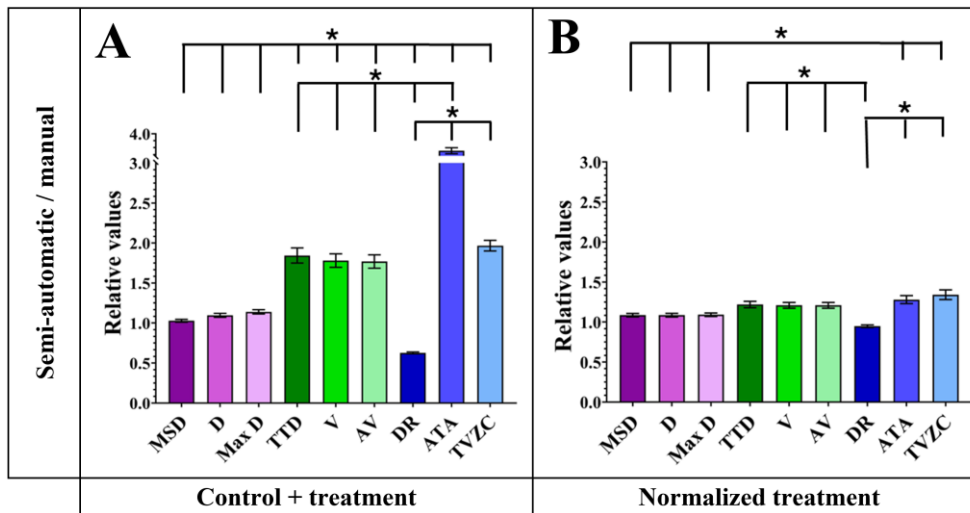


Figure 9. Sensitivity of migration parameters to various tracking methods. A) Semi-automatic / manual, all datasets. B) Semi-automatic / manual, treatment values normalized to control. (MSD = mean squared displacement; D = displacement; Max D = maximal displacement; TTD = total traveled distance; V = velocity; AV = average velocity; DR = directionality ratio; ATA = average turning angle; TVZC = turning vector zero crossing. Mean $\pm$ SEM; ANOVA + Tukey, $p < 0.01$.)

Displacement-based parameters were largely insensitive to the choice of tracking method. Across all datasets (Figure 9A, purple columns), semi-automatic/manual ratios for MSD, D, and MaxD remained close to unity. Among these, MSD exhibited the lowest ratio, indicating that mean squared displacement is the most robust against methodological variance. These findings highlight

that displacement-based metrics primarily reflect large-scale trajectory organization and therefore remain minimally affected by small frame-to-frame positional noise inherent to semi-automatic tracking. After normalization to control values (Figure 9B), even the small remaining differences between manual and semi-automatic tracking were further reduced, reflecting the proportional cancellation of noise when both numerator and denominator contain similar fluctuations.

In contrast, **path-based parameters** displayed pronounced sensitivity to tracking methodology. Total traveled distance, velocity, and average velocity quantified by semi-automatic tracking were nearly double the values obtained manually (Figure 9A, green columns). This systematic inflation arises from pixel-level fluctuations: the semi-automatic algorithm adjusts the tracking window at each frame, interpreting minor shape changes or local intensity variations as actual displacement. As a result, cumulative path length and thus all path-based parameters are substantially overestimated, while net displacement remains comparable to manual assessments.

When treatment values were normalized to their corresponding controls (Figure 9B), these discrepancies decreased markedly. Because the same type of noise affects both control and treated trajectories, ratio-based normalization cancels much of the inflated path length, reducing – but not fully eliminating – the method-dependent bias.

Directionality-based parameters exhibited the most pronounced method-specific differences. Semi-automatic tracking consistently generated lower DR values and higher ATA and TVZC values, indicative of artificially elevated turning frequencies (Figure 9B, blue columns). These distortions originate from the same micro-fluctuations affecting path-based parameters: noise-induced pseudo-movements appear as frequent small-angle changes, thereby exaggerating turning behavior and underestimating directional persistence. Consequently, directionality-based parameters were the most susceptible to artefacts introduced by semi-automatic tracking, even after normalization.

5.1.3.2. Effect of noise filtering on semi-automatic tracking

To mitigate the artefacts arising from frame-to-frame fluctuations in the semi-automatic tracking output, we applied a noise-filtering procedure in which all displacement steps below a predefined threshold were removed before parameter calculation (Methods 4.3.1.2). This step

aimed to eliminate small apparent movements generated by the algorithm, rather than by true cellular motion, and thereby improve concordance with manually annotated trajectories.

The optimal threshold ($\sigma = 24 \mu\text{m}$) was determined empirically by evaluating how different σ values affected the ratio between semi-automatic and manual readouts (Figure 10). The selected threshold corresponded to the point at which the ratios of key displacement- and path-based parameters converged to unity within a $\pm 10\%$ window. Importantly, threshold values optimized strictly for the $\pm 10\%$ criterion of these two parameter groups would have driven other descriptors outside this acceptable range; therefore, σ was chosen to achieve the best overall balance across all eight migration parameters.

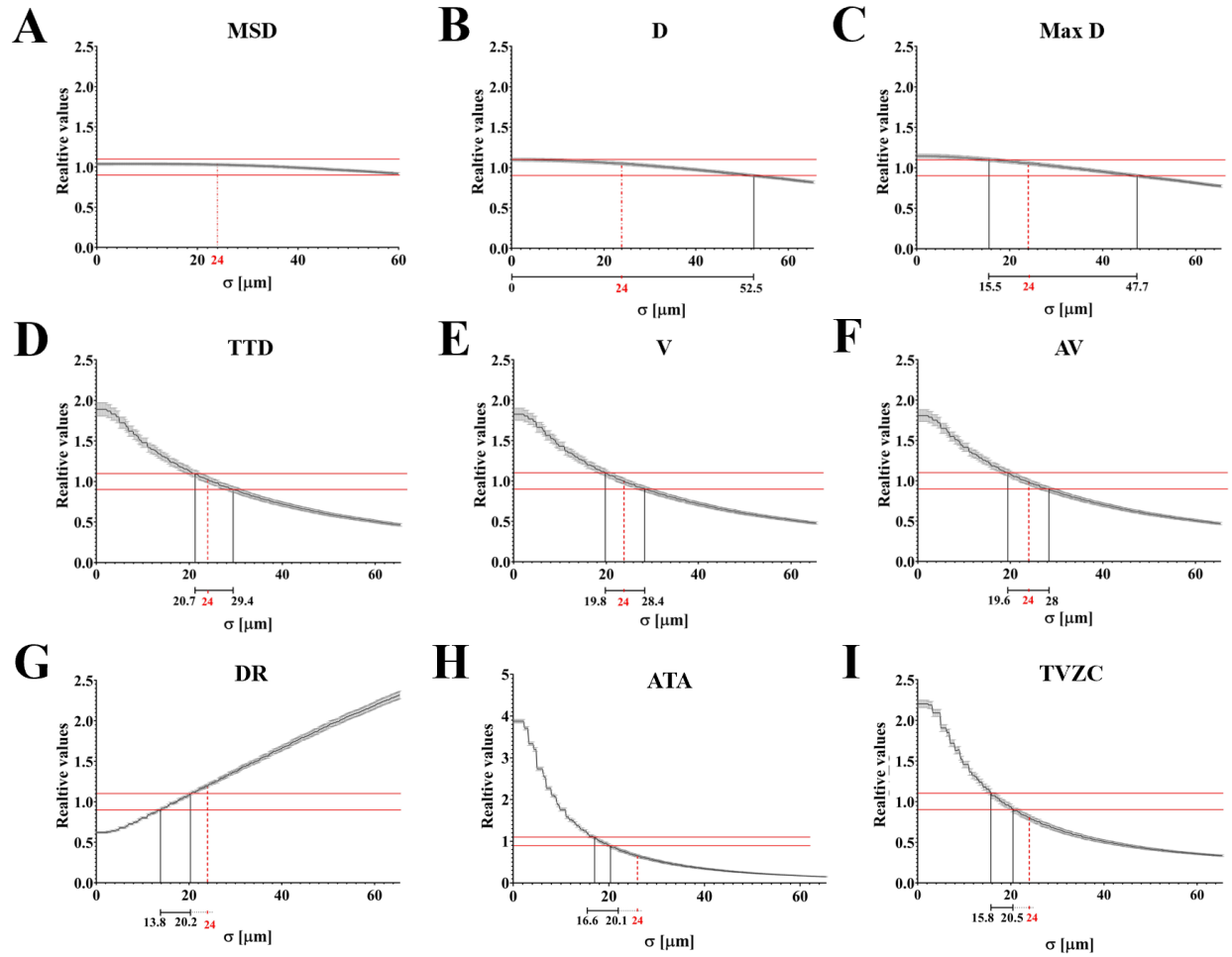


Figure 10. Effect of varying the noise-filter threshold (σ) on the ratio of semi-automatic to manual migration parameters. Horizontal red bands denote the $\pm 10\%$ agreement interval (ratio = $1 \pm 10\%$). Vertical black lines mark the σ range fulfilling this criterion; the chosen σ is indicated by a vertical dotted red line.

We applied the chosen σ value to the full dataset and recalculated all parameters using the generated noise-filtered semi-automatic trajectories. This enabled a direct evaluation of how noise removal alters the agreement between tracking methods, both at the level of absolute parameter values and normalized treatment responses. The impact of noise filtering on parameter sensitivity is summarized in Figure 11.

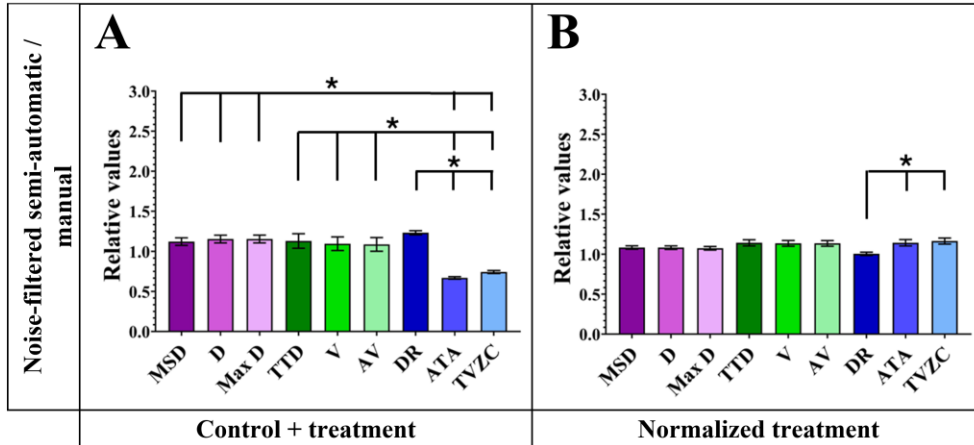


Figure 11. Sensitivity of migration parameters to various tracking methods. A) Noise-filtered semi-automatic / manual, all datasets. B) Noise-filtered semi-automatic / manual, normalized treatment values. (MSD = mean squared displacement; D = displacement; Max D = maximal displacement; TTD = total traveled distance; V = velocity; AV = average velocity; DR = directionality ratio; ATA = average turning angle; TVZC = turning vector zero crossing. Mean $\pm$ SEM; ANOVA + Tukey, $p < 0.01$.)

After thresholding, **displacement-based parameters** remained essentially unchanged (Figure 11A, purple columns), demonstrating their intrinsic robustness and limited susceptibility to fine-scale positional artefacts.

For **path-based parameters**, noise filtering markedly reduced the artificial inflation of path length introduced by semi-automatic tracking. As shown in Figure 11A (green columns), TTD, V, and AV converged toward a ratio of ~ 1 relative to manual measurements, with no significant differences from displacement-based descriptors. When treatment responses were normalized to control values (Figure 11B), the effect of filtering was smaller, consistent with the expectation that normalization already eliminates much of the shared noise component.

Among **directionality-based parameters**, filtering increased DR toward unity, reflecting improved agreement between total path length and displacement. By contrast, ATA and TVZC fell below corresponding manual values (Figure 11A, blue columns), indicating that the filtering

process removes not only spurious reorientations but also some genuine small-scale turning events captured through manual annotation. Despite this reduction, the overall discrepancy between methods was substantially reduced, underscoring the stabilizing effect of noise removal.

5.1.3.3. Comparison of semi-automatic vs noise-filtered semi-automatic tracking

To visualize the impact of noise filtering directly, we compared the unfiltered and filtered semi-automatic outputs (Figure 12).

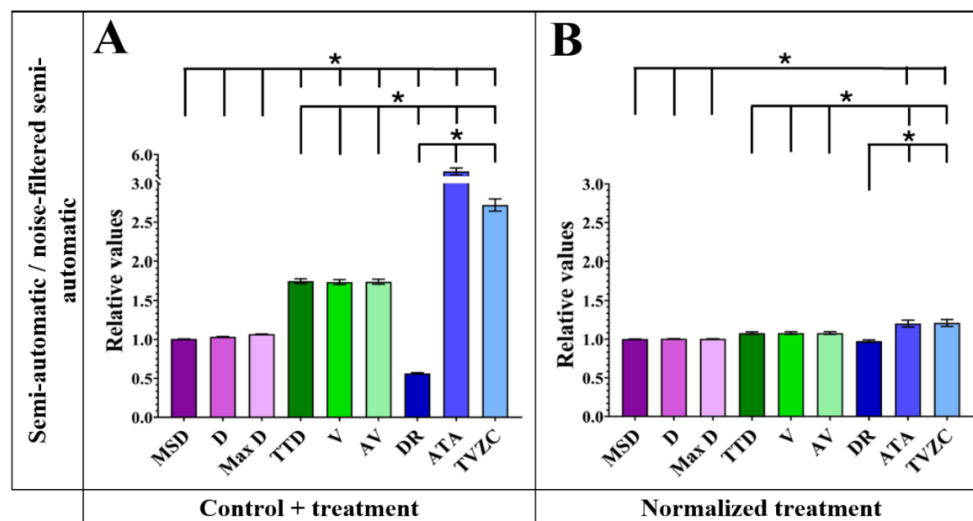


Figure 12. Sensitivity of migration parameters to various tracking methods. A) Semi-automatic / noise-filtered semi-automatic, all datasets. B) Semi-automatic / noise-filtered semi-automatic, normalized treatment values. (MSD = mean squared displacement; D = displacement; Max D = maximal displacement; TTD = total traveled distance; V = velocity; AV = average velocity; DR = directionality ratio; ATA = average turning angle; TVZC = turning vector zero crossing. Mean $\pm$ SEM; ANOVA + Tukey, $p < 0.01$.)

This comparison mirrors the manual vs semi-automatic comparison but shows more significant differences, because the exact same cells were analyzed in both cases (Figure 12). This eliminates biological variability and isolates the contribution of tracking artefacts, highlighting the importance of noise reduction.

5.1.4. Repeatability of migration parameters

To evaluate the repeatability of the nine migration parameters across tracking methods, variability was quantified using the coefficient of variation (CV). For each cell line and each control or treatment condition, the standard deviation (SD) was expressed as a percentage of the

corresponding mean value, yielding 293 CV values per tracking method. These CV values were averaged for every migration parameter, and the standard error of the mean (SEM) was calculated. Statistical analysis was performed using ANOVA followed by Tukey's multiple comparisons test.

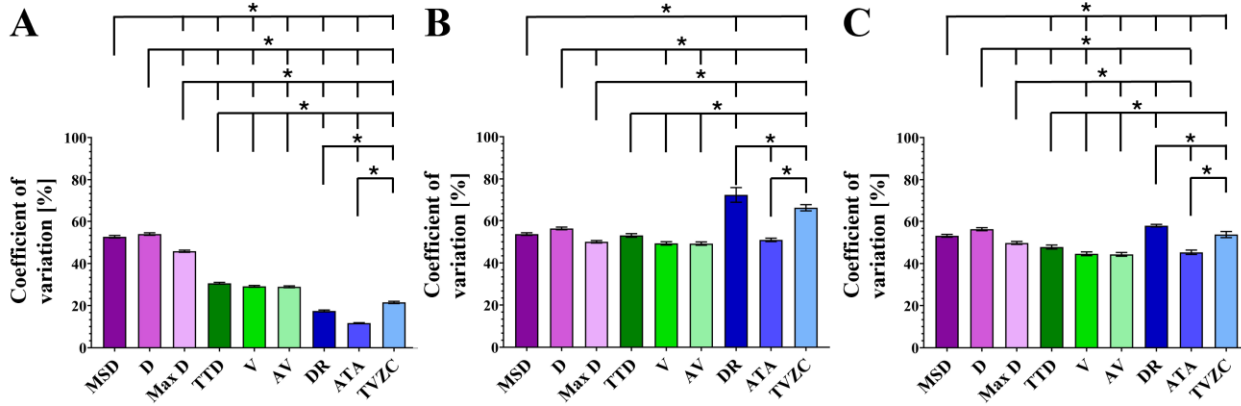


Figure 13. Repeatability of migration parameters across tracking methods. A) Semi-automatic tracking; B) Semi-automatic tracking after noise removal; C) Manual tracking. (MSD = mean squared displacement; D = displacement; Max D = maximal displacement; TTD = total travelled distance; V = velocity; AV = average velocity; DR = directionality ratio; ATA = average turning angle; TVZC = turning vector zero crossing. Mean $\pm$ SEM; ANOVA with Tukey's post-hoc test, $p < 0.01$.)

In all tracking cases, **displacement-based parameters** (MSD, D, Max D) demonstrated similar CV values, largely consistent across tracking methods (Figure 13, purplish columns). These parameters showed moderate repeatability, with 50 - 60% CV in all cases.

Path-based parameters (TTD, V, AV) showed the lowest CVs under semi-automatic tracking (Figure 13A, greenish columns). This artificially high repeatability is attributable to noise-induced micro-movements that accumulate uniformly across trajectories, inflating the total path length and reducing cell-to-cell variability. After applying noise filtering (Figure 13B), CV values increased and approached those of displacement-based parameters, confirming that the apparent repeatability under unfiltered conditions was driven by algorithmic noise rather than biological similarity. Under manual tracking (Figure 13C, greenish columns), path-based parameters showed repeatability comparable to displacement-based metrics, too, reinforcing that genuine biological variability.

Directionality-based parameters (DR, ATA, TVZC) exhibited a distinct repeatability pattern. Under semi-automatic tracking (Figure 13A, bluish columns), ATA showed exceptionally low CV values due to its strong sensitivity to noise-generated angular fluctuations, while DR and TVZC also appeared relatively stable. After noise filtering (Figure 13B), the variability of DR and TVZC

increased markedly, indicating that the previously low CVs were driven largely by noise-induced over-segmentation of directional changes. Under manual tracking (Figure 13C), DR and TVZC remained the least repeatable parameters overall, consistent with their dependence on subtle, cell-intrinsic fluctuations in turning behavior.

In summary, despite methodological differences, manual tracking revealed a relatively uniform CV across most migration parameters, converging around 40 - 60%. This high degree of variability most likely reflects genuine biological heterogeneity: cells within the same population frequently display markedly different migratory behaviors under both control and treatment conditions. As such, the repeatability analysis underscores that biological noise, rather than methodological imprecision, is the dominant source of variability in single-cell migration measurements.

5.1.5. Cell morphology correlates with motility features

Given the substantial heterogeneity across the cell lines included in the reference dataset, we next investigated how intrinsic cellular properties influence the quantitative migration readouts. While migration parameters primarily capture dynamic behavioral features, static morphological and population-level traits may critically modulate motility and thus contribute to the variability observed across conditions and tracking approaches.

To systematically assess these influences, nine cell-line-specific descriptors were extracted from control recordings of all cell lines. Nine morphological and population-based parameters were derived using ImageJ-based segmentation – cell size (CS), cell number (CN), circularity (CI), aspect ratio (AR), solidity (SOL), convexity (CONV), coverage (COV), colonization (COL), and colony size (COLS). In addition, we included a dynamic descriptor, the cell line-specific velocity (CLSV), calculated as the mean velocity of manually tracked control cells, representing the characteristic motility baseline of each cell line.

5.1.5.1. The correlation between cell line-specific and migration parameters across three tracking methods

First to achieve a systematic comparison of how cellular features relate to migration parameters under each analysis pipeline, correlation analyses were performed separately for semi-automatic (Figure 14A), noise-filtered semi-automatic (Figure 14B), and manual tracking (Figure 14C).

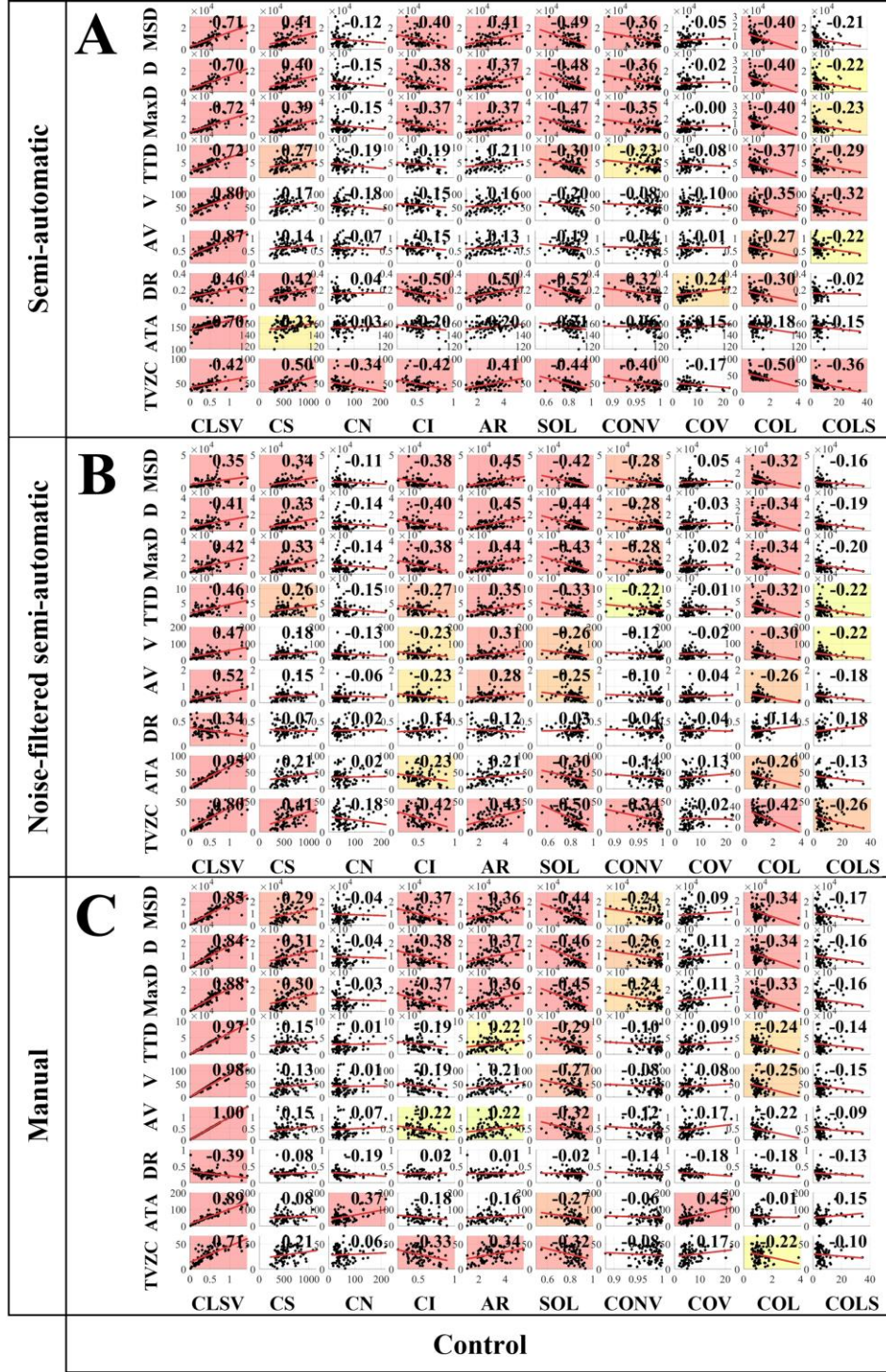


Figure 14. Correlation between migration parameters (control conditions) and cell line-specific parameters. A) Semi-automatic tracking; B) Semi-automatic tracking after noise filtering; C) Manual tracking. (CLSV = cell line-specific velocity; CS = cell size; CN = cell number; CI = circularity; AR = aspect ratio; SOL = solidity; CONV = convexity; COV = coverage; COL = colonization; COLS = colony size; $0 < p < 0.05$: background-color: red to yellow; fitted line (red) and R values)

Using **semi-automatic tracking**, all migration parameters exhibited a strong positive correlation with cell line-specific velocity (CLSV), indicating that faster cell lines consistently appeared more motile across metrics (Figure 14A). Among the other cell line features, colonization (COL) exhibited the most consistent negative correlation with migration behavior. Cell lines forming dense, cohesive colonies migrated less efficiently, likely due to increased cell-cell adhesion restricting movement even at the colony periphery. Displacement-based metrics (MSD, D, MaxD) correlated positively with cell size (CS) and showed characteristic associations with shape descriptors: circularity (CI), solidity (SOL), and convexity (CONV) correlated negatively, whereas aspect ratio (AR) correlated positively. These patterns collectively indicate that elongated, less compact cells (mesenchymal-like) display greater net translocation, while round, compact cells (amoeboid-like) exhibit more limited displacement. Notably, TAZC was the directionality-related parameter most strongly influenced by cell morphology and colony organization, reflecting its sensitivity to small fluctuations in turning behavior captured by the semi-automatic algorithm.

When **semi-automatic trajectories were subjected to noise filtering**, the overall correlation landscape remained largely consistent with the unfiltered case (Figure 14B), demonstrating that most associations between morphology and motility reflect genuine biological properties rather than algorithm-generated artefacts. The main exception was the disappearance of correlations involving the DR, indicating that DR values in unfiltered semi-automatic data were partially driven by noise-induced angular fluctuations.

Correlation patterns derived from **manually tracked** trajectories (Figure 14C) closely mirrored those obtained after noise filtering, confirming that noise-filtered semi-automatic tracking accurately replicates biologically meaningful relationships. As expected, average velocity (AV) and CLSV were perfectly correlated, since CLSV was calculated directly from these manual measurements. Moreover, manual tracking revealed weaker dependence of path-based parameters on morphological descriptors and colonization (CI, AR, COL) compared to displacement-based metrics, underscoring that path-length-related measures are more tightly coupled to intrinsic motility than to cell shape.

5.1.5.2. The impact of cell parameters on discrepancy derived from different tracking methods

To determine how intrinsic cell line–specific properties influence the agreement between different tracking approaches, we quantified the systematic discrepancies between manual, semi-automatic, and noise-filtered semi-automatic tracking. For each cell line and treatment combination, ratios were calculated between migration parameters obtained from semi-automatic (with or without noise filtering) and manual tracking. This allowed us to isolate differences arising specifically from the tracking method rather than from biological variability.

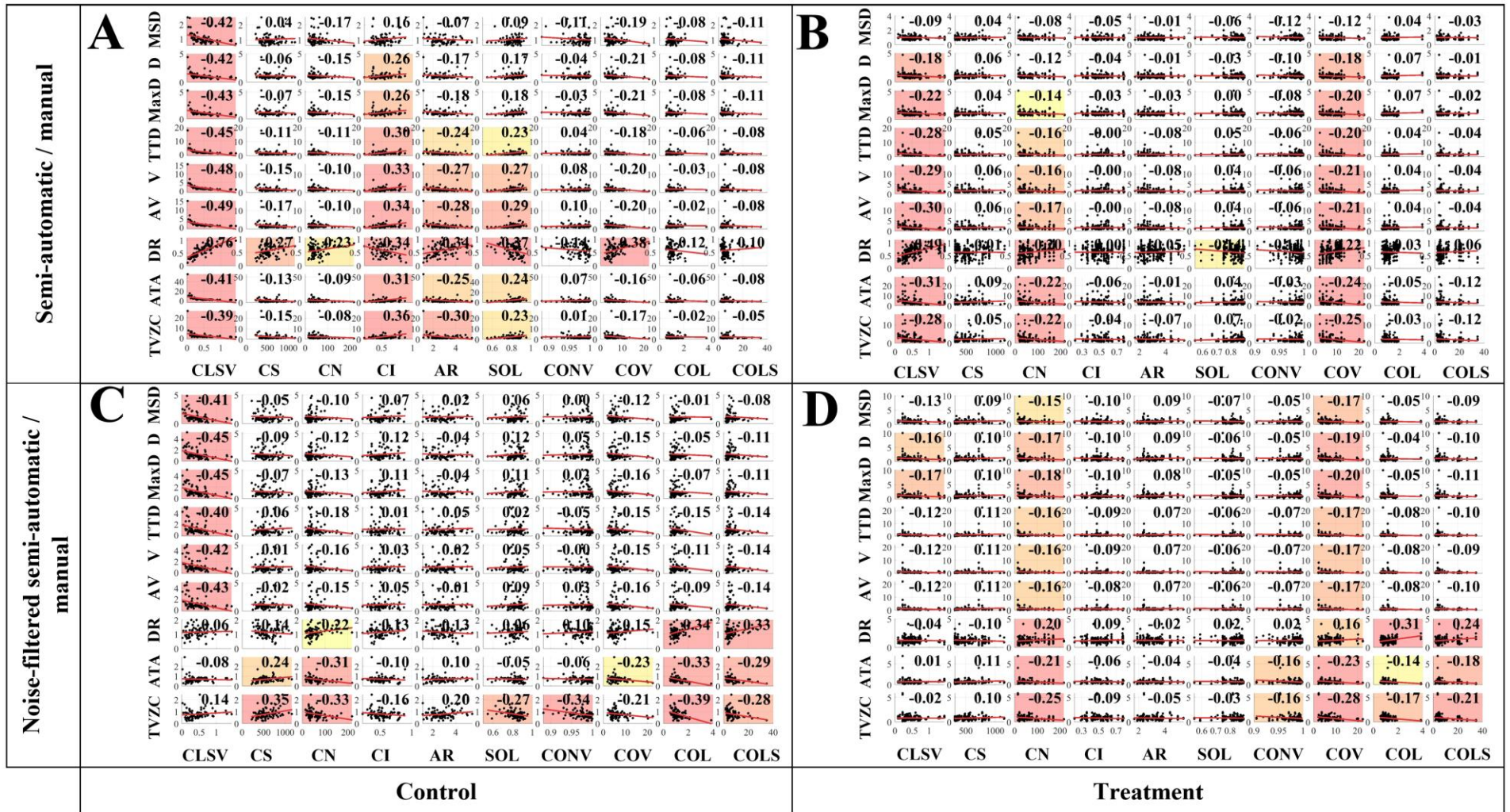


Figure 15. Correlation between cell line-specific parameters and the ratio of the migration parameters derived from semi-automatic and manual tracking. A) Control measurements; B) Treatment measurements; C) Control measurement, after noise removal; D) Treatment measurements, after noise removal. (CLSV = cell line-specific velocity; CS = cell size; CN = cell number; CI = circularity; AR = aspect ratio; SOL = solidity; CONV = convexity; COV = coverage; COL = colonization; COLS = colony size; $0 < p < 0.05$: background-color: red to yellow; fitted line (red) and R values)

When comparing **semi-automatic and manual tracking** under **control conditions**, several intrinsic cell-line characteristics showed clear associations with the degree of discrepancy between tracking methods (Figure 15A). The strongest and most consistent relationship was observed with CLSV: faster-moving cell lines exhibited markedly smaller differences between semi-automatic and manual tracking across most migration parameters (with the exception of DR). This indicates that increased motility improves the reliability of semi-automatic tracking, likely because biological movement dominates over frame-to-frame noise.

Morphology-derived descriptors also contributed to method-dependent discrepancies. CI and SOL displayed positive correlations with the semi-automatic/manual ratio, whereas AR exhibited a negative correlation. These trends indicate that round, compact cell morphologies (high CI and SOL, low AR) tended to produce larger deviations between the two methods, whereas elongated morphologies resulted in more consistent tracking outcomes. This suggests that the semi-automatic algorithm struggles more with smooth, homogeneous cell outlines where boundary detection is ambiguous, and performs better when elongated cell shapes provide clearer structural features for segmentation.

Among the migration descriptors, MSD was largely insensitive to these shape-related biases, consistent with its general robustness across tracking approaches.

Following **noise filtering of semi-automatic** trajectories under **control conditions** (Figure 15C), nearly all correlations with morphological descriptors (CI, SOL, AR) diminished or disappeared. Path-based and directionality-based parameters also showed reduced correlation with CLSV. Together, these findings demonstrate that most shape-dependent discrepancies originated from frame-to-frame noise inherent to the semi-automatic algorithm rather than from systematic algorithmic failure.

Following noise removal, only modest discrepancies remained across parameters, with ATA and TVZC retaining weak correlations with colony-related descriptors (COL, COLS), reflecting their sensitivity to subtle angular fluctuations.

Under **treatment conditions**, additional cell line descriptors emerged as strong predictors of discrepancies between **semi-automatic and manual tracking** (Figure 15B). Specifically, cell number (CN) and coverage (COV) displayed pronounced negative correlations with the semi-

automatic/manual ratio, indicating that lower cell density was associated with larger method-dependent deviations.

This effect likely arises from the increased presence of dying or non-motile cells in treated samples. During manual tracking, such cells are typically excluded by the observer, whereas the semi-automatic algorithm continues to follow them, producing artificially low motility values. As a result, the semi-automatic/manual ratio becomes inflated, particularly when CN and COV are low.

After noise filtering under treatment conditions (Figure 15D), correlations between discrepancies and CLSV largely disappeared, again demonstrating that algorithmic noise disproportionately affects rapidly moving cells. By contrast, correlations with CN and COV persisted, indicating that discrepancies under treatment conditions also reflect contributions from dying or non-motile cells, an issue that noise filtering alone cannot resolve. Additionally, after noise removal, discrepancies in directionality-related parameters (ATA, TVZC) showed associations with coverage and colonization behavior, indicating that directional descriptors remain partially affected by cell crowding and collective organization.

To mitigate artefacts caused by **non-viable cells**, a filtering procedure was introduced in which cells that ceased movement during the final three hours, or those displaying abrupt artefactual displacements ($> +2$ SD in at least two consecutive frames), were removed. Although this approach reduced CN/COV-associated discrepancies, it did not eliminate them (Figure 16), highlighting the challenge of reliably excluding dead or floating debris based solely on simple thresholds.

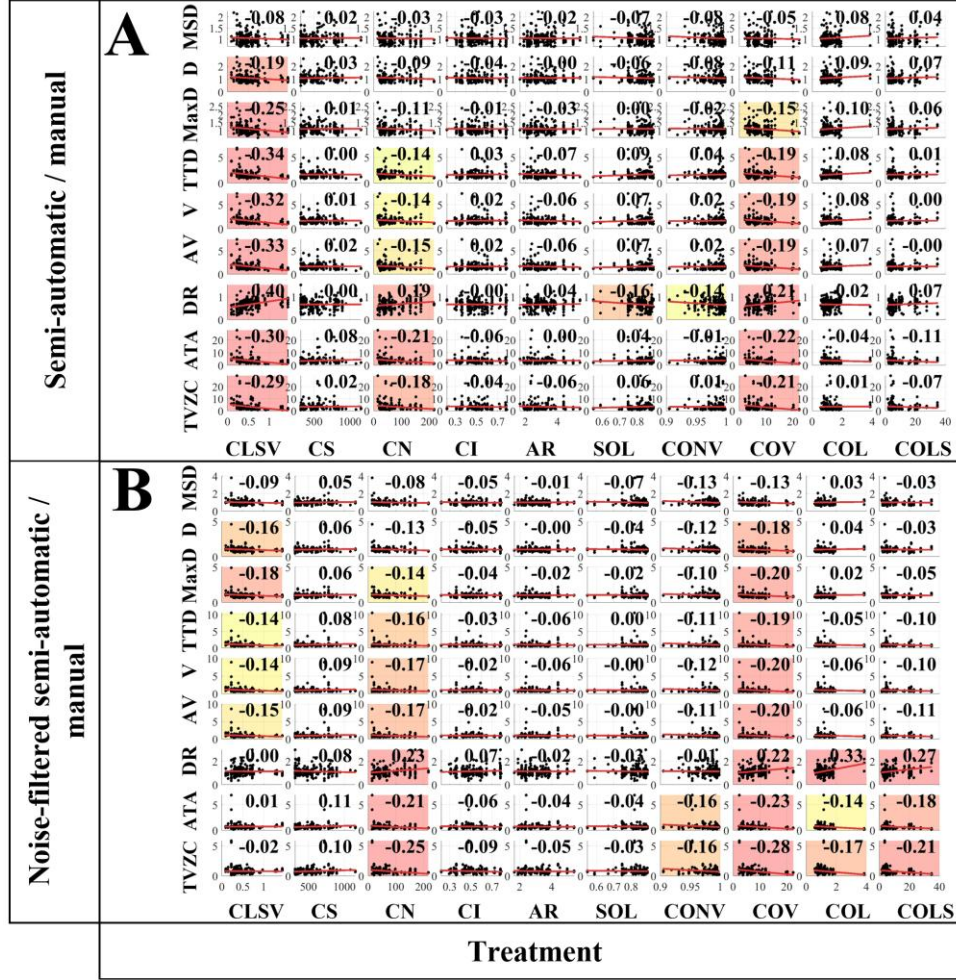


Figure 16. Correlation between cell line-specific features and the ratio of migration parameters derived from semi-automatic and manual tracking after removal of dead cells. A) Treatment measurements. B) Treatment measurements after noise filtering. (CLSV = cell line-specific velocity; CS = cell size; CN = cell number; CI = circularity; COV = coverage; COL = colonization; COLS = colony size; $0 < p < 0.05$: background-color: red to yellow; fitted line (red) and R values)

5.1.5.3. Relationship between cell-line-specific parameters and the relative standard deviation of migration metrics

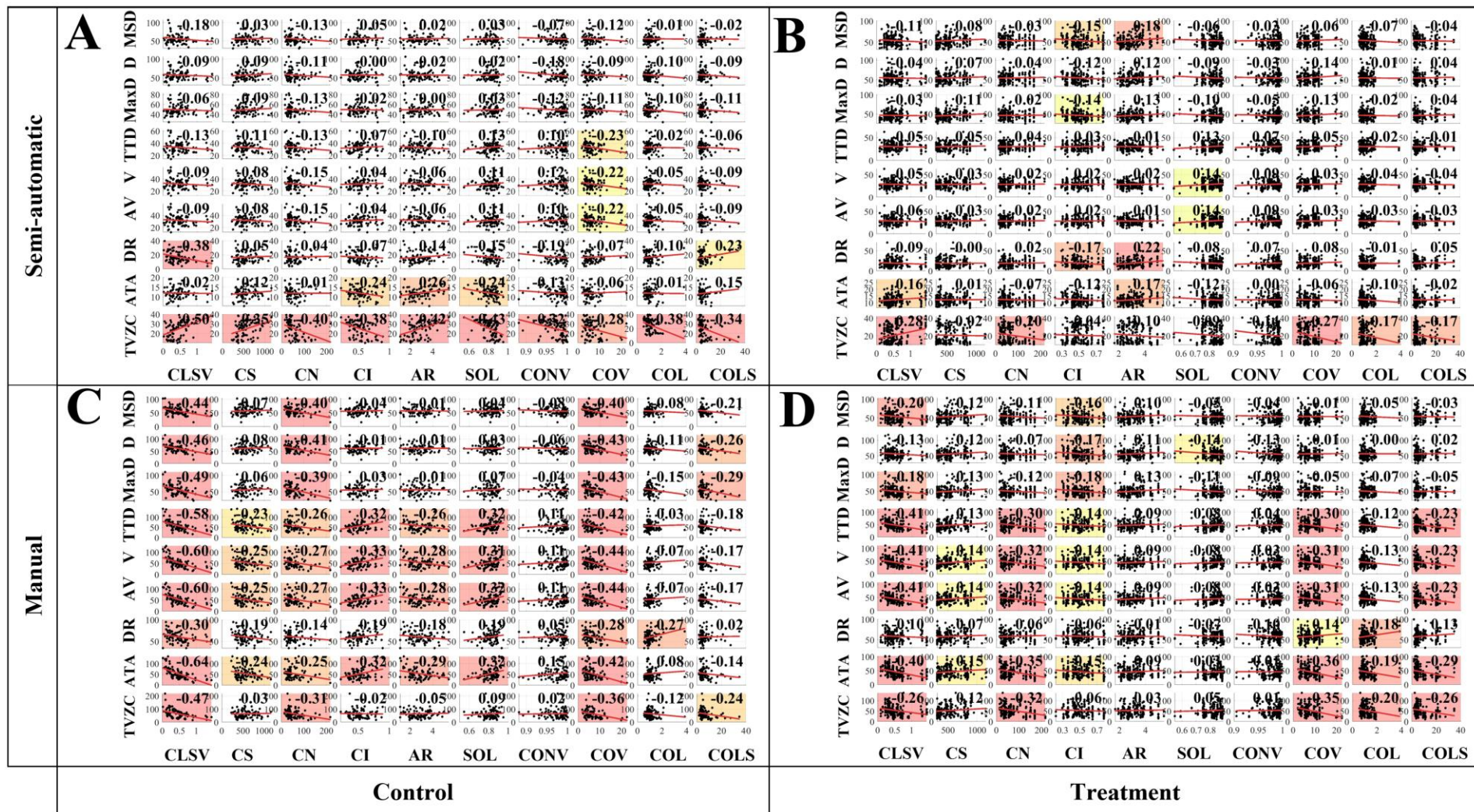
To determine whether the repeatability of the migration descriptors – quantified as the coefficient of variation (CV) – is influenced by intrinsic cellular properties, we calculated CV values for each migration parameter across all cell lines and treatment conditions. These CV values were then correlated with the cell-line-specific descriptors described earlier, and analyses were carried out separately for semi-automatic and manual tracking (Figure 17).

Across both control and treatment datasets (Figure 17A-B), CV values obtained using **semi-automatic tracking** exhibited minimal dependence on any of the cell line-specific descriptors. This indicates that the repeatability of migration parameters under this tracking approach is largely unaffected by cell-intrinsic morphological variation or density-related characteristics.

An exception to this trend was the TVZC, whose repeatability showed notable correlations with several cell-specific features, including CS, CI, SOL, and colony-forming properties (COL, COLS). This heightened sensitivity likely reflects the fact that TVZC captures fine-grained directional fluctuations, which are more susceptible to subtle variations in cell shape and local microenvironment.

In contrast, migration parameters derived from **manual tracking** displayed a markedly stronger dependence on intrinsic cell-line characteristics (Figure 17C-D). The CV values correlated robustly with CLSV, CN, and COV, and to a lesser extent with shape descriptors including CI, AR, SOL, and COLS.

This pattern likely stems from the inherently subjective nature of manual tracking: observers may subconsciously favor cells that are easier to follow, typically those that are larger, faster, or more isolated, thereby introducing systematic biases in measurement repeatability. As a result, variability in manual annotations becomes more strongly coupled to the underlying cellular phenotype.



5.2. Comparative analysis of *in vitro* migration assays

Having established how quantitative migration descriptors behave in 2D single-cell time-lapse assays – and how their sensitivity is influenced by treatment effects, tracking methodology, noise, and intrinsic cellular properties – we next sought to place these observations into a broader experimental context. Single-cell tracking offers a uniquely direct readout of migratory behavior, as it quantifies cell movement independently of proliferation, collective effects, or cell-cell interactions. In contrast, several widely used population-based migration assays inherently couple motility with additional biological processes.

Gap-closure assays, such as the scratch and zone-exclusion (Z-E) assays, measure the rate at which a cell population repopulates an initially cell-free area. Although frequently interpreted as migration assays, their readouts are strongly shaped not only by motility but also by proliferation dynamics, cell size, and contact-mediated behaviors. Moving toward more physiologically relevant conditions, the transwell assay represents a semi-3D approach in which cells migrate through membrane pores toward a chemoattractant. Here, cell size, shape, deformability, and the physical ability to traverse confined spaces become major determinants of the measured response.

To understand how these commonly applied assays relate to and diverge from 2D single-cell motility measurements, we systematically analyzed scratch, Z-E, and transwell assays across the same melanoma cell lines.

5.2.1. Single-cell migration dynamics

5.2.1.1. *Baseline single-cell migratory behavior of melanoma cell lines*

To initiate the comparative assessment of different migration assay formats, we first characterized the baseline 2D single-cell migratory behavior of the five melanoma cell lines. Guided by the systematic analysis of migration parameters presented in the previous sections, we selected a representative set of descriptors that capture complementary and biologically distinct aspects of cell motility. To ensure comprehensive coverage, at least one parameter from each major functional category – displacement-based, path-based, and directionality-based – was included (Figure 18).

Specifically, displacement-related behavior was quantified using mean squared displacement (MSD) and displacement (D), both evaluated through their area under the curve (AUC) to reflect cumulative progression over time. Path-based motility was represented by the average velocity (AV), while trajectory linearity was assessed using the directionality ratio (DR). This parameter set was chosen to provide an integrative overview of migration speed, net translocation, trajectory tortuosity, and directional stability across cell lines.

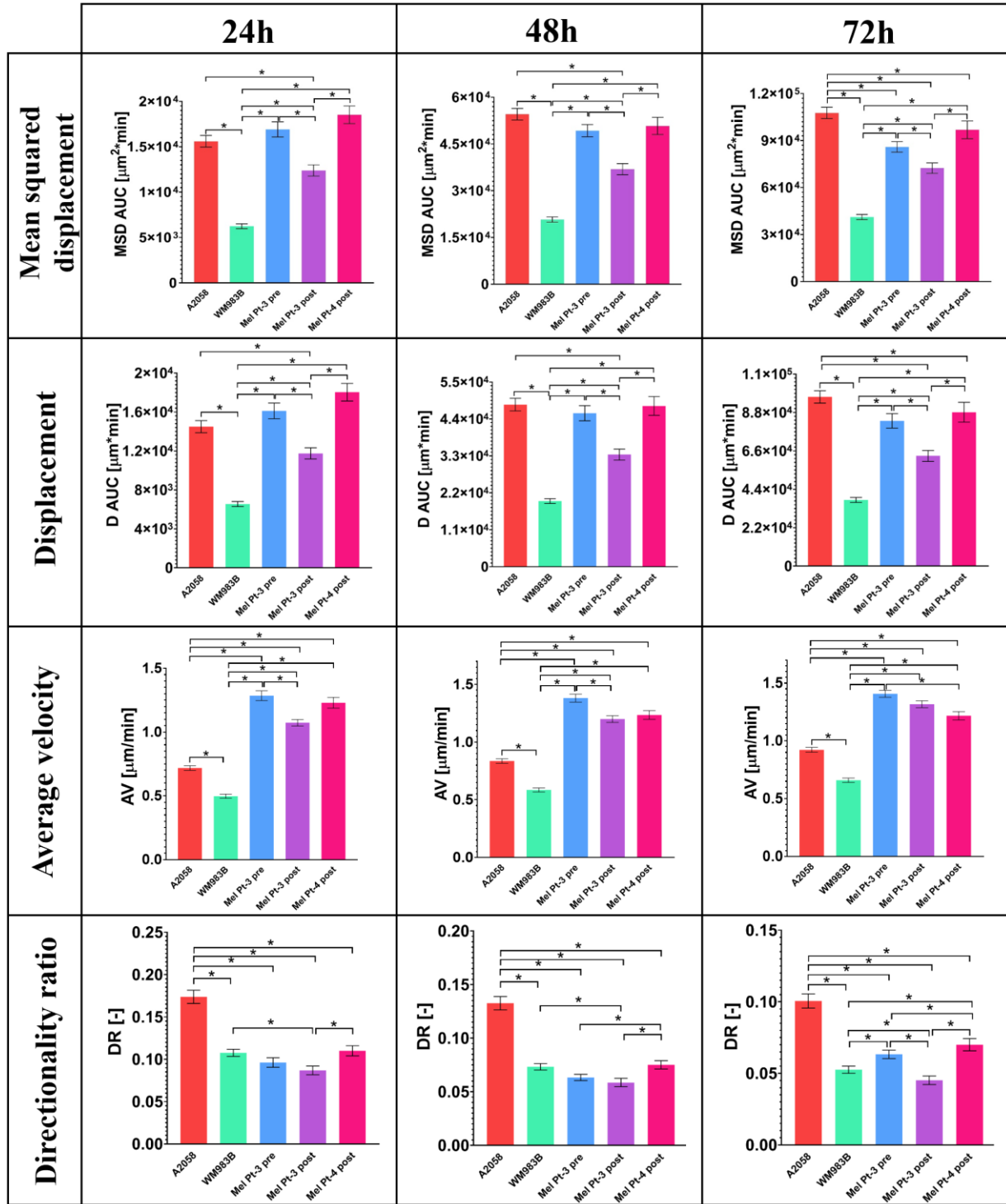


Figure 18. Single-cell migration dynamics of melanoma cell lines. Cells were tracked for 72 h using semi-automatic videomicroscopy. Mean squared displacement (MSD), displacement (D), average velocity (AV), and directionality ratio (DR) were quantified; AUC values were used for D and MSD. Data represent mean $\pm$ SEM ($N \approx 200$ –270 cells/condition). Kruskal–Wallis test with Dunn’s post hoc comparison; $p < 0.05$.

Mean squared displacement (MSD), which reflects the combined influence of motility speed and directional persistence, revealed marked temporal differences in migratory efficiency across the five lines (Figure 18, first row). At 24 h, Mel Pt-4 post cells displayed the highest MSD, closely followed by Mel Pt-3 pre. Although A2058 cells ranked third at early time points, their MSD increased steadily and surpassed both Mel Pt-4 post and Mel Pt-3 pre by 48 h, indicating a progressive enhancement of migration efficiency. Mel Pt-3 post cells exhibited moderate MSD values throughout, whereas WM983B cells consistently showed the lowest MSD, characteristic of slow, low-persistence motility. These trends illustrate that MSD, owing to its integration of displacement over time and relative insensitivity to short-term tracking noise, captures meaningful temporal changes in migration behavior that instantaneous parameters may fail to resolve.

Cumulative **displacement (D)** exhibited the most distinct progression patterns among cell lines (Figure 18, second row). Mel Pt-4 post cells showed the greatest net translocation during the first 24 h. However, A2058 cells continued to accumulate displacement prominently at later time points, ultimately surpassing all other lines. Both Mel Pt-3 pre and Mel Pt-3 post exhibited moderate, relatively stable displacement trajectories, while WM983B cells traveled the shortest distances overall. The divergence between early (24 h) and late (48-72 h) displacement rankings highlights that migration is highly dynamic, and that extended time-lapse observation is essential to accurately characterize a cell line's long-term motility phenotype.

Analysis of **average velocity (AV)** revealed that Mel Pt-3 pre cells migrated the fastest, followed by Mel Pt-3 post and Mel Pt-4 post cells, which exhibited comparable velocities (Figure 18, third row). A2058 cells displayed intermediate motility, whereas WM983B cells consistently showed the lowest average speed. Importantly, the velocity-based ranking did not fully align with the displacement outcomes, most notably for A2058 cells. This divergence highlights that velocity and displacement capture distinct aspects of migratory behavior and must be interpreted together to obtain a comprehensive view of cell motility.

The **directionality ratio (DR)** distinguished clear differences in trajectory linearity (Figure 18, fourth row). A2058 cells displayed the highest DR, indicating the most persistent and linear migration among the examined lines. This finding also explains the previously noted discrepancy between displacement-based parameters and AV in this cell line, as high directional persistence enables substantial net translocation despite only moderate average velocity. In contrast, WM983B,

Mel Pt-3 pre, Mel Pt-3 post, and Mel Pt-4 post cells exhibited lower DR values, reflecting more curved and less persistent trajectories.

5.2.1.2. Effects of nintedanib on single-cell migration dynamics – rationale for dose selection

Before evaluating how nintedanib modulates single-cell migratory behavior, we first established treatment concentrations that exert minimal effects on cell viability. To this end, nintedanib sensitivity was quantified in all five melanoma cell lines using a total-protein–based Sulforhodamine B (SRB) assay across a 1–8 μM concentration range. From these measurements, IC_{50} values were determined as the concentrations reducing total cell mass by 50% relative to untreated controls. In parallel, relative proliferation rates were calculated by comparing cell numbers at 24 h in the videomicroscopy recordings to their corresponding 0 h values (Section 4.3.3.2).

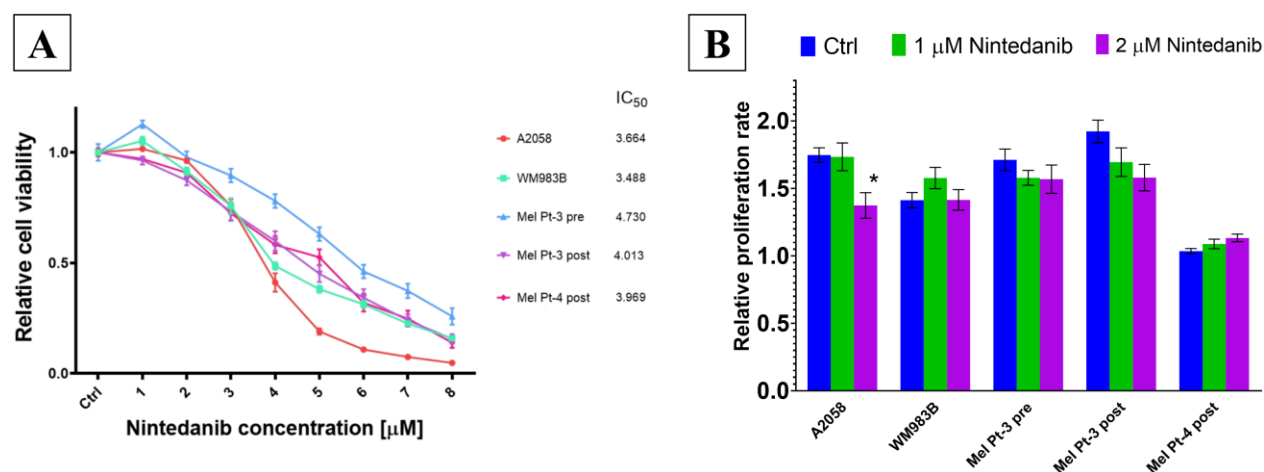


Figure 19. Assessment of nintedanib sensitivity across cell lines. A) Dose–response curves obtained from 72 h SRB assays across 1–8 μM nintedanib, with fitted IC_{50} values (mean $\pm$ 95% CI, three independent experiments). B) Proliferation rates measured under 1 μM and 2 μM nintedanib over 24 h (A2058, WM983B, Mel Pt-3 pre, Mel Pt-3 post) or 4 h (Mel Pt-4 post).

Across all cell lines, IC_{50} values exceeded both 1 μ M and 2 μ M concentrations, indicating that these doses fall within the sub-cytotoxic range. Because our objective was specifically to examine migration-related effects while avoiding confounding influences from drug-induced cytotoxicity or proliferation arrest, concentrations below the IC_{50} were selected. Consistent with this rationale, proliferation measurements extracted from time-lapse recordings confirmed that at 1-2 μ M, nintedanib induced only minimal changes in proliferation rates for most cell lines (Figure 19B). The only notable exception was A2058, which showed a detectable reduction in proliferation at 2 μ M, whereas all other lines remained largely unaffected.

Having established that 1 μ M and 2 μ M nintedanib act primarily as migration-modulating rather than cytotoxic doses, we proceeded to characterize their impact on 2D single-cell motility. As in the untreated baseline comparison, four complementary descriptors – MSD, D, AV, and DR – were quantified to capture distinct and mechanistically informative dimensions of cell migration (Figure 20).

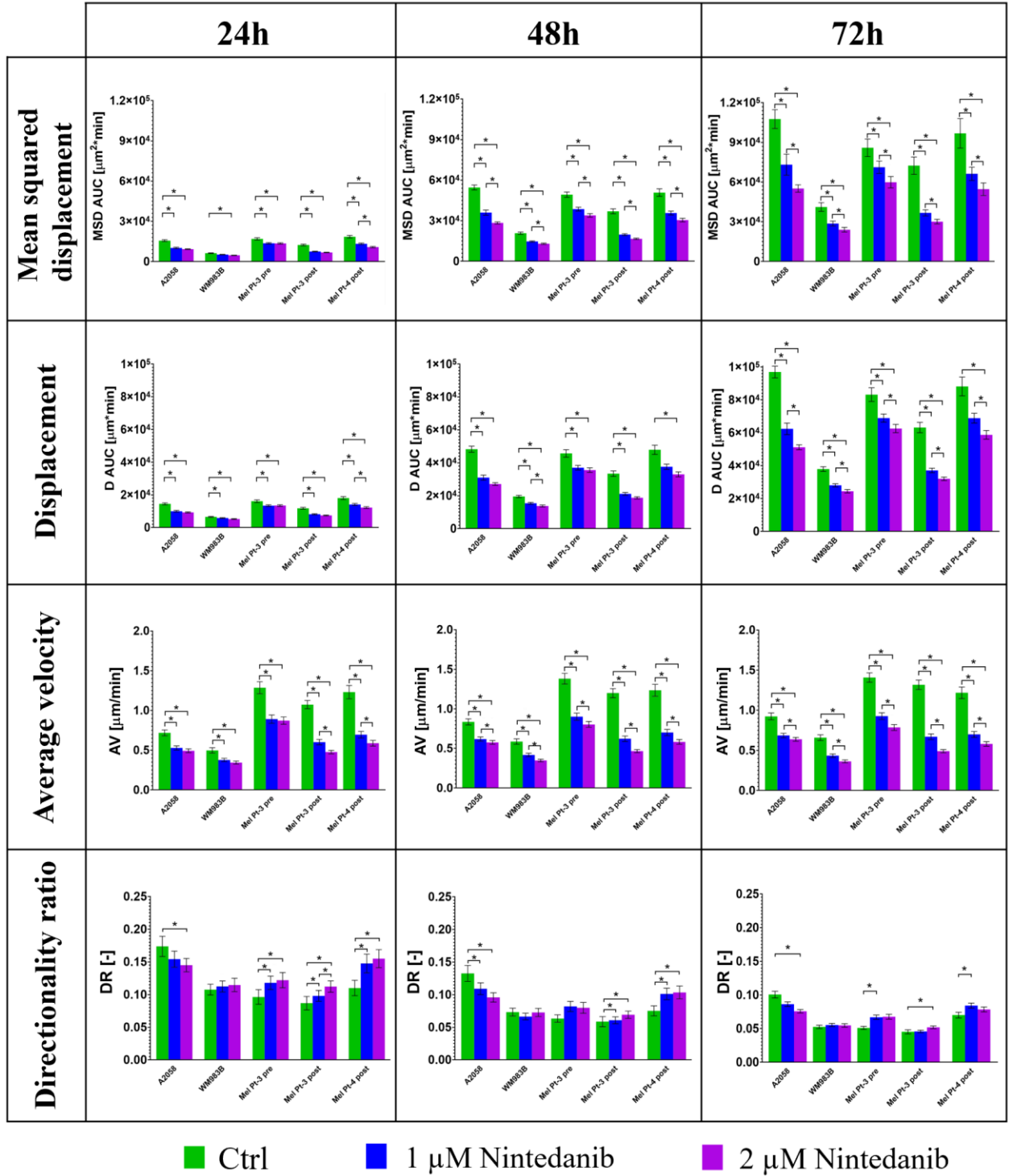


Figure 20. Evaluating nintedanib's effect on cell migration by single-cell tracking. Cells were treated with 1 or 2 μM nintedanib and monitored over 72 h using semi-automatic tracking. Migration parameters – MSD, D, AV, and DR – were extracted from x-y trajectories. For D and MSD, AUC values were used for quantitative comparison. Data represent mean $\pm$ 95% CI ($N \approx 240\text{--}340$ cells/condition; 3 plates $\times$ 3 wells $\times$ 2 fields). Statistical analysis: Kruskal–Wallis with Dunn's multiple-comparison test ($p < 0.05$).

To complement these summary metrics, the full temporal evolution of MSD and D is shown in Figure 21, illustrating the progressive development of inhibitory effects across the 24 hours time course.

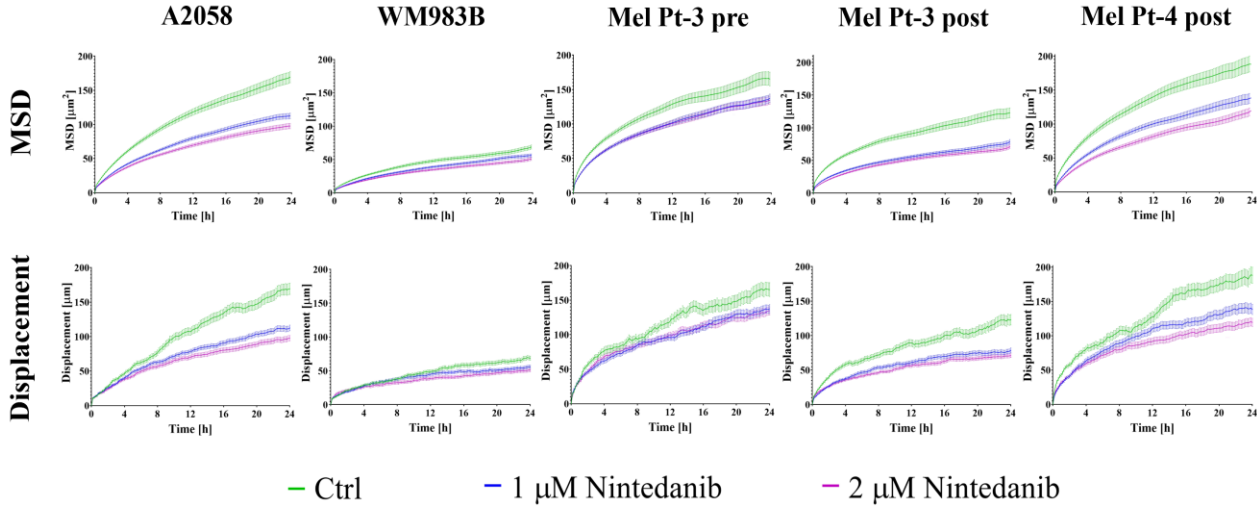


Figure 21. Time-resolved single-cell migration parameters under nintedanib treatment. Cells were treated with 1 or 2 μM nintedanib for 24 hours. Semi-automatic tracking was used to compute D and MSD curves for each cell line. Data represent mean $\pm$ SEM ($N \approx 240\text{--}340$ cells/condition).

Nintedanib induced a clear, dose-dependent reduction in **mean squared displacement (MSD)** across all melanoma cell lines (Figures 20 and 21). Significant decreases were already evident at 24 h for both concentrations, with further suppression emerging by 48-72 h (Figure 20). The smallest inhibition occurred in WM983B, the intrinsically least motile cell line, whereas Mel Pt-3 post cells showed the strongest reduction; its MSD at 72 h was approximately halved compared with untreated controls. Although A2058 cells were strongly affected relative to their own baseline, their MSD values under treatment remained comparable to or even higher than those of several other lines because their untreated motility substantially exceeded that of all other models.

Time-resolved MSD curves (Figure 21) confirmed that 2 μM consistently produced stronger and earlier declines, reflecting impaired long-range migration and diminished directional persistence.

Cumulative **displacement (D)** showed an inhibitory pattern that closely paralleled the MSD results. Both the dose dependence and temporal progression were highly similar, as expected given the strong coupling between these two displacement-based descriptors. D was consistently reduced at both 1 and 2 μM , with suppression deepening over time (Figures 20 and 21).

Nintedanib also significantly reduced **average velocity (AV)** in a dose-dependent manner; in most cell lines, this inhibition was more pronounced than the suppression observed for displacement-based parameters (Figure 20). A2058 cells, however, constituted a notable exception. Despite showing a strong reduction in displacement, their AV decreased only modestly, the second smallest decline across all lines (after WM983B). This decoupling demonstrates that velocity and displacement can be differentially affected by treatment, reinforcing earlier conclusions that migration parameters capture mechanistically distinct facets of motility. Importantly, the unique response pattern in A2058 supports our prior observation that parameter behavior is highly contingent on the specific cell line $\times$ treatment combination.

The effect of nintedanib on the **directionality ratio (DR)** was more heterogeneous than on the other parameters (Figure 20). In four of the five melanoma models, DR increased at 24 h, indicating straighter, more persistent migration early after treatment. This initial increase typically diminished by 48-72 h, suggesting a transient adaptation rather than a sustained directional phenotype. In contrast, A2058 cells showed a pronounced and progressive reduction in DR, indicating increased turning frequency and loss of directional persistence. This decrease in trajectory linearity provides a mechanistic explanation for their disproportionately large reduction in displacement despite only moderate velocity inhibition: erratic movement reduces the efficiency of net translocation, amplifying the functional impact of even subtle speed changes.

5.2.2. Gap closure dynamics in scratch and Z-E assays

5.2.2.1. *Baseline gap-closure behavior across melanoma cell lines*

The two population-based gap-closure assays – scratch and zone-exclusion (Z-E) – differ fundamentally in how the cell-free region is generated and maintained. These methodological differences strongly influence the reproducibility of the initial gap, the baseline variability, and ultimately the interpretability of closure kinetics. Prior to performing a quantitative comparison, it is essential to first illustrate the characteristic morphology and temporal evolution of the gaps generated by each assay type. To this end, representative videomicroscopic images from both the scratch and Z-E assays are shown for all five melanoma cell lines (Figure 22). In each panel, the initial gap boundaries at $t = 0$ are marked in red, while the corresponding boundaries at subsequent time points are outlined in green. These examples provide a visual overview of the intrinsic

geometric differences between scratch- and insert-generated gaps and highlight the distinct closure behaviors observed across cell lines.

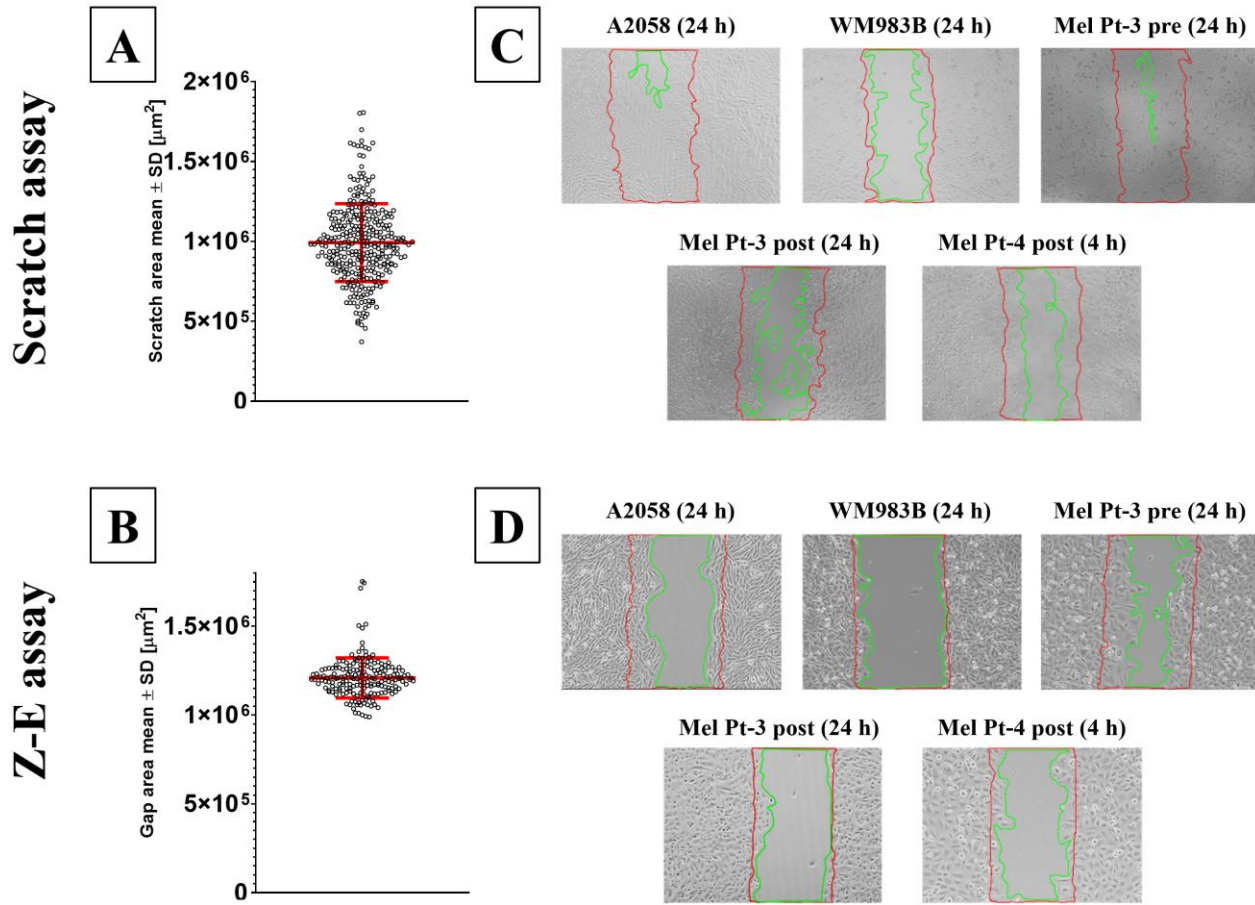


Figure 22. Baseline gap formation and representative gap-closure images in melanoma cell lines. A) Distribution of initial scratch areas ($n = 180$); red lines = mean $\pm$ SD. B) Distribution of initial Z-E areas created with silicone inserts ($n = 60$); red lines = mean $\pm$ SD. C) Scratch assay: scratches were manually introduced into confluent monolayers, and gap closure was monitored every 4 h for 72 h. Red boundaries mark the initial wound edges; green boundaries outline the remaining gap at later time points. D) Zone-exclusion assay: uniform circular gaps were produced using silicone inserts and imaged every 4 h for 72 h. Red boundaries denote initial gap edges following insert removal; green boundaries mark the gap contours during closure.

In the **scratch assay**, manual gap generation using a pipette tip resulted in substantial variability in initial gap size and frequently produced irregular, frayed wound edges (Figure 22C). Across the 180 scratch regions analyzed, the starting areas were widely dispersed around the mean, reflecting differences in the applied pressure, angle of scratching, and tip positioning. Although gaps outside the mean $\pm$ 2 SD range were excluded, considerable heterogeneity persisted (Figure 22A),

consistent with known limitations of pipette-based scratch generation. Gap-closure dynamics differed markedly among cell lines. Mel Pt-4 post cells closed the scratch most rapidly, completing full closure within 24 h; hence, an early time point (4 h) is shown in Figure 22 to allow visualization of the gap. At the opposite end of the spectrum, WM983B cells exhibited minimal closure during the first 24 h, consistent with their intrinsically low migratory capacity in single-cell assays (Figure 22C).

By comparison, in the **zone-exclusion (Z-E) assay**, cell-free gaps were generated through removal of silicone inserts, yielding highly uniform, smooth-edged exclusion zones (Figure 22D). Owing to the standardized geometry of the inserts, initial gap sizes exhibited excellent reproducibility: of 60 regions analyzed, only two fell outside the mean $\pm$ 2 SD range, and exclusion of these outliers resulted in a nearly homogeneous distribution of starting gap areas (Figure 22B). Across cell lines, gap-closure patterns largely paralleled those observed in the scratch assay. However, closure tended to occur more slowly in the Z-E assay, consistent with the larger starting gap and the absence of debris-induced cues that may influence collective migration in the scratch setting (Figure 22D).

To quantitatively evaluate migratory capacity in both assays, the relative gap area was calculated at each time point by normalizing the measured cell-free area to its corresponding 0 h value. Figure 23 summarizes the distribution of initial gap sizes for each assay type, the temporal evolution of relative gap closure, and the resulting AUC values that reflect cumulative closure efficiency.

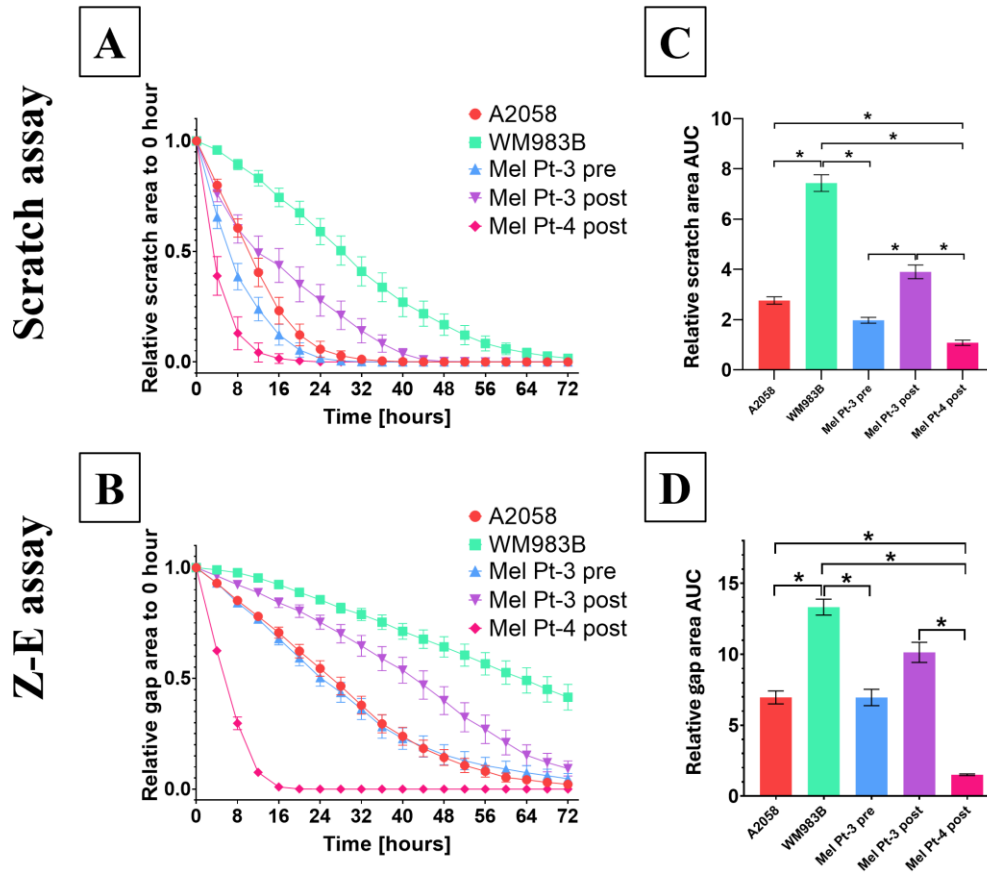


Figure 23. Quantitative assessment of gap-closure dynamics in scratch and Z-E assays. A) Relative scratch area over time for each cell line (mean $\pm$ SEM; N = 24 across four independent experiments). B) Relative Z-E area over time (mean $\pm$ SEM; N = 12 across four independent experiments). C) Area under the curve (AUC) for relative scratch-area reduction (mean $\pm$ SEM). D) AUC for relative Z-E-area reduction (mean $\pm$ SEM). Replicates with initial gap sizes outside the mean $\pm$ 2 SD range were excluded. Statistical analysis: Kruskal–Wallis with Dunn’s multiple-comparison test ($p < 0.05$).

In the **scratch assay**, pronounced cell line-specific differences were observed in gap-closure kinetics. Mel Pt-4 post cells displayed the fastest closure, fully sealing the wound within approximately 16 h. A2058 and Mel Pt-3 pre cells also exhibited rapid closure, completing the scratch by ~28 h. Mel Pt-3 post required ~44 h to reach complete closure. WM983B, consistent with its known low motility, displayed only gradual closure and achieved full gap resolution only near the 72 h endpoint (Figure 23A).

These kinetics were reflected in the corresponding AUC values: faster-migrating lines exhibited lower AUCs, indicative of more rapid and efficient reduction of the relative scratch area over time (Figure 23C).

Gap-closure behavior in the **Z-E assay** followed similar cell-line-specific trends. Mel Pt-4 post cells again migrated most efficiently, closing the Z-E gap within ~16 h. A2058 and Mel Pt-3 pre cells substantially reduced the gap between 28-72 h, although the Z-E gap consistently closed more slowly than scratches. Mel Pt-3 post cells demonstrated partial but incomplete closure by 72 h, while WM983B showed minimal progress throughout the experiment (Figure 23B).

The slower overall progression in the Z-E assay, compared with the scratch assay, was reflected in higher AUC values (Figure 23D). This difference is likely attributable to the larger, more geometrically regular initial gap produced by silicone inserts and to procedural distinctions: unlike scratch assays, Z-E assays do not involve mechanical injury or the presence of cellular debris, both of which can transiently modulate migratory behavior.

5.2.2.2. Impact of nintedanib on collective gap-closure dynamics

Building upon the baseline characterization of gap-closure behavior in both scratch and Z-E assays, we next examined how nintedanib modulates collective migration in these population-based systems. To first illustrate the qualitative impact of treatment on gap morphology and closure progression, representative images of nintedanib-treated scratch and Z-E assays are shown in Figure 24. As before, initial gap boundaries are marked in red, while green outlines trace the remaining cell-free area at subsequent time points, allowing direct visual comparison with untreated conditions.

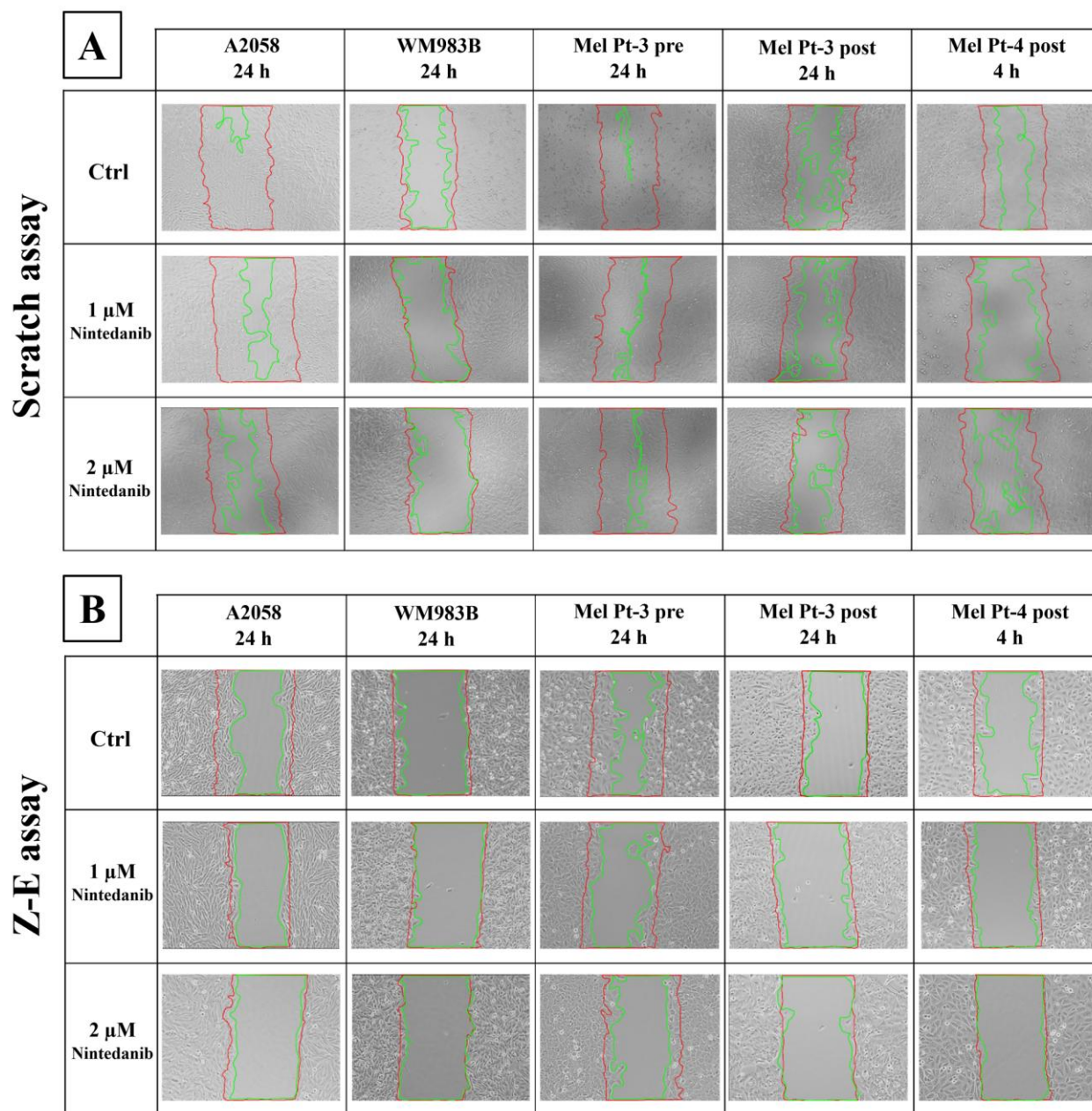


Figure 24. Representative images of gap-closure assays in nintedanib treatment. A) Scratch assay: Scratches were manually introduced into confluent monolayers, and gap closure was monitored every 4 hours for 72 hours. Red lines mark the initial wound edges; green lines indicate the boundaries of the remaining cell-free area over time. B) Zone-exclusion assay: Cell-free gaps were generated using silicone inserts and imaged at 4-hour intervals for 72 hours. Red lines denote initial gap edges after insert removal; green lines outline the gap boundaries at subsequent time points.

As in the baseline measurements, Mel Pt-4 post cells closed the scratch extremely rapidly, reaching full closure within 24 h; therefore, an earlier time point (4 h) is shown to enable

visualization of the gap. Across the remaining cell lines, nintedanib produced varying degrees of inhibition. In all responsive lines, the cell-free area remained visibly larger under treatment compared with the corresponding controls, reflecting reduced collective migration in a largely dose-dependent manner. In line with its intrinsically low motility, WM983B showed only modest visible effects in these representative images, consistent with its overall limited responsiveness to the drug. In the Z-E assay, gap closure progresses more slowly, and the initial gaps are larger and geometrically more uniform, making treatment-induced differences less apparent at the early time points displayed here. Nevertheless, subtle widening of the gap under nintedanib treatment can still be observed.

Although these representative images highlight treatment-induced delays in closure, quantitative analysis is necessary to accurately assess the magnitude of inhibition. Therefore, the relative gap area was calculated at each time point using the same normalization strategy applied in untreated conditions. Treatment effects were evaluated by examining the temporal closure trajectories and their corresponding AUC values (Figure 25).

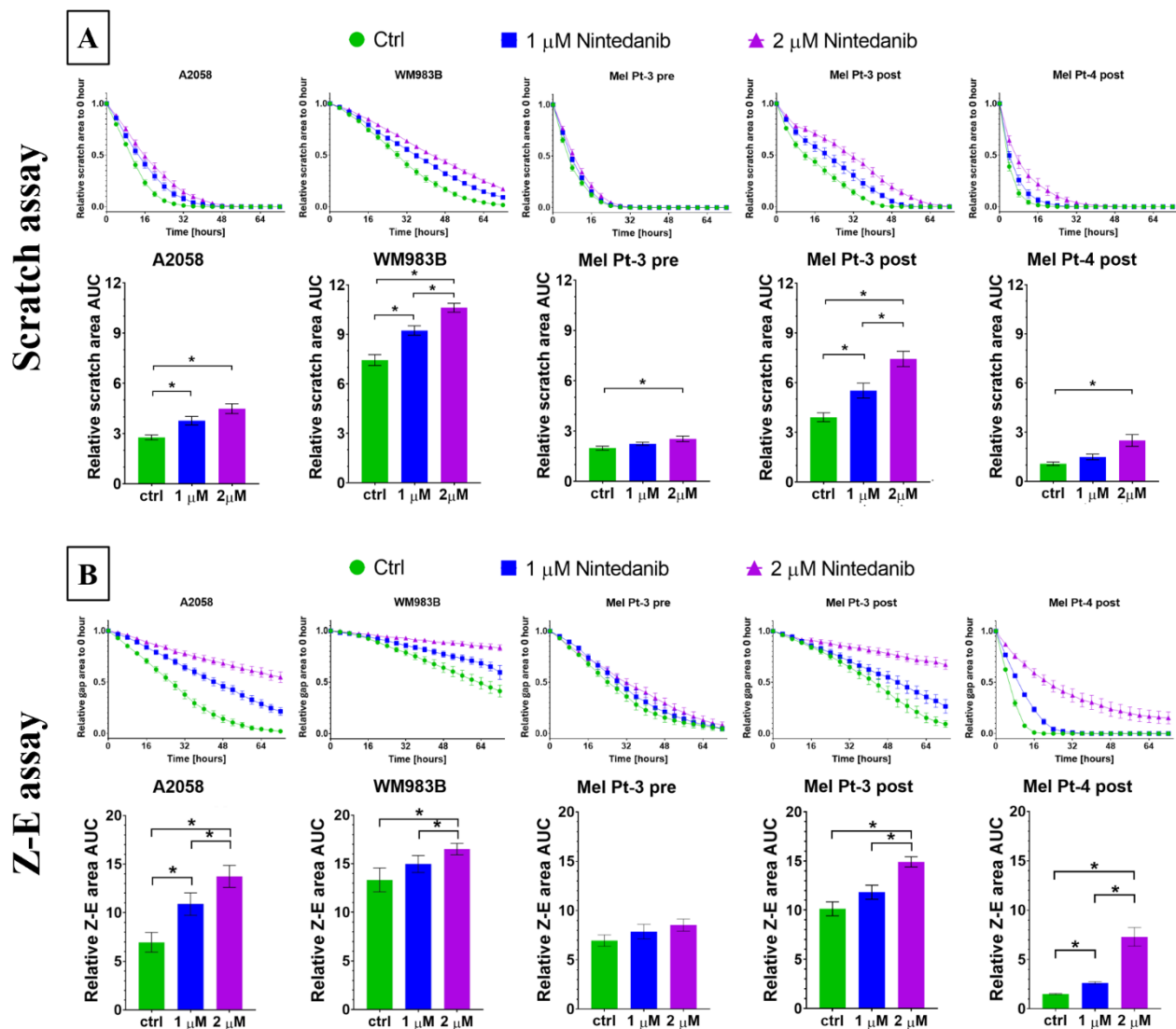


Figure 25. Evaluating nintedanib's effect on gap-closure assays. Relative scratch (A) and Z-E (B) gap areas were normalized to the initial gap size at $t = 0$ and tracked for 72 h. AUC values quantify closure efficiency under control, 1 μ M, and 2 μ M nintedanib conditions. Data: mean $\pm$ 95% CI; scratch: $N = 24$; Z-E: $N = 12$ across four independent experiments. Outliers outside ± 2 SD of the initial gap size were excluded. Statistical analysis: Kruskal–Wallis with Dunn's multiple-comparison test ($p < 0.05$).

In the **scratch assay**, nintedanib reduced collective migration across all melanoma cell lines, although the detectability of inhibition was strongly shaped by the intrinsic closure dynamics. Mel Pt-4 post was the fastest-closing line, and although it exhibited limited inhibition overall, the higher dose (2 μ M) produced a clearly detectable delay, whereas 1 μ M showed virtually no effect due to the extremely short observation window before full closure. Mel Pt-3 pre, the second fastest migrating line, displayed the weakest measurable treatment response. This pattern indicates that,

in this cell line, the limited detectability of inhibition reflects not only the rapid closure that restricts the time available to observe drug effects but also an intrinsically low responsiveness to nintedanib (Figure 25).

A2058 closed the scratch by approximately 32 h, allowing sufficient temporal resolution to distinguish between concentrations, and clear dose-dependent inhibition was observed. In the slower-closing lines (Mel Pt-3 post and WM983B), both nintedanib concentrations significantly delayed closure, with 2 μ M consistently exerting stronger inhibition. These findings collectively indicate that the sensitivity of the scratch assay to pharmacological inhibition depends heavily on the baseline closure rate: rapidly closing cell lines provide a narrow window for detecting treatment effects, whereas slower-closing lines permit more robust resolution of dose-dependent inhibition.

In the **zone-exclusion (Z-E) assay**, the inhibitory effects of nintedanib were more consistently detectable across most melanoma cell lines. Mel Pt-3 pre again showed only minimal response at either drug concentration, closely paralleling the weak inhibition observed in the scratch assay. This concordant outcome across both assay formats suggests that the limited treatment effect in this cell line is not merely the consequence of rapid gap closure restricting the temporal window for inhibition to manifest, but also reflects an intrinsically low biological responsiveness to nintedanib (Figure 25).

In the remaining four cell lines, the extended closure time permitted clear discrimination of dose-dependent effects. Treatment with 2 μ M nintedanib consistently produced marked delays, while 1 μ M exerted moderate but measurable inhibition. Compared to the scratch assay, the Z-E format captured a more uniform dose response. This improved sensitivity likely stems from the larger, geometrically consistent initial gap, which provides a broader dynamic range and avoids the rapid saturation characteristic of manually created wounds.

In summary, across both assay formats, nintedanib reduced collective migration in a dose-dependent manner, with inhibitory effects detectable as early as 24 h. Importantly, the Z-E assay offered superior sensitivity overall. As described in the baseline characterization, Z-E gaps are larger, more geometrically uniform, and close more slowly than scratches, providing a broader temporal window for drug effects to manifest. Moreover, unlike scratch assays, Z-E assays avoid confounding influences associated with mechanical wounding, such as the release of intracellular content or debris from damaged cells, which can transiently modulate motility and obscure

pharmacological inhibition. These methodological advantages contribute to the clearer detection of dose-dependent responses in the Z-E format.

5.2.3. *Quantification of chemotactic migration using the transwell assay*

5.2.3.1. *Baseline chemotactic migration capacity across melanoma cell lines*

To evaluate directed, chemotaxis-driven migration under a serum gradient, transwell assays were performed using 8- μ m pore inserts. Cells were seeded into serum-free medium in the upper chamber and allowed to migrate toward 1% FBS in the lower chamber. Migration was quantified after 24 hours for all cell lines, except for Mel Pt-4 post, which required a shorter incubation period (4 hours) because rapid membrane coverage impeded reliable quantification at later time points. To enable direct comparison between cell lines, migration rates were calculated by normalizing the number of migrated cells to the corresponding incubation time. Representative microscopy images of the membrane undersides are also provided to illustrate qualitative differences in migratory behavior (Figure 26).

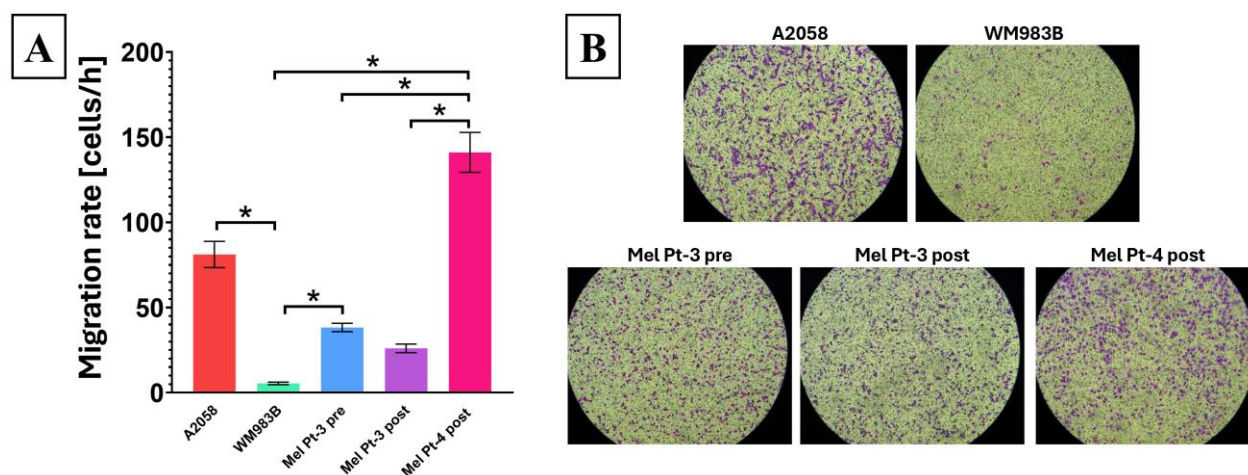


Figure 26. Chemotactic migration measured by the transwell assay. A) Migration rates toward 1% serum through 8- μ m pores after 24 hours (4 hours for Mel Pt-4 post). Data represent mean $\pm$ SEM from ≥ 3 independent experiments (minimum N = 9). Statistical analysis: Kruskal–Wallis with Dunn’s multiple-comparison test ($p < 0.05$). B) Representative images of migrated cells on the underside of the membrane (24 hours for all lines except Mel Pt-4 post, measured at 4 hours).

Consistent with the strong directional and mechanical constraints of the assay, the transwell setup revealed the largest divergence in migratory behavior across the evaluated cell lines,

highlighting phenotypic differences that were less pronounced in gap-closure or 2D single-cell assays (Figure 26A-B). Mel Pt-4 post cells displayed the highest migratory capacity: even within the shortened 4-hour incubation, they produced a dense, nearly confluent layer of cells on the underside of the membrane, as reflected both in quantitative measurements and in the representative microscopy images. Among the cell lines assessed over 24 hours, A2058 cells exhibited the most efficient chemotactic migration, followed in decreasing order by Mel Pt-3 pre, Mel Pt-3 post, and finally WM983B cells. These qualitative trends observed in the images closely paralleled the quantitative migration rates, further confirming the robustness of the assay readout.

5.2.3.2. Nintedanib-induced modulation of chemotactic transwell migration

The impact of nintedanib on chemotaxis-driven migration was next evaluated using the same transwell system. Cells were treated with either 1 or 2 μ M nintedanib and seeded into the serum-free upper chamber, while the lower chamber contained 1% serum supplemented with the corresponding drug concentration. Migration was quantified after 24 hours (or 4 hours for Mel Pt-4 post due to rapid membrane coverage), and treatment effects were expressed as the ratio of migrated treated cells relative to untreated controls (Figure 27).

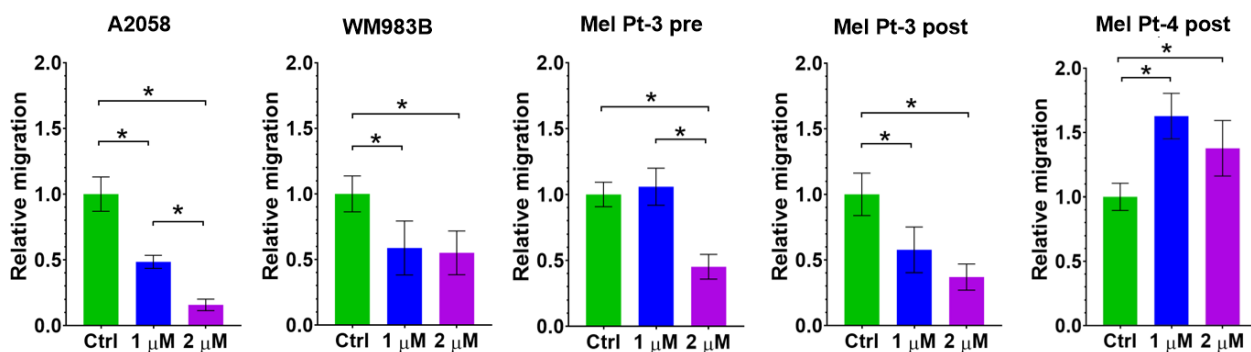


Figure 27. Nintedanib-mediated inhibition of chemotactic migration in the transwell assay. Cells were seeded in serum-free medium containing 1 or 2 μ M nintedanib and allowed to transmigrate toward 1% serum supplemented with the same drug concentration. Migration was assessed after 24 hours (A2058, WM983B, Mel Pt-3 pre, Mel Pt-3 post) or 4 hours (Mel Pt-4 post). Relative migration was calculated as treated/control. Data represent mean $\pm$ 95% CI from ≥ 3 independent experiments (minimum N = 9). Statistical analysis: Kruskal–Wallis with Dunn’s multiple-comparison test ($p < 0.05$).

As observed in the baseline assessment, the transwell assay yielded the most heterogeneous responses to nintedanib across the migration platforms examined. Nintedanib significantly reduced

chemotactic migration in A2058, WM983B, and Mel Pt-3 post cells at both concentrations. A2058 and Mel Pt-3 pre exhibited clear dose-dependent inhibition, although migration of Mel Pt-3 pre was unaffected at 1 μ M.

A notable exception was Mel Pt-4 post; in contrast to results from single-cell and gap-closure assays, this cell line showed an increase in transwell migration under both nintedanib concentrations. This unexpected behavior suggested the possibility of a treatment-induced morphological shift that facilitates more efficient passage through the 8- μ m pores.

To explore this hypothesis, we examined cell size (CS) and circularity (CI) under control and nintedanib-treated conditions after 24 hours (or after 4 hours in Mel Pt-4 post), using measurements derived from single-cell videomicroscopy (Figure 28). As detailed earlier, these recordings provide morphology measurements that are not confounded by cell-cell contacts or local density effects.

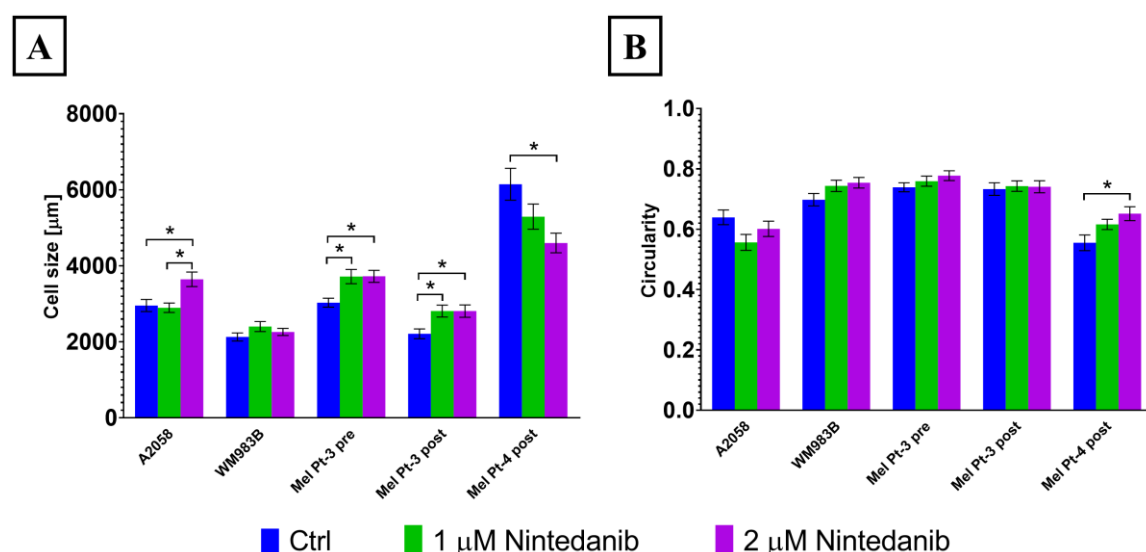


Figure 28. Effects of nintedanib on cell size and circularity. A) Comparison of control cell size (CS) with values under 1 or 2 μ M nintedanib at 24 h (A2058, WM983B, Mel Pt-3 pre, Mel Pt-3 post) and 4 h (Mel Pt-4 post). B) Comparison of control cell circularity (CI) with values under 1 or 2 μ M nintedanib at the same time points.

Across most cell lines, nintedanib treatment induced a modest increase in CS with minimal accompanying changes in CI. Such enlargement would be expected to make passage through the narrow pores more difficult, further amplifying the inhibitory effect reflected in the relative migration values compared with the other assay formats. In contrast, Mel Pt-4 post cells exhibited a strikingly different response, showing a reduction in CS together with increased CI, resulting in

smaller, rounder cells under treatment. Such a morphological shift would enhance the ability of these cells to traverse the narrow pores of the transwell membrane, thereby explaining the apparent increase in migration despite the drug's general anti-migratory profile (Figure 28).

Altogether, these findings highlight a key limitation of the transwell system. The measured "migration rate" reflects not only motile behavior but also physical attributes such as cell size, shape, and deformability, which may themselves be altered by treatment. Consequently, treatment-induced increases or decreases in transwell migration cannot be interpreted solely as changes in intrinsic motility without parallel morphological evaluation.

5.2.4. Cross-assay comparative evaluation of migratory behavior

5.2.4.1. Comparative analysis of baseline migration outputs across assay formats

Although each migration assay captures a distinct aspect of cell motility, it remained unclear whether these approaches provide consistent information regarding the relative migratory capacity of the cell lines. To address this, we next compared the performance of the five melanoma cell lines in the control condition across all assay types: single-cell tracking, scratch assay, Z-E assay, and transwell migration. Our aim was not only to evaluate absolute migration outcomes within each platform, but also to determine whether the assays yield comparable rank-order relationships between cell lines. Such cross-assay ranking consistency is particularly relevant when selecting experimental approaches or interpreting results from different migration platforms.

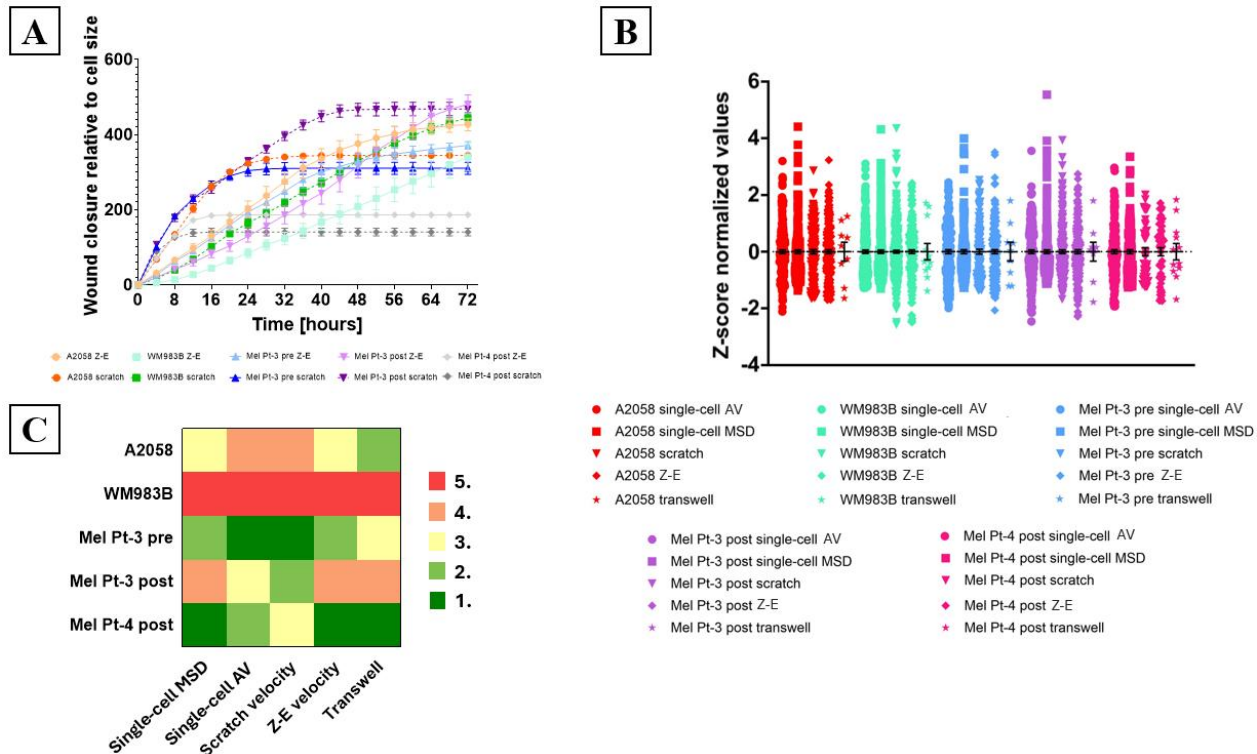


Figure 29. Comparison of the different migration assays. A) Wound closure normalized to cell size ($(A_0 - A_t) / A_{cell}$) in scratch and zone-exclusion (Z-E) assays (mean $\pm$ SEM). B) Z-score-normalized 24-hour migration metrics derived from single-cell MSD and AV, scratch assay closure velocity, Z-E closure velocity, and transwell migration rates. C) Heat map summarizing relative migration rankings across the five assay formats at 24 hours.

Because gap-closure kinetics depend not only on intrinsic migratory capacity but also on initial gap dimensions and the physical size of the cells populating the wound edge, the relative **gap-area curves were normalized to the mean cell size (CS)** obtained from the single-cell migration experiments. This normalization provides a size-independent descriptor of collective migration (Figure 29A). The normalized metric increases over time as the cell-free area diminishes: cell lines with higher migratory potential show an earlier and steeper rise, followed by a plateau as closure nears completion.

Importantly, even after normalization, the distinction between the two assay formats remained evident. The scratch assay consistently exhibited faster closure dynamics than the Z-E assay. However, normalization also altered the relative ordering of cell line-specific closure capacities, indicating that differences in cell size substantially influenced the unnormalized gap-closure trajectories. This highlights the value of incorporating cell-size normalization when interpreting

migration-driven gap-closure measurements, as it provides a more size-independent assessment of motility. Nevertheless, it should be noted that proliferation dynamics continue to contribute to closure behavior and cannot be fully accounted for by size normalization alone.

To derive a CS-independent measure of closure rate, we quantified the slope of the exponential growth phase of each normalized curve by fitting a linear model to its initial rising segment. These size-corrected closure velocities were then used to generate a comparative ranking of the wound-healing capacities of the five melanoma cell lines across both assay formats (Figure 29C). Because this ranking provides a clearer basis for interpreting assay-specific behaviors, the detailed analysis is presented alongside the results shown in Figure 29C.

To evaluate **reproducibility** and the internal distribution of migration values within each assay type, all migration metrics were transformed using **Z-score normalization** (Figure 29B). This approach expresses each observation as the number of standard deviations from the mean of its respective assay, thereby standardizing assays with different measurement units and ranges, reducing the influence of absolute scale differences, and enabling comparison of relative variability within each method.

Importantly, Z-score normalization does not allow direct comparison across assay formats, because the underlying measurements represent fundamentally different biological aspects (e.g., chemotaxis, gap closure, tortuosity, free 2D motion).

After normalization, variability between methods and cell lines appeared modest. Interestingly, Mel Pt-4 cells displayed the smallest deviation from the assay means across most methods, possibly reflecting their intrinsically consistent migratory performance.

To directly compare migratory capacity across distinct assay types, we generated **relative migration rankings** based on 24-hour metrics: single-cell mean squared displacement (MSD AUC), single-cell average velocity (AV), scratch closure velocity, Z-E closure velocity, and transwell migration rate (Figure 29C). This ranking approach enabled a direct evaluation of cell-line ordering across assays despite the differing measurement scales.

We first considered the two single-cell parameters – MSD and AV – which were derived from the same underlying time-lapse recordings. As discussed in earlier sections, these metrics capture mechanistically distinct aspects of migration. MSD depends strongly on both motility and directional persistence, whereas AV reflects instantaneous step-to-step speed and is largely

direction-independent. Accordingly, the resulting rankings differed markedly between these two descriptors.

Turning to population-based assays, we next examined how normalization to cell size affected the rankings obtained from the scratch and Z-E assays. After normalization, the Z-E-based ranking remained largely unchanged relative to the non-normalized curves, indicating that Z-E closure kinetics are only modestly influenced by intrinsic differences in cell size (CS). This is likely because Z-E gaps are larger, more uniform, and often remain partially open even at 72 h, reducing the impact of local crowding or cell packing density. In contrast, the scratch-assay ranking changed substantially after CS normalization. Scratch wounds close rapidly and involve narrow migration fronts where larger cells occupy a disproportionate fraction of the available space. As a result, closure kinetics in the scratch format are more sensitive to cell size, both because larger cells span a greater fraction of the wound and because full closure can occur quickly, limiting the assay's dynamic range. This explains why, even though the scratch and Z-E assays share the same conceptual readout (gap closure), their normalized rankings diverged.

Interestingly, the Z-E ranking most closely aligned with single-cell MSD, with both methods identifying Mel Pt-4 post as the fastest cell line, followed by Mel Pt-3 pre and then Mel Pt-3 post. By contrast, the scratch-assay ranking aligned more closely with AV. Consistent with this, Mel Pt-3 pre was ranked as the fastest line by AV and by the scratch assay, while Mel Pt-3 post fell lower in both rankings.

These comparisons allow us to revisit a key message from our single-cell analyses: different migration descriptors do not provide interchangeable readouts of motility. Despite being extracted from identical trajectories, they often yield divergent rankings, whereas migration assays based on entirely different methodologies show greater agreement. Thus, the variability introduced by the choice of parameter can equal or exceed the variability introduced by the choice of assay. This highlights the need for deliberate selection and interpretation of quantitative descriptors in single-cell migration studies.

The transwell assay produced rankings most similar to Z-E and MSD. Mel Pt-4 post again appeared as the most migratory line, while the ordering of A2058 and Mel Pt-3 pre shifted relative to 2D assays, likely reflecting size- and morphology-dependent effects on pore traversal.

Across all five metrics and all four assay formats, WM983B was consistently identified as the least migratory cell line, demonstrating extremely low motility in both single-cell and population-based contexts.

5.2.4.2. Comparison of the nintedanib treatment results of the migration assays, machine learning-based assay comparison

To systematically assess how effectively each migration assay detects nintedanib-induced suppression of cell motility, we calculated a unified inhibition metric (Inhibition%, Eqs. (2), (5), (6)) for all cell lines and assay formats. This metric quantifies the relative reduction in migratory output compared with untreated controls, enabling direct cross-assay comparison despite the fundamentally different biophysical principles underlying single-cell tracking, gap-closure assays, and transwell migration. Higher Inhibition% values reflect stronger drug-induced impairment of migration (Figure 30A). To complement this quantitative evaluation, we implemented a machine-learning approach to determine how well each assay discriminates between control, 1 μ M, and 2 μ M nintedanib conditions. A random forest classifier was trained on quantitative features extracted from single-cell tracking, scratch, and Z-E assays; the transwell assay was excluded because its endpoint-only readout does not provide the temporal information needed for feature construction. The model was trained using the 15 most discriminative features and evaluated using stratified cross-validation to quantify classification performance (Figure 30B).

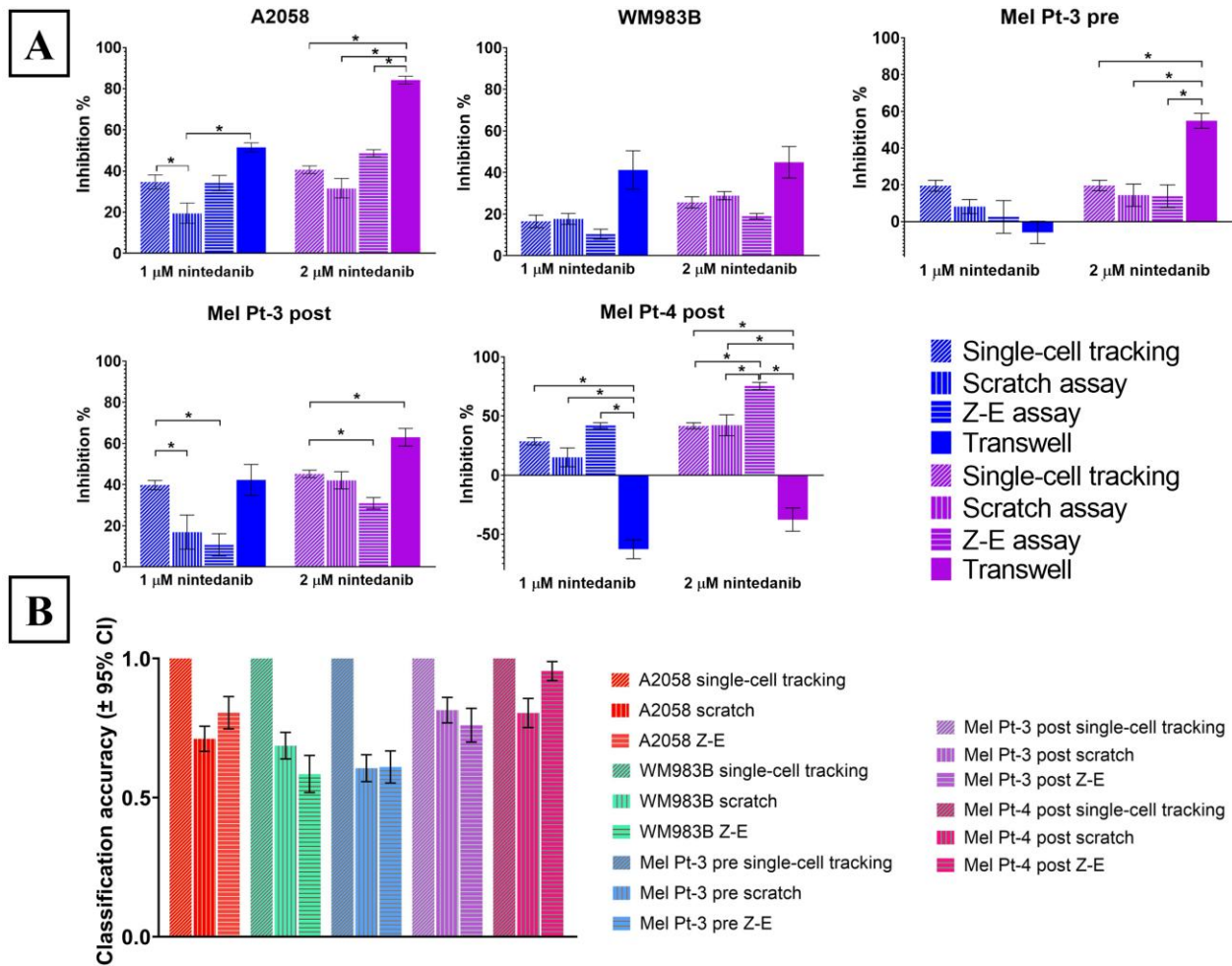


Figure 30. Comparison of migration assays under nintedanib treatment. A) Inhibition% values were computed using: 24-h MSD AUC for single-cell tracking; AUC of relative scratch-area reduction for the scratch assay; AUC of relative Z-E–area reduction for the zone-exclusion assay; and relative migrated cell number (treated/control) for the transwell assay. Data = mean $\pm$ SEM from ≥ 3 independent experiments. Kruskal–Wallis with Dunn’s multiple-comparison test ($p < 0.05$). B) Classification accuracy of a random forest model trained to distinguish control, 1 μ M, and 2 μ M nintedanib-treated samples based on quantitative features extracted from single-cell tracking, scratch, and Z-E assays. Error bars: 95% CI from cross-validation.

Examining inhibition patterns across the four migration assays (**Inhibition%**), nintedanib exerted migration-inhibitory effects across all melanoma cell lines in every experimental system except for a single assay-cell line combination: Mel Pt-4 post in the transwell setup, where an unexpected increase in migration was detected (Figure 30A).

Notably, in the single-cell tracking assay, the lower concentration (1 μ M) often produced disproportionately larger differences compared with 2 μ M, indicating that these cells were already highly sensitive to minimal nintedanib exposure; this heightened sensitivity may amplify detectable differences in migratory behavior relative to the other assays. When comparing the single-cell migration results to the two gap-closure assays, the degree of inhibition varied modestly and in a cell-line-dependent manner. In A2058 cells, inhibition at 1 μ M appeared more pronounced than in the scratch assay. Similarly, in Mel Pt-3 pre cells, inhibition tended to be higher than in either gap-closure assay, albeit without reaching statistical significance. For Mel Pt-3 post cells, the detected inhibition was again greater relative to both gap-closure assays. In contrast, for Mel Pt-4 post cells, inhibition measured in the Z-E assay fell below that observed in the scratch assay.

Between the two gap-closure assays, the most consistent divergence was again seen in Mel Pt-4 post cells, where the Z-E assay repeatedly revealed stronger inhibition than the scratch assay. This pattern likely reflects assay-intrinsic geometric factors: the larger initial gap and longer closure time in the Z-E system require more extensive collective migration, meaning that any reduction in migratory capacity becomes more pronounced as more cells must contribute to gap narrowing. Consequently, the inhibitory effect of nintedanib – particularly in cell lines with inherently larger morphology, such as Mel Pt-4 – becomes more detectable in the Z-E assay because the increased spatial demand accentuates even modest reductions in effective migratory output.

Finally, the transwell assay produced some of the most pronounced inhibitory effects across the entire experimental panel, particularly at 2 μ M nintedanib in A2058, Mel Pt-3 pre, and Mel Pt-3 post cells, underscoring the strong capacity of nintedanib to suppress directed chemotactic migration under these conditions. However, Mel Pt-4 post exhibited a paradoxical increase in migration under both concentrations, highlighting that transmigration through pores introduces additional biological constraints (cell size, deformability) that can dominate over motility changes (as described in Section 5.2.3.2).

Evaluating **machine-learning-based classification** performance, single-cell tracking achieved the highest accuracy, reaching 100% when cell-intrinsic morphological descriptors were included—features that are uniquely accessible in this assay format. Scratch and Z-E assays showed greater cell-line dependence, with classification accuracy correlating strongly with the

magnitude of inhibition observed in the Inhibition% analysis. The lowest accuracies occurred in Mel Pt-3 pre, consistent with its minimal measurable inhibition across assays. In contrast, the Z-E assay in Mel Pt-4 post, a case with strong, statistically significant inhibition, achieved accuracies exceeding 90%, demonstrating that clearer biological separation translates directly into improved classifier performance (Figure 30B).

In summary, the comparative analyses show that the apparent magnitude of nintedanib-induced migration inhibition depends strongly on the assay format, as each method captures distinct biophysical aspects of motility. Single-cell tracking provided the most sensitive and mechanistically informative readout, while gap-closure and transwell assays revealed assay-specific constraints that shaped the measured responses. Despite these differences, all assays consistently detected inhibitory effects in most cell lines, with cell line $\times$ assay $\times$ treatment interactions contributing to variation in effect size.

6. DISCUSSION

6.1. Quantitative insights from video-microscopic single-cell migration analysis

Single-cell migration assays provide a direct and information-rich means of quantifying cell motility, as they allow individual cells to be tracked independently of collective interactions or tissue-level constraints. Although such assays cannot reproduce the full spatial and mechanical complexity of *in vivo* environments, their highly controlled nature makes them uniquely suited for dissecting intrinsic migratory behavior and evaluating how pharmacological perturbations alter cell movement (Masuzzo et al., 2016; Svensson et al., 2018). To investigate how analytical decisions influence the interpretation of single-cell motility, we performed a systematic, large-scale quantitative analysis of videomicroscopic datasets encompassing multiple cell lines and treatment conditions.

From each tracked individual cell trajectory, we calculated a comprehensive set of migration descriptors spanning three major parameter groups: displacement-based (mean squared displacement, MSD; displacement, D; maximal displacement, MaxD), path-based (total traveled distance, TTD; velocity, V; average velocity, AV), and directionality-based metrics (directionality ratio, DR; average turning angle, ATA; turning vector zero crossing, TVZC). To ensure comparability across experiments and to avoid biases arising from arbitrary endpoint selection, time-dependent parameters (MSD, D, MaxD, TTD, and V) were condensed into single values by calculating the area under the curve (AUC). This approach provides an integrated measure of motility over the entire observation window and reduces sensitivity to fluctuations at individual time points. By summarizing temporal dynamics into a unified metric, AUC facilitates more robust statistical comparisons between conditions and enables direct cross-parameter evaluation. This advantage is rarely utilized in conventional migration analyses (Masuzzo et al., 2016).

This multidimensional analytical approach revealed that these parameter classes capture distinct and partially independent facets of motility. As a consequence, their responses to identical biological perturbations often diverged, and in several cases, individual descriptors even changed in opposite directions following the same pharmacological treatment. Such discrepancies highlight that the quantitative outcome of a migration experiment is determined not only by the biological effect of the treatment but also by the analytical lens through which motility is assessed. Importantly, these divergent parameter responses also carry significant biological implications. A

treatment may appear to “inhibit” migration according to one descriptor while simultaneously enhancing a different, equally relevant aspect of cell behavior. In practical terms, a pharmacological agent could reduce instantaneous speed yet increase directional persistence, leading to cells that move more slowly but still progress farther from their point of origin over time (Sawyer et al., 2003; Huang et al., 2005; Weiger et al., 2013). In a metastatic context, such a shift would be detrimental rather than beneficial: slower but more directionally persistent tumor cells may have an increased likelihood of reaching and breaching vascular structures.

Within this framework, the observed association between discordance and cell line–specific velocity in semi-automatic tracking warrants particular consideration. A plausible explanation is that semi-automatic algorithms operate with a reduced signal-to-noise ratio in inherently slow-moving cells. Under these conditions, minor sources of technical variability, such as segmentation jitter, fluctuations in image intensity, or subtle stage drift, may contribute disproportionately to the reconstructed migration path. As a result, modest biological effects, including small reductions in velocity, can be masked or distorted, leading to artificially inflated path lengths and reduced net displacements, which in turn increases the likelihood of discordant parameter responses. In contrast, fast-moving cells exhibit a substantially higher true-movement-to-noise ratio, rendering semi-automatic tracking more robust and less susceptible to such artefacts.

To further clarify the origin of the observed discrepancies between migration parameters, we next examined their behavior under controlled, noise-free conditions using a two-dimensional random-walk simulation framework (Masuzzo et al., 2016; Codling et al., 2008). In this system, speed and directionality could be modulated in combination, allowing us to model four combined treatment scenarios (simultaneously increasing or decreasing both features, or shifting them in opposite directions), as well as two single-parameter manipulations in which only speed or only directionality was altered.

These simulations revealed distinct sensitivity profiles. Path-based parameters were consistently the most responsive, detecting even subtle speed changes regardless of whether directionality was modified. In contrast, displacement-based descriptors – particularly MSD – were substantially less sensitive and required more pronounced divergences from control to show significant differences. When speed and directionality were changed in the same direction, displacement-based parameters detected deviations earlier; however, when the two were modified

oppositely, detection was delayed, especially when increased speed was accompanied by reduced persistence or more rotational trajectories. Finally, the single-parameter simulations provided mechanistic clarity: changes in speed alone produced strong responses in both displacement- and path-based descriptors, whereas altering directionality primarily affected directionality-based metrics, with minimal impact on path-based parameters and only delayed effects in displacement-based ones. Altogether, this simulation analysis demonstrates that the inherent mathematical properties of migration descriptors dictate their sensitivity to different types of motility alterations (Huang et al., 2005; Sawyer et al., 2003).

Because quantitative migration outcomes also depend on the accuracy and reproducibility of trajectory extraction, we next assessed how different tracking approaches influence the obtained motility parameters. Manual and semi-automatic tracking produced broadly comparable qualitative patterns; however, the magnitude and statistical detectability of treatment effects differed markedly between methods. Consistent with our earlier findings on parameter sensitivity, displacement-based descriptors were relatively insensitive to tracking-related fluctuations. In contrast, path-based parameters showed substantial discrepancies between manual and semi-automatic tracking, indicating that these metrics are highly sensitive not only to biological motility changes but also to tracking precision.

Detailed inspection revealed that the source of this divergence was systematic noise inherent to semi-automatic tracking. As cell shape and boundary intensity fluctuated over time, the algorithm frequently shifted the detected centroid by small amounts, even when the cell itself did not move (Piccinini et al., 2016). These artificial micro-displacements accumulated along the trajectory, inflating instantaneous velocity estimates and path length, while leaving net displacement largely unaffected. To correct this, we implemented a customized noise-filtering pipeline, removing sub-threshold positional jitter while preserving true biological movement. Applying this filtering procedure substantially reduced the differences between semi-automatic and manual tracking for path-based parameters, confirming our theory. Importantly, when comparing treatment groups to their respective controls, this artefactual noise largely cancels out, but for cross-method comparisons or absolute quantification of speed, noise filtering proved essential.

Directionality-based parameters showed an additional layer of complexity. In the simulations, they appeared largely insensitive to isolated changes in speed or persistence; yet in the experimental

datasets, they were highly susceptible to even minimal tracking jitter, which manifested as artificial fluctuations in turning angles or zero-crossing events. After noise filtering removed these micro-displacements, the semi-automatic and manual estimates often shifted in the opposite direction, indicating that directionality descriptors can be disproportionately shaped by methodological artefacts when cells migrate without a guiding stimulus. In practice, this makes directionality-based parameters unreliable for assessing subtle treatment effects under non-directed conditions (Masuzzo et al., 2016; Svensson et al., 2018). Among them, the directionality ratio showed the greatest consistency and remains the most usable within this parameter group, but it too requires cautious interpretation

To evaluate reproducibility, we calculated the coefficient of variation (CV) for all migration parameters across experiments and tracking methods. Semi-automatic tracking, influenced by persistent positional noise, initially produced artificially low CV values for path-based parameters; however, after applying noise filtering, CVs converged to consistent levels (~50%) across all descriptor classes. Directionality-based parameters showed the poorest repeatability, displaying high variability and detecting treatment effects only at substantial divergences from control conditions. These findings indicate that directionality metrics are unreliable in non-directed single-cell migration assays, whereas displacement- and path-based descriptors provide more stable and interpretable readouts. Biological heterogeneity likely further contributes to the limited reproducibility of directionality measures.

To better understand the biological sources of variability in migration quantification, we next examined how intrinsic cell-specific features – including projected cell size, circularity, solidity, aspect ratio, convexity, cell number, coverage, colonization, and colony size – influence motility readouts and tracking precision. In addition to baseline cell line-specific velocity, several morphology descriptors showed strong and systematic associations with migration parameters. Larger and more elongated cells exhibited higher net displacement and faster movement, whereas round, highly solid cells displayed slower and less persistent trajectories. These relationships are consistent with established biological principles: elongated, mesenchymal-like morphologies typically support more productive forward translocation, whereas compact, amoeboid-like cells often generate short-range, less persistent movements (Panková et al., 2010; George et al., 2023). Colony-forming behavior was inversely correlated with motility: cell lines that preferentially formed compact, cohesive colonies displayed substantially reduced migratory activity, consistent

with a growth strategy that favors maintenance and expansion of the colony through local proliferation rather than dispersal.

Cell morphology also influenced the reproducibility and accuracy of trajectory extraction across tracking methods. In baseline conditions, rounder cells with lower solidity generated larger discrepancies between methods, reflecting the challenge of reliably segmenting and centroid-tracking low-motility cells that display minimal frame-to-frame displacement and lack distinct edge features. As a result, semi-automatic tracking frequently introduced small, non-biological positional fluctuations, causing trajectory jitter and inflating differences from manually curated tracks. In contrast, elongated cells provide clearer morphological landmarks and exhibit larger effective displacements, enabling more consistent and robust tracking performance across all methods. Importantly, applying our noise-filtering pipeline effectively eliminated these morphology-dependent discrepancies, confirming that they originated largely from segmentation-related jitter rather than true biological differences.

Under treatment conditions, correlations shifted: cell number and coverage became the dominant factors influencing tracking reliability, and these effects persisted even after noise filtering. We hypothesize that the presence of immobile, dying, or apoptotic cells, which are more impactful at lower densities, contributes to this residual variability in semi-automatic tracking.

Finally, the degree to which migration parameters were repeatable was itself shaped by cell-intrinsic morphology, particularly in manually tracked datasets. This suggests that manual tracking may introduce unconscious operator bias, with evaluators tending to select “cleaner,” more easily trackable cells over rounder or morphologically ambiguous ones. Consequently, morphology not only affects true biological motility but also subtly influences which cells are preferentially included in manually curated analyses.

6.2. Differences between migration assays

To place single-cell migration into a broader experimental context, we next examined additional commonly used migration assays that capture different facets of motility. Scratch assays rely on mechanically creating a wound in a confluent monolayer; however, the associated cell damage releases intracellular factors that can influence migratory behavior and introduce variability. Zone-exclusion assays overcome these limitations by using silicone inserts to generate uniform, debris-free cell-free areas, allowing collective inward movement to be assessed under more standardized conditions. In contrast, transwell assays require cells to migrate through vertical pores toward a chemoattractant, introducing a semi-3D, confinement-dependent component in which cell size, deformability, and mechanical adaptability critically influence the outcome. Because these assay formats interrogate distinct biological processes, they naturally yield different quantitative outcomes and may reveal different facets of treatment effects (Kramer et al., 2013; Pijuan et al., 2019). To systematically compare their sensitivities and interpretational limits, we evaluated all four migration assays (including single-cell tracking) using the same panel of melanoma cell lines and the multikinase inhibitor nintedanib as a model anti-migratory treatment.

In single-cell migration assays, we extracted multiple quantitative descriptors from each trajectory, representing three mechanistically distinct categories: displacement-based (mean squared displacement, MSD; displacement, D), path-based (average velocity, AV), and directionality-based metrics (directionality ratio, DR). A key observation was that the relative ranking of melanoma cell lines depended strongly on the parameter class used for evaluation. For example, Mel Pt-4 post appeared the most motile according to displacement-based metrics, whereas path-based analysis ranked Mel Pt-3 pre higher. Likewise, A2058 exhibited a pronounced divergence between AV and displacement-based readouts, attributable to its exceptionally high directionality ratio compared with the other lines. These findings further reinforce that reliance on a single descriptor, such as velocity, the most commonly used parameter in the literature, can yield an incomplete or even misleading representation of migratory capacity (Masuzzo et al., 2016; Svensson et al., 2018).

Under nintedanib treatment, the multidimensional nature of these readouts became even more informative. Across all cell lines, the drug reduced migration in a concentration-dependent manner; however, the pattern of inhibition differed markedly between parameters. Notably, A2058 was the

only line in which nintedanib decreased directionality, whereas in all other cell lines the treatment increased directional persistence. This unique response in A2058 resulted in disproportionately stronger inhibition of displacement-based parameters compared to the path-based metrics, unlike in the other cell lines. Such orthogonal responses further illustrate that pharmacological perturbations can differentially modulate distinct components of motility (Molnár et al., 2019; Schelch et al., 2018).

Beyond descriptive quantification, single-cell tracking provided the most powerful phenotypic discrimination in machine-learning analysis: it achieved 100% classification accuracy in distinguishing treatment conditions across all melanoma cell lines. This superior performance likely reflects the integration of both motility descriptors and morphology-related features (e.g., cell size, shape), which can only be quantified with high fidelity in single-cell assays, where cells migrate in isolation and are not subjected to crowding, compression, or contact-mediated shape constraints present in collective formats. These intrinsic phenotypic attributes proved critical for detecting drug-induced changes and were not retrievable in scratch, zone-exclusion, or transwell assays. Importantly, the generalizability of such high classification performance depends on the strength and consistency of the underlying biological signal. In systems with low intrinsic motility or subtle treatment effects, classification accuracy is expected to decrease. Nevertheless, the relative performance of different assay formats, particularly the higher discriminative power of information-rich single-cell measurements, is likely to be preserved across experimental models.

Gap-closure assays are among the most widely used approaches for assessing collective migration, yet our results demonstrate that their reliability and interpretability differ markedly between formats. The traditional scratch assay produced highly variable initial gap geometries and irregular wound edges, substantially compromising reproducibility. Despite this inconsistency, scratch wounds closed faster than zone-exclusion (Z-E) gaps. This seemingly enhanced migratory response likely reflects two assay-specific factors: the smaller initial gap size in scratch assays and the release of paracrine signals from mechanically damaged cells (Kramer et al., 2013; Pijuan et al., 2019). The latter can stimulate migration and thereby confound both baseline measurements and treatment-dependent effects.

Normalizing gap-closure dynamics to cell size was essential, as closure speed is inherently influenced by how much area a single cell occupies. Larger cells fill the cell-free region more

rapidly than smaller ones. Although this adjustment is seldom performed in the literature (Kramer et al., 2013), our results demonstrate that size normalization substantially improves the interpretability of gap-closure assays by reducing morphology-driven bias and allowing more meaningful comparisons across cell lines and treatments.

Importantly, the two gap-closure methods yielded markedly different rankings of cell-line motility. In scratch assays, cell size strongly influenced closure kinetics; rank order changed substantially after size normalization, highlighting the susceptibility of this format to non-migratory confounders. In contrast, rankings in the Z-E assay remained largely unchanged after normalization, as the ample gap space minimized geometric constraints imposed by cell size. These rank discrepancies underscore the fundamental methodological differences between the two assays. Interestingly, scratch assay rankings showed greater similarity to the single-cell AV metric, whereas Z-E rankings more closely resembled the single-cell MSD. Thus, parameters measured by two conceptually similar gap-closure assays were less correlated with each other than with entirely different classes of single-cell descriptors, reinforcing how strongly quantification choices shape perceived migratory behavior.

Under nintedanib treatment, both gap-closure assays detected migration inhibition, yet the magnitude and detectability of drug effects varied across cell lines. This pattern was mirrored in machine-learning classification performance: Mel Pt-3 pre showed consistently poor classification accuracy in both formats, whereas Mel Pt-4 post was robustly distinguishable from controls in the Z-E assay. These differences likely reflect cell-line-specific sensitivity to nintedanib, with stronger inhibitory effects producing clearer separation in classification space.

Transwell assays assess directed migration under vertical confinement, requiring cells to actively deform and squeeze through pores. Consequently, cell size, shape, and mechanical flexibility play a disproportionately large role in determining whether a cell successfully traverses the membrane (Denais et al., 2016; Justus et al., 2014). In our experiments, this assay produced the largest differences in apparent migratory capacity across cell lines, reflecting its strong dependence on multiple biophysical variables rather than motility alone. Nintedanib treatment produced the clearest inhibitory effects in the transwell format: for most melanoma cell lines, the number of cells passing through the membrane was markedly reduced, consistent with impaired migration. However, an exception was observed in Mel Pt-4 post, where nintedanib unexpectedly increased

the number of transmigrated cells relative to control. This paradoxical result illustrates a key limitation of transwell assays: treatment-induced changes in cell morphology or cytoskeletal organization can directly alter pore passage efficiency, thereby inflating or diminishing the measured “migration” signal independently of true motility. Indeed, only by integrating morphology measurements from the single-cell migration assays were we able to interpret this phenomenon. These analyses indicated that nintedanib reduced the size and increased the circularity of Mel Pt-4 post cells in a manner that facilitated pore transit, despite not increasing intrinsic motility. Thus, while transwell assays are highly sensitive for detecting treatment effects, they capture a complex composite of motility, cell mechanics, and morphology, and must therefore be interpreted with particular caution (Justus et al., 2014; Pijuan et al., 2019).

6.3. Summary of methodological insights, limitations, and recommendations

Cell migration can be quantified through a wide array of *in vitro* assays and computational descriptors, each capturing only a specific facet of this inherently multidimensional process. In this work, we demonstrated how strongly the perceived migratory phenotype depends on the analytical and methodological framework through which it is examined. By systematically comparing multiple migration parameters, tracking strategies, and assay formats, we showed that different quantitative readouts, although often treated as interchangeable, frequently diverge, respond differently to pharmacological perturbations, and may even lead to contradictory biological interpretations if used in isolation.

Our results highlight that meaningful interpretation of migration behavior requires understanding what each parameter or assay actually measures, how sensitive it is to changes in speed, directionality, or morphology, and under which conditions methodological artefacts may distort the outcome. The comparative analyses presented here outline the circumstances under which each descriptor class and assay format performs optimally, where their limitations lie, and how they should (or should not) be used to characterize treatment effects.

The following summary tables consolidate these methodological insights, providing practical guidance for selecting appropriate migration metrics and assay systems (Tables 5 and 6). Together, these recommendations aim to support more reproducible, interpretable, and biologically grounded quantification of cell migration across experimental settings.

Parameter type	Displacement-based	Path-based	Directionality-based
Parameters	MSD, D, MaxD	TTD, V, AV	DR, ATA, TVZC
Interpretation	Net translocation away from the starting point	Total travelled distance and instantaneous movement speed	Turning behavior and persistence
Strengths	Robust; low sensitivity to tracking noise; consistent across methods	Highly sensitive to subtle treatment effects; captures the dynamics of motility	Provides mechanistic insight into how trajectories are organized and helps interpret cases where displacement- and path-based parameters diverge
Limitations	Insensitive to changes to treatment effect; strongly influenced by cell-line morphology	Susceptible to noise introduced by the tracking method; requires noise filtering	Low sensitivity to small changes; noisy; difficult to interpret in isolation
Recommended use cases	Comparing across experiments, mixed tracking methods, and detecting large treatment effects	Detecting weak treatment effects, analyzing motility dynamics	Interpreting the mechanistic basis of treatment effects; understanding how treatments alter trajectory shape

Table 5. Comparative overview of migration parameter categories derived from 2D single-cell migration analyses. Interpretation, strengths, limitations, and recommended contexts of use are summarized based on a previously established reference dataset encompassing multiple cell lines and treatment conditions.

Assay	Biological question addressed	Strengths	Limitations / when to be cautious	Recommended use
Scratch wound	Collective migration; injury-driven response; composite readout (migration + proliferation + signaling)	Simple; rapid setup; faster apparent closure than Z-E; consistent inhibition trends	Variable gap size; uneven edges; damage-induced paracrine signaling; size-dependent closure → may overestimate migration	Preliminary screening; high throughput; interpret cautiously; consider size normalization
Zone-exclusion (Z-E)	Collective migration without injury; controlled gap closure	Uniform gaps; reproducible; reduced damage artifacts; clearer inhibition in some lines	Slower closure; longer incubation; cell-line dependent effects → may underestimate subtle responses	Controlled collective migration studies; when reproducibility is critical
Single-cell tracking	Intrinsic motility; speed, persistence, morphology; migration isolated from proliferation	Rich dataset; multiparametric; robust inhibition detection; enables morphology–migration linkage	Labor-intensive; low scalability; lacks collective context → limited generalizability to tissue behavior	Mechanistic analysis; disentangling migration components; morphology-dependent effects
Transwell	Chemotaxis/invasion; migration under confinement; pore traversal	Captures directional migration; quasi-3D constraint; strong inhibition detection	Highly morphology-dependent; size/deformability bias; pore filtering → may mask true migration effects	Invasion/chemotaxis studies; interpret with morphology and size readouts

Table 6. Comparative summary of migration assay performance. Strengths, limitations, and recommended contexts of use are summarized for each assay type based on our experimental results with five melanoma cell lines under nintedanib treatment.

7. THESIS POINTS

- I. Quantitative methodological advances in single-cell migration analysis
 - 1) I quantified how displacement- and path-based migration parameters respond to the same pharmacological perturbations and demonstrated that they can exhibit opposite trends. Despite being derived from identical trajectories, these parameters may align more closely with other migration assay readouts than with each other, indicating that they capture mechanistically distinct facets of motility and that meaningful biological interpretation requires their integrated evaluation rather than reliance on any single metric (Bányai et al., 2026; Németh et al., 2025).
 - 2) I systematically benchmarked the three major classes of migration parameters and identified their distinct performance characteristics. Path-based metrics were the most responsive but also the most noise-sensitive; whereas displacement-based metrics, especially MSD, were more robust but require larger effect sizes; and directionality-based descriptors were generally unreliable under non-directed conditions, with DR retaining the highest explanatory value (Bányai et al., 2026).
 - 3) I analyzed the impact of intrinsic cellular properties, including morphology, confluency, local cell density, and coverage, and showed that these factors substantially influence both the absolute magnitude and variability of migration parameters. I further demonstrated that these features differentially affect the manual and semi-automatic tracking outputs (Bányai et al., 2026).

- II. Cross-assay benchmarking of migration assays using melanoma cell lines and pharmacological inhibition
- 4) I compared gap-closure assay formats and demonstrated that their outcomes are strongly influenced by assay geometry and collective mechanics. Scratch assays were confounded by heterogeneous wound edges and wounding artefacts, while Z-E assays offered superior reproducibility and sensitivity. I also showed that normalization for cell size is essential due to its strong effect on closure kinetics (Németh et al., 2025).
 - 5) I evaluated transwell assays across multiple cell lines and showed that they produce the largest divergence in apparent migratory behavior between assay formats. I further demonstrated that pharmacological treatments can, under certain conditions, increase apparent migration due to treatment-induced changes in cell size and morphology (Németh et al., 2025).
 - 6) I demonstrated that single-cell tracking provided the most informative and mechanistically interpretable assessment of drug effects by capturing both motility parameters and cell-intrinsic morphological features. When combined with machine-learning analysis, developed in cooperation with Dr. Köllöd Csaba, this approach achieved the highest classification accuracy among all assay formats (Németh et al., 2025).

8. LIST OF PUBLICATIONS

Journal publications of the thesis:

- 1) Bányai, G. L., Naszlady, M. B., Juhász, J., Horváth, A., Hegedűs, B. & Garay, T. (2026). Revisiting single-cell migration parameters from 2D video microscopic measurements. *European Journal of Cell Biology*, 105, 151524.
- 2) Németh, A., Bányai, G. L.*, Andrászek, M., Dobos, N. K., Köllöd, C., Garay, T. (2025). Comparison of *in vitro* migration assays evaluating nintedanib's migration inhibitory effects on melanoma cells. *Scientific Reports*, 15, 42599.

Further journal publications:

- 1) Németh, A., Ekström, K., Crescitelli, R., Bagge, R. O., Bányai, G. L., Rák, Á., Cserey, G., Bölükbas, S., Visnovitz, T., Hegedűs, B., Garay, T. Machine learning-enhanced analysis of extracellular vesicle profiles in pleural effusions for cancer diagnosis. *MedComm – Under revision*.
- 2) Németh, A., Bányai, G. L., Dobos, N. K., Kós, T., Gaál, A., Varga, Z., Buzás, E. I., Khamari, D., Dank, M., Takács, I., Szász, A. M., Garay, T. (2024). Extracellular vesicles promote migration despite BRAF inhibitor treatment in malignant melanoma cells. *Cell Communication and Signaling*, 22(1), 282. <https://doi.org/10.1186/s12964-024-01660-4>
- 3) Jász, B., Balogh, T., Ambrus, S., Schmidt, P., Bányai, G. L., Körmendi, S., Jász, M. (2024). Pure rotation in the temporomandibular joint during jaw opening? A digital motion analysis. *BDJ Open*, 10(1), 32. <https://doi.org/10.1038/s41405-024-00213-8>

Proceedings papers:

- 1) Bányai, G. L. (2025). Exploring diffusion-driven variations in extracellular vesicles from cancer patients using microfluidic systems. *PhD Proceedings – Annual Issues of the Doctoral School, Faculty of Information Technology and Bionics*, 20, 23–26.
- 2) Bányai, G. L. (2024). Exploring the correlation between migration parameters and cell characteristics. *PhD Proceedings – Annual Issues of the Doctoral School, Faculty of Information Technology and Bionics*, 19, 23–26.
- 3) Bányai, G. L. (2023). Comparison of commonly used single-cell migration parameters. *PhD Proceedings – Annual Issues of the Doctoral School, Faculty of Information Technology and Bionics*.

- 4) Bányai, G. L. (2022). Quantitative analysis of single-cell trajectories. PhD Proceedings – Annual Issues of the Doctoral School, Faculty of Information Technology and Bionics, 17, 17.

Conference posters:

- 1) Bányai, G. L., Merényi, A., Szabó, A., Németh, A., Gaál, A., Pongor, C., Garay, T. (2025). Utilizing microfluidic techniques to differentiate cancer patient samples based on extracellular vesicle profiles. ISEV2025, Vienna, Austria.
- 2) Németh, A., Ekström, K., Crescitelli, R., Bagge, R. O., Bányai, G. L., Rák, Á., Cserey, G., Bölükbas, S., Visnovitz, T., Hegedűs, B., Garay, T. (2025). Diagnostic potential of pleural fluid-derived extracellular vesicles in differentiating pleural-associated diseases using machine learning. ISEV2025, Vienna, Austria.
- 3) Németh, A., Kós, T., Bányai, G. L., Gaál, A., Varga, Z., Dank, M., Takács, I., Szász, A. M., Garay, T. (2022). Effects of extracellular vesicles on vemurafenib sensitivity in syngeneic melanoma cell lines. ISEV2022, Lyon, France.
- 4) Németh, A., Kós, T., Bányai, G. L., Gaál, A., Varga, Z., Dank, M., Takács, I., Szász, A. M., Garay, T. (2022). Can extracellular vesicles transmit vemurafenib resistance in melanoma cells *in vitro*? EACR Congress 2022, Seville, Spain.
- 5) Bányai, G. L., Dobos, N. K., Németh, A., Merényi, A., Szabó, A., Gaál, A., Pongor, C., Garay, T. (2025). Microfluidic profiling of extracellular vesicle fractions reveals systemic changes in cancer patients. HSEV2025, Budapest, Hungary.
- 6) Bányai, G. L., Merényi, A., Szabó, A., Gaál, A., Pongor, C., Garay, T. (2025). Identifying tumor-associated differences in extracellular vesicle populations using microfluidic techniques. Semmelweis Scientific Days 2025, Budapest, Hungary.
- 7) Németh, A., Simon, A., Bányai, G. L., Garay, T. (2025). Extracellular vesicle pretreatment enhances melanoma cell migration despite dual MAPK pathway inhibition. PhD Scientific Days 2025, Budapest, Hungary.
- 8) Bányai, G. L., Szabó, A., Gaál, A., Pongor, C., Garay, T. (2024). Microfluidic techniques for extracellular vesicle analysis in clinical diagnostics. EACR Conference “Liquid Biopsies”, Lyon, France.
- 9) Bányai, G. L., Szabó, A., Gaál, A., Pongor, C., Merényi, A. G., Garay, T. (2024). Advancing extracellular vesicle characterization: Microfluidic approaches for cancer diagnostics. UKEV2024, Newcastle upon Tyne, United Kingdom.

- 10) Bányai, G. L., Szabó, A., Gaál, A., Pongor, C., Kós, T., Garay, T. (2024). Microfluidic-based methods for the analysis of extracellular vesicles in liquid biopsies. HSEV, Budapest, Hungary.
- 11) Kvasznicza, J., Magyar, R., Bányai, G. L., Szőcs, L., Németh, A., Garay, T. (2024). Cyclodextrins affect extracellular vesicle production in melanoma cells *in vitro*. HSEV2024, Budapest, Hungary.
- 12) Németh, A., Bányai, G. L., Dobos, N. K., Kós, T., Gaál, A., Varga, Z., Buzás, E. I., Khamari, D., Dank, M., Takács, I., Szász, A. M., Garay, T. (2024). Extracellular vesicles promote migration despite BRAF inhibitor treatment. HSEV2024, Budapest, Hungary.
- 13) Bányai, G. L., Naszlady, M. B., Juhász, J., Garay, T. (2024). Analysis of migration parameter sensitivity. Applied Mathematics Conference (AMK), Szeged, Hungary.
- 14) Bányai, G. L., Szabó, A., Gaál, A., Pongor, C., Kós, T., Garay, T. (2023). Microfluidics as a tool for analyzing the size distribution of plasma-derived microsomal fractions. ASEV – Small New World 2.0, Graz, Austria.
- 15) Choi, J., Görög, D., Gaál, A., Khamari, D., Buzás, E. I., Németh, A., Garay, T., Bányai, G. L. (2023). Investigating the effects of extracellular vesicles on proliferation and migration in syngeneic colorectal cancer cell lines. ASEV – Small New World 2.0, Graz, Austria.
- 16) Németh, A., Bányai, G. L., Dobos, N. K., Kós, T., Gaál, A., Varga, Z., Szász, A. M., Garay, T. (2023). Extracellular vesicles can promote migration and transfer vemurafenib resistance among melanoma cells. ASEV – Small New World 2.0, Graz, Austria.
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- 18) Bányai, G. L., Naszlady, M. B., Németh, A., Molnár, E., Rittler, D., Hegedűs, B., Garay, T. (2022). Is an increase or decrease in cell migratory activity merely a consequence of parameter selection? Semmelweis Symposium 2022, Budapest, Hungary.
- 19) Németh, A., Bányai, G. L., Dobos, N. K., Kós, T., Gaál, A., Varga, Z., Buzás, E. I., Khamari, D., Dank, M., Takács, I., Szász, A. M., Garay, T. (2022). Extracellular vesicles in vemurafenib resistance in melanoma cells. Semmelweis Symposium 2022, Budapest, Hungary.

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