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Automated Tracking of Tumor Invasion
In Three Dimensional Extracellular Matrix Analogs and a Novel Stochastic Analysis of the Cell Trajectories

by

Zoe NM Demou

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

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HOUSTON, TEXAS
April, 2001
Μεσάνυχτα και ταξιδεύεις δύος πλευρικά!
Σκιάζεσαι μήπως στο γιαλό τα φώτα σε προδίνουν,
μα πρώτα πλώρα μόνο εσύ πατάς στοχαστικά,
κρατώντας στα χεράκια σου το λύχνο του Αλλαδίνου.

Νίκος Καββαδίας

Midnight and you are travelling without any sides!
You fear the lights in the sea may betray you,
but thoughtfully you step ahead alone,
holding in your little hands the Alladin’s lamp.

Nikos Kabbadias
Στους Γονείς μου
Μαλαματή και Νικόλαος

To my Parents

Malamati and Nikolaos
ABSTRACT

AUTOMATED TRACKING OF TUMOR INVASION IN THREE DIMENSIONAL EXTRACELLULAR MATRIX ANALOGS AND A NOVEL STOCHASTIC ANALYSIS OF THE CELL TRAJECTORIES

by

Zoe NM Demou

Tumor cell migration and invasion of body tissues are prerequisite mediators for lymphatic or hematogenous cancer dissemination. To date, there is insufficient understanding of what triggers the metastatic cascade, and of how the interplay among cell receptors, the cellular and acellular components of the extracellular matrix and proteolytic enzymes mediate cancer migration, invasion, proliferation and survival. In addition to the inherent complexity of each one of the aforementioned phenomena is the lack of an experimental technique capable of dissecting the mechanisms that mediate the dynamic invasive and migratory behavior at the cellular level and with respect to the properties of the cell environment.

The goal of my thesis was to develop an automated system for cell tracking in three dimensions and use it to model the dynamics of cancer invasion and migration. Therefore the hardware and software were designed for a fully automated optical 3D cell tracking system that quantified long-term invasion and migration of cancer cells infiltrating 3D extracellular matrix analogs. The quantitative analysis of the cell trajectories employed a novel formulation of the continuous Markov model that evaluated the potential for invasive or lateral motion and cell stops. The infiltration of human HT-1080 fibrosarcoma and human MDA-MB-231 adenocarcinoma cells, was monitored in
plain or Matrigel-containing collagen type I gels. Parameters such as the speed subpopulations, the persistence of motion in certain directions, the turning frequency of the cells, the preferred directions of motion, and the invasion depth profiles over time quantified infiltration at the cellular level. Distinct migratory and invasive phenotypes significantly dependent on the gel composition were identified for the two cell types. The HT1080 cell line expressed a high motility phenotype and well-preserved lateral motion on the plain collagen gel surface. The basement membrane components transformed the HT1080 cells to robust invaders by significantly enhancing the matrix infiltration and the turning frequency. The low motility, slow invasion and low turning behavior of MDA-MB-231 cells indicated that their invasiveness may depend on matrix-degrading activity.

To the best of my knowledge this is the first study employing a detailed set of quantitative descriptors to demonstrate that tumor invasion and migration are dynamic processes of individual cells that depend significantly on the cell type and the tumor microenvironment.
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My beloved parents Malamati and Nikolaos Demou to whom this thesis is dedicated, for a lifetime of unconditional love and support and that very first letter I still cherish concluding:

Ασε τι βάρκα στο κύμα να πλέει,
μη λογαριάζεις τιμόνι, πανί,
άκρη η ζωή μας δεν έχει,
και είναι πιο όμορφοι οι άγνωστοι πάντα γιαλοί.

Ε. Ρούδης
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I. INTRODUCTION

A. Cell Motility in Physiological Phenomena and Neoplasia

Several aspects of cancer such as genetic, developmental, biochemical, metabolic, immunological, endocrinological, and viral, are scientifically challenging and crucial to understand the disease. However, the most prominent feature of this hydra is the capability to disseminate in the body in a devastating manner that is usually halted only by the demise of the host. Metastatic tumors are the major cause of treatment failure and mortality for cancer patients. Apart from the neoplastic processes of tumor invasion and angiogenesis, cell movement is essential in several physiological phenomena, such as embryonic tissue development, inflammatory responses, tissue repair, bone remodeling, hormone-dependent tissue turnover, and tissue engineering applications, i.e. colonization of biomedical implants (1-3). Inside the organisms cells penetrate tissue barriers including basement membranes, interstitial tissue stroma, and cell-cell junctions and often migrate several hundreds times their bodylength away from their origin. The non-convective spread of cancer cells into adjacent tissues or at distant metastatic sites of the organism is one of the most dramatic patterns of cell displacement and a major reason for interest in cellular motility.

B. The Metastatic Cascade

Metastases are not random events but rather the output of complex biochemical and biophysical interactions between cancer cells and their host tissue, of which substantial part relates to the migration and invasion of the extracellular matrix (ECM). Metastasis is orchestrated by a variety of gene products such as: (i) cell-cell and cell-ECM receptors,
(ii) proteolytic enzymes, (iii) cell motility factors, (iv) organ-specific receptors, (v) growth factors to support the growth of the metastatic tumors, and (vi) angiogenesis factors to develop the neovasculature needed for supplying nutrients (4).

There exist several kinds of cancer but two general types of tumor: benign and malignant. Benign tumors are local growths of slowly dividing cells that cohere in arrangements resembling normal tissues. Early tumors always maintain the characteristics of the surrounding tissue, herein the diagnostic terminology reflects their histological character e.g., a pancreatic adenocarcinoma is a glandular epithelial cancer of the pancreas (5). Malignant tumors, in contrast, usually grow fast and their cells tend to lose their tissue specific phenotype and become irregularly arranged. Malignant growths contain an excess of connective tissue that often constitutes the main part of the tumor and possess a very prominent characteristic, the ability to invade and initiate the metastatic cascade (6, 7).

A simplified description (8, 9) of the metastatic cascade is shown in Figure 1. More specifically, malignancy begins when cancer cells detach from the primary tumor and penetrate the surrounding connective barriers. As the size of a tumor increases due to cell division in the primary tumor, cells at the periphery progress into adjacent tissues, individually or in groups by insinuating the surrounding structures, infiltrating and proteolytically destroying components of their microenvironment. Invasion may be massive making the affected organ grossly enlarged, distorted and hard. When the invasion is slow, streams of cells hesitantly infiltrate while preserving anatomical continuity of the adjacent tissues. The onset of metastasis is marked by tumor cells invading vascular or lymphatic vessels. Cancer cells surviving the mechanical forces of
the blood flow or the attack of the immune system disseminate eventually through the body. Metastasis is accomplished when the tumor cells extravasate and lodge in the tissue of a new organ that provides a favorable environment for their growth.
Figure 1. The metastatic cascade.
(A) Onset of malignancy. (B) Onset of metastasis. (C) Journey in the blood or the lymphatic vessels. (D) Extravasation into a new host site. (E) Proliferation and formation of the metastatic tumor. Invasion and migration of tumor cells play critical roles for metastasis to occur.
Malignant invasion is another multifaceted aspect of cancer. Tumor cells invade either as individual cells or as cell aggregates (10). Invasion of tumor clusters in vivo has been observed for epithelial carcinomas and melanomas. In this case, the aggregates maintain intercellular cohesion (11) and invade through tissue discontinuities (fiber bundles, nerves, blood vessels) (12). Individual cells can become motile and leave the primary tumor when receptors establishing homotypic adhesion, such as cadherins and catenins, are mutated or downregulated. In addition, loss of cell-cell contact may induce alterations at the intercellular and intracellular signaling cascades and the structure of the cytoskeleton that may further encourage the release of cells from the primary tumor (13) (14). The limiting step during stromal invasion is to overcome the matrix barrier and to a smaller extent, the intercellular interactions with host cells or other neoplastic cells. The individual cancer cells employ a slow, adhesion-dependent, fibroblast like mechanism of locomotion (15, 16), or a faster ameboid crawling (17).

To date, there is no concrete explanation of what triggers the metastatic cascade, and of how the interplay among cell receptors, ECM cellular and acellular components and proteolytic enzymes mediate cell migration, proliferation and survival. Apart from the inherent complexity of each one of the aforementioned phenomena, additional parameters are inserted from the microenvironment composition and the cellular phenotype that obviously mediate the expression level and the affinity of the implicated factors.
C. **The Extracellular Matrix**

The structure and function of higher organisms relies on discrete organs, each composed of two major specialized tissues: the basement membrane and the interstitial matrix (stroma) (18). The ECM structural elements are architecturally arranged into a meshwork that not only presents a passive physical barrier to the metastasizing cells but is also the stage of dynamic physicochemical interactions and signaling cascades during most of the steps of the malignant dissemination. Immunohistochemical localization studies *in vivo* and *in vitro* suggested that tumor cells affect the expression of ECM components that are involved in the regulation of tumor invasion (19, 20).

The basement membranes are synthesized by epithelial, endothelial and stromal cells as multilayer structures consisting of: (a) basal and (b) reticular lamina. Their major functions are tensile strength, cell attachment, and ultrafiltration. The major layer, the basal lamina is a 50-100 μm thick bilayer structure containing: (i) the lamina lucida (lamina rara), in direct contact with the cell membranes, and (ii) the lamina densa, that neighbors the reticular lamina. The basal lamina contains mainly collagen type IV, laminin, entactin (nidogen) and heparan sulfate proteoglycans especially in the lamina lucida. The components of the reticular lamina depend on the tissue of origin and can include lamina densa extensions, collagen type VII fibrils or microfibrils that may facilitate anchoring to the underlying matrix.

The matrix stroma contains collagen fibers (mainly type I and III collagens), fibronectin, elastin, glycosaminoglycans and proteoglycans various stromal cells such as fibroblasts, smooth muscle cells etc. Tumor growth is associated with alterations in the stromal ECM, and malignant tumors often induce increased expression of type I and III collagens.
to the adjacent stroma. The formation of tumor stroma is thought to inhibit tumor progression and is often viewed as a nonspecific host attempt to wall off the tumor.

1. Fibrilar Collagens

The typical collagen molecule is a long, stiff, triple-stranded structure composed of three collagen polypeptide chains (e.g. two collagen α1(I) chains and one α2(I) chain for collagen type I) (18, 21). Collagen chains have a characteristic sequence of repeated Gly-X-Y aminoacid triplets and numerous proline and hydroxyproline residues that allow the 3 chains to intertwine into a stable, ropelike superhelix of 1.5 nm diameter. Collagen chains are flanked at both ends with nonhelical telopeptides. Lysine and hydroxylysine residues in the telopeptides allow the triple helices to assemble into ordered collagen fibrils of 10-300 nm diameter that can further pack into even larger collagen fibers of 500-3000 nm. Other collagen molecules decorate the surface of the fibrils and help maintain the inter-fibril cohesion or anchor collagen to other ECM components. The collagen fibrils exhibit extreme tensile strength and remarkable proteolytic resistance. Exclusively, a single enzyme, the mammalian interstitial collagenase, (CNaseI), cleaves the native collagen triple helices at a specific site about 75 nm from the carboxyterminus. Subsequently the collagen fibrils are susceptible to denaturation at 37°C and to less specific proteases.

2. Collagen type IV

The collagen type IV molecule is found in most tissues as a 400 nm long trimer of two α1(IV) chains and one α2(IV) chain twisted in a helix (18). This helix is interrupted by
approximately 26 nonhelical stretches, it contains also a small nonhelical N-terminal domain, an extensive globular C-terminal domain (NC1 domain). Collagen type IV is a very flexible molecule and very susceptible to proteases with broad specificity. However, a specific type IV collagenase cleaves at about 120 nm from the N-terminus. The collagen IV helices are arranged into a mesh-like network by: (i) crosslinking the N-termini of four trimers oriented in anti-parallel fashion, (ii) crosslinking of C-terminal globular NC1 between two anti-parallel trimers, and by (iii) lateral aggregation between the central triple helical areas. The first two crosslinks create a chicken wire-like network with 800 nm long sides. The aggregation of the central parts creates a compact structure with sides of 200 nm in a hexagonal configuration. This structure confers barrier qualities to the basal lamina and creates a major obstacle for invading malignant cells.

Recently, four more \( \alpha \) chains of collagen IV, \( \alpha_3(IV) \), \( \alpha_4(IV) \) (22 and \( \alpha_5(IV) \) (23), \( \alpha_6(IV) \) (24) have been identified. Although \( \alpha_1-\alpha_2(IV) \) chains exist in all basement membranes, immunohistochemical studies revealed that \( \alpha_3-\alpha_6(IV) \) chains are restricted in specific human tissues or are associated with pathological conditions (22, 23, 25).

The other types of collagen differ in the length of the monomers, the number, location and site of the globular domains, the 3D configuration and the affinity for cells and for other ECM components.
3. **Elastin**

Elastin, similar to collagens, has abundant Gly and Pro residues but little hydroxyproline and hydroxylysine (18). The 90 kDa random coiled monomers crosslink extensively via lysine residues to form a highly elastic network. The elastin network exhibits good proteolytic resistance in general, however it is susceptible to specific elastases (26).

4. **Laminin**

Laminin is the most abundant noncollagenous glycoprotein of the basal lamina. Several epithelial, endothelial and endodermal normal and neoplastic cells synthesize laminin that mediates attachment, spreading, proliferation, differentiation, survival, morphology, and migration of various cells (18). The laminin family has various members with structures depending on the tissue of origin. The most commonly used laminin *in vitro* is extracted from the EHS murine tumor. This laminin is a trimer of a 400 kDa A chain, a 220 kDa B1 chain and a 210 kDa B2 chain. Several laminin peptides (27) have been associated with cell attachment and chemotaxis such as the YIGSR (28) (29-31), IKVAV (32-34) and the fragment E8 (35, 36).

5. **Fibronectin**

Fibronectin is a family of 500 kDa dimers linked with disulfide bonds at their C-terminus (18). The fibronectin molecule is a multidomain structure containing homologous repeats of type I (or finger) domain, type II (or "kringle-like") domain and type III domain. Fibronectin is a major attachment, spreading, and migration promoting substrate for several cell lines and its involvement in embryonic development, wound healing, and
metastasis has been the subject of several studies. The multimeric fibronectin molecule contains: several cell recognition sequences for normal and neoplastic cells such as GRGDS (with absolute requirement for the RGD tripeptide)(35, 37, 38), REDV (39), CSI (40), promoting attachment, spreading and motility, and also protein binding domains for e.g. collagen (41), gelatin (42), fibrin (41), actin (43), and heparin (proteoglycan) (44).

D. Mechanism of Invasion

The cellular mechanism of invasion has been characterized as three-step process involving: (i) tumor cell attachment to the matrix by tumor cell surface receptors, (ii) local ECM degradation by tumor-associated proteolytic enzymes, and (iii) tumor cell locomotion into the regions modified by proteolysis (45, 46).

1. Cell Surface Receptors

The cellular mechanism of migration is based on various protein networks that mediate cell adhesion to the substrate. At least three structural elements are required for cell migration: ECM ligands, the corresponding cell membrane receptors, and the cytoskeleton. The integrins form a large family of cell adhesion receptors, which mediate cell-matrix and cell-cell adhesion (47). They are heterodimers composed of a larger α and a smaller β subunit. Both subunits contain a large relatively small cytoplasmic domain, a membrane-spanning region, and a large extracellular domain. The two extracellular domains of the integrin molecule, associate noncovalently to form a functional receptor. To date, 24 αβ heterodimers of 18 α and 8 β subunits have been
identified. Eighteen of these receptors recognize and bind to ECM proteins while others interact with cell surface proteins. Some integrins can mediate both interactions (see Figure 2). The amino terminus of all the α subunits contains about 60 amino-acid that have been predicted to form a four-stranded β-sheet (the β propeller model) (48). This domain forms the ligand-binding pocket together with the β subunit. However, nine of the 18 α subunits contain a highly conserved, autonomously folded I domain, of ~200 amino acids, inserted between the third and fourth repeat of the propeller (47). In these integrins the I domain is responsible for the ligand binding. Such integrins include the four collagen-binding β1-associated subunits (α1, α2, α10, and α11), and the β1 leukocyte integrins (αL, αX, αD, and αM).

The specificity and functions of various integrins are overlapping since several of them have a common ligand, or each receptor may have affinity for several ligands (promiscuous). Cell surface receptors of the integrin family interconnect reversibly to the cytoskeleton, including the actin-containing microfilaments and to the ECM proteins. Integrons α1β1 and α2β1 are considered the major collagen receptors for the cell. α1β1 prefers collagen type IV and laminin-1 (49), while integrin α2β1 binds to several ECM components such as collagens, laminin-1, and tenascin. The major ligand for α3β1 is the basement membrane component laminin-5 and also laminin-1 and fibronectin (50). Another integrin with affinity for laminin is the α6β4 (51). All αV integrins recognize the RGD motif in their ligands.

Integrins transduce signals from the cell environment to control cell movement, morphology, cell growth, and gene expression. Additionally, integrins can be regulated from the inside of the cell to modulate their binding affinity to ligands (52, 53). Proteins
interacting with integrins include components of the cell cytoskeleton (i.e. the Rho family of GTPases through their effects on both the actin and the microtubule cytoarchitecture) (54) (55), and various kinases (i.e. SH2-B, SIRP, and JAK2) (56, 57). When binding to ligands, the integrins move laterally in the cellular membrane to form clusters called focal adhesion sites. These sites contain several cytoskeletal proteins and signaling molecules (58, 59). The focal adhesion following the integrin-ligand interaction, leads to organization of the cytoskeleton and recruitment of signaling molecules and cytoskeletal proteins (i.e. tenascin, a-actinin, talin, vinculin, and focal adhesion kinase (FAK)), to the site of the cluster (54, 60). The roles of integrins in cell migration and attachment are described in a following section (section E1).

Another interesting function of integrins is to control cell invasion through ECM by regulating expression and activation of matrix metalloproteinases (MMPs). MMPs are not constitutively expressed by cells in vivo (see also section D2), but their expression is induced by growth factors, cytokines, and by alterations in the cell-matrix interactions (61). Several studies (62) showed that recognition of matrix molecules by cell surface integrins and the subsequent degradation of the matrix play important role in cell invasion (integrin-guided proteolysis), i.e. uPA expression and localization of its receptor (63), in osteoclasts ligation of the collagen binding α1β1 mediated downregulation of collagen, while α2β1 lead to increased MMP-1 mRNA (64). Additionally, models have been developed for integrin-guided degradation of fibrillar collagen. Cell interaction with 3D collagen gels via α2β1 and mediated production of MMP-1 that degraded collagen exposing the RGD site (65). Recognition of the RGD site by αVβ3 upregulated
gelatinase-A (MMP-2) that proceeded to total degradation of the collagen (66). Finally, integrins can be cellular receptors of MMPs, i.e. in fibrosarcoma cells, the adhesion of \( \alpha 5\beta 1 \) to fibronectin leads to induction of MMP-2 secretion and upregulation of membrane type 1-MMP (MT1-MMP) that activates latent MMP-2 at the cell surface (67).
Figure 2. The integrin family of cell adhesion receptors. At present, 24 αβ combinations are known. The boxes at the top indicate the ECM ligands of the receptor and the boxes below, the plasma component ligands and the cell surface counter-receptors. The α subunits containing the I domain are circled [figure adopted from (47)].
2. The ECM Degradation

In general, the ECM can be degraded during physiologic and pathologic conditions by four classes of proteolytic enzymes: matrix metalloproteinases, serine proteinases, cysteine proteinases and aspartic proteinases (68). An increased expression or activation of matrix degrading enzymes such as various matrix metalloproteinases (MMPs), urokinase type plasminogen activator, elastase (69) and a number of cathepsins (70, 71) have been associated with tumor invasion. A more detailed description follows for the MMPs and the plasminogen activation system.

a. Matrix Metalloproteinases

Matrix metalloproteinases (MMPs) are a family of zinc-dependent endopeptidases capable of degrading essentially all the ECM components. Several physiologic processes, such as fetal tissue development and tissue repair involve ECM remodeling by MMPs (68). However, excessive damage of the ECM by MMPs occurs in pathological conditions such as periodontitis (73), autoimmune disorders (74), dermal photoaging (75), rheumatoid arthritis (76), osteoarthritis (77) and chronic ulcerations (78). Additionally, degradation of the ECM is essential for the growth, invasion and metastasis of malignant tumors, and for tumor-induced angiogenesis. The tumor cells either express their needed MMPs or induce their expression to host cells such as fibroblasts, endothelial cells, macrophages or leukocytes (45, 79). Recent evidence associates the MMPs with tumor survival (80).

Eighteen mammalian MMPs have been identified so far and categorized according to their substrate specificity into: collagenases, stromelysins, gelatinases, membrane type
MMPs (MT-MMPs) and other MMPs. Table 1 summarizes the known MMPs, the cells that produce them and their action and substrates (68). MMPs have a characteristic multidomain structure consisting of (68): (i) a signal peptide, (ii) a propeptide, which is essential for maintaining the proMMP in a latent form, (iii) a catalytic domain containing the highly conserved Zn$^{2+}$–binding site (HexGHxxGxxHS/T), (iv) a proline-rich hinge region that links the catalytic domain to (v) the hemopexin-like domain, which determines the substrate specificity of the MMP. The catalytic domain of gelatinases contains additionally three repeats of the fibronectin-type II domain, involved in binding of the enzymes to gelatin. MT-MMPs contain a transmembrane domain of 20 hydrophobic amino acids in the C-terminal end of the hemopexin domain followed by a 24 amino-acid intracellular domain (81).

Collagenase-1 (MMP-1), collagenase-2 (MMP-8), and collagenase-3 (MMP-13) are the major secreted neutral proteinases that degrade native fibrillar collagens of types I, II, III, V, and XI (68). The cleavage site of the fibrillar collagen is between Gly775 and Leu/Ile776 of the α chains, generating N-terminal 3/4 and C-terminal 3/4 fragments (82). These fragments rapidly denature to gelatine at body temperature and become susceptible to degradation by other MMPs (gelatinases) (83).
<table>
<thead>
<tr>
<th>Collagenases</th>
<th>(MMP)</th>
<th>Producer Cells</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Collagenase-1</td>
<td>(MMP-1)</td>
<td>tumor, fibroblasts, osteoblasts, endothelial, keratinocytes, hepatocytes, chondrocytes, macrophages</td>
<td>Collagen III</td>
</tr>
<tr>
<td>Collagenase-2</td>
<td>(MMP-8)</td>
<td>melanoma, leukocytes, chondrocytes, synovial fibroblasts, endothelial</td>
<td>Collagen I</td>
</tr>
<tr>
<td>Collagenase-3</td>
<td>(MMP-13)</td>
<td>breast carcinoma, melanomas, squamous cell carcinoma, chondrosarcomas</td>
<td>Fibronectin, laminin, osteoclast core protein, fibrillin-1, serine proteinase inhibitors</td>
</tr>
<tr>
<td>Stromelysins</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Stromelysin-1</td>
<td>(MMP-3)</td>
<td>fibroblasts, epithelial</td>
<td>Collagen IV, V IX, X, gelatin, proteoglycans, fibronectin, lammin fibrillin-1, collagenase inhibitor, tumor necrosis factor (TNF)-α precursor, myelin basic protein, (IL)-1β</td>
</tr>
<tr>
<td>Stromelysin-2</td>
<td>(MMP-10)</td>
<td>fibroblasts, epithelial</td>
<td>Collagen IV, V IX, X, gelatin, proteoglycans, fibronectin, lammin fibrillin-1</td>
</tr>
<tr>
<td>Stromelysin-3</td>
<td>(MMP-11)</td>
<td>breast cancer, uterus, placenta, mammary gland</td>
<td>u1-proteinase inhibitor, u1-antitrypsin</td>
</tr>
<tr>
<td>Metalloelastase</td>
<td>(MMP-12)</td>
<td>Macrophages in placenta and lung (pulmonary emphysema), fibroblasts (intestine, skin)</td>
<td>elastin, collagen IV, gelatin I, fibronectin, laminin, vitronectin, proteoglycans, myelin basic protein, u1-antitrypsin</td>
</tr>
<tr>
<td>Matriksin</td>
<td>(MMP-7)</td>
<td>malignant epithelial: gastrointestinal, prostate and breast, normal glandular epithelial, small intestinal crypts, skin, airways</td>
<td>Fibronectin, laminin, nidogen, collagen IV, proteoglycans, β4 integrin</td>
</tr>
<tr>
<td>Gelatinases</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Gelatinase A (72 kDa)</td>
<td>(MMP-2)</td>
<td>fibroblasts, keratinocytes, endothelial, chondrocytes, osteoblasts, monocytes, various transformed cells</td>
<td>Collagen IV, V, VII, X, XI, XIV, gelatin, native collagen I elastin, proteoglycan core proteins, myelin basic protein, fibronectin,fibrillin-1, precursors of (TNF)-α and (IL)-1β</td>
</tr>
<tr>
<td>Gelatinase B (92 kDa)</td>
<td>(MMP-9)</td>
<td>Alveolar macrophages, polymorphonuclear leukocytes, osteoclasts, keratinocytes, invading trophoblasts, various transformed cells</td>
<td>Collagen IV, V, VII, X, XI, XIV, gelatin, N-terminal telopeptide of collagen I, elastin, proteoglycan core proteins, myelin basic protein, fibronectin,fibrillin-1, precursors of (TNF)-α and (IL)-1β</td>
</tr>
<tr>
<td>Membrane-type MMPs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MT1-MMP</td>
<td>(MMP-14)</td>
<td>malignant epithelial, dermal fibroblasts, osteoclasts</td>
<td>Activates latent MMP 2 at the cell membrane, cleaves collagen I, III, gelatin, fibronectin, laminin-1, vitronectin, cartilage proteoglycans, fibrillin-1</td>
</tr>
<tr>
<td>MT2-MMP</td>
<td>(MMP-15)</td>
<td>Human placenta, brain, heart</td>
<td>Activates proMMP-2 and proMMP-13, degrades laminin, fibronectin, tenasin</td>
</tr>
<tr>
<td>MT3-MMP</td>
<td>(MMP-16)</td>
<td>Lung, placenta, kidney, ovary, intestine, prostate, spleen, heart, skeletal muscle (membrane bound and soluble)</td>
<td>Activates proMMP-2, hydrolyzes gelatin, casein, collagen III, fibronectin</td>
</tr>
<tr>
<td>MT4-MMP</td>
<td>(MMP-17)</td>
<td>Breast carcinoma, breast cancer cell lines, brain, leukocytes, colon, ovary, tests</td>
<td>Unknown</td>
</tr>
<tr>
<td>MT5-MMP</td>
<td>(MMP-24)</td>
<td>Brain tumors, brain, kidney, pancreas, lung (membrane bound and soluble)</td>
<td>Activates MMP-2</td>
</tr>
<tr>
<td>Other MMPs</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>MMP-19</td>
<td></td>
<td>Mammary gland, liver</td>
<td>Unknown</td>
</tr>
<tr>
<td>Enamelysin</td>
<td>(MMP-20)</td>
<td>Odontoblasts, dental tissues</td>
<td>Degrades amelogenin</td>
</tr>
<tr>
<td>MMP-23</td>
<td></td>
<td>Ovary</td>
<td>Unknown</td>
</tr>
</tbody>
</table>
MMPs in Tumor Invasion

MMPs have long been associated with the spread of cancer. In fact, several MMPs were first purified and cloned from tumor cell lines or tumor tissues (84-88). Several studies revealed positive correlation between MMP expression and invasive and metastatic potential of malignant tumors of the colon, lung, head and neck, basal cell, breast, thyroid, prostate, ovarian and gastric carcinomas (61, 69). In malignant tumors, most MMPs are produced by normal stromal cells rather than tumor cells (90). Tumor cells can also secrete factors, such as extracellular MMP inducer, which enhances expression of MMP-1, MMP-2 and MMP-3 by fibroblasts. Additionally, tumor-infiltrating inflammatory cells and stromal cells secrete various growth factors and cytokines capable of modulating MMP expression (91-93).

In general, invasion of malignant tumors involves interplay between tumor cells, stromal cells, and inflammatory cells. All of these cells secrete distinct but often overlapping types of MMPs that may complement each other’s substrate specificity and form a network of MMP cascades in which a single MMP cleaves a certain native or partially degraded ECM component, or activates other latent MMPs (89, 90).

Transcriptional Regulation and Activation of MMPs

Most MMPs are not constitutively expressed in vivo, but rather regulated at the transcriptional level (MMP-1, -3, -7, -9, -10, -12, -13, and -19) by external signals, e.g. growth factors, cytokines, oncogenes, hormones, cell-matrix and cell-cell contacts. Additionally, modulation of MMP mRNA half-life by growth factors and cytokines has also been documented (94).
The majority of MMPs are secreted as latent precursors (zymogens) that are proteolytically activated in the extracellular space (95). Latent MMPs are retained in the proform by a “cysteine switch” formed by covalent interaction of the conserved cysteine in the propeptide with the catalytic zinc. Various compounds, e.g. organomercurials, can react with cysteine converting it to a nonbinding form, exposing the catalytic site, and resulting in autocatalytic cleavage of the propeptide (96). The propeptide of most MMPs can also be cleaved by a number of other extracellular proteinases, e.g. plasmin and other MMPs. Activation of latent MMP-2 and MMP-13 at the cell membrane by membrane type 1 MMP (MT1-MMP) provides a potent way of directing their activity to the pericellular environment.

**Inhibition of MMP Activity**

The proteolytic activity of MMPs is regulated by: (i) zymogen activation, (ii) inhibition by specific inhibitors, i.e., tissue inhibitors of metalloproteinases (TIMPs), and by (iii) nonspecific proteinase inhibitors, e.g. α1-proteinase inhibitor and α2-macroglobulin (68). TIMPs are expressed by various cell types and mediate tissue development, angiogenesis, cancer cell invasion, and metastasis by regulating MMP activity and stimulating cell growth (97). Overexpression of TIMPs by cancer cells or by the host tissue reduces the invasive and metastatic potential of tumor cells. To date, four tissue inhibitors of metalloproteinases (TIMPs) with common structural features have been identified (98): TIMP-1, -2, -3, -4. TIMPs compete for the zinc-binding catalytic site of the MMPs. TIMP-1 and TIMP-2 can bind to the hemopexin domain of latent MMP-2 and MMP-9 respectively. Their activity is summarized in Table 2 (68).
Currently synthetic MMP inhibitors are in clinical trials to evaluate their ability to inhibit the tumor growth and invasion *in vivo* (99-101). Gene delivery of TIMPs into malignant cells may inhibit tumor invasion and survival (102). Furthermore, inhibition of the MMPs may be also possible at the transcriptional level.

**Table 2.** Tissue Inhibitors of Metalloproteinases and Inhibition Substrates

<table>
<thead>
<tr>
<th>Tissue Inhibitors of Metalloproteinases</th>
<th>Substrate</th>
</tr>
</thead>
<tbody>
<tr>
<td>TIMP-1</td>
<td>most MMPs except MMP-2 and MT1-MMP</td>
</tr>
<tr>
<td>TIMP-2</td>
<td>most MMPs except MMP-9</td>
</tr>
<tr>
<td>TIMP-3</td>
<td>MMP-1, -2, -3, -9, -13</td>
</tr>
<tr>
<td>TIMP-4</td>
<td>MMP-2, -9, -7</td>
</tr>
</tbody>
</table>
b. **Plasminogen Activation System in Cell Adhesion, Migration, and Invasion**

The extracellular zymogen plasminogen is catalytically converted to the serine proteinase plasmin by either of two serine proteinases, the urokinase- and tissue-type plasminogen activators (uPA and tPA respectively) (103, 104). uPA seems to be more pertinent in tumor biology, while tPA generates plasmin for fibrinolysis in blood vessels. Additionally, roles in the plasminogen activation system are played by the two major plasminogen activator inhibitors the serpins PAI-1 and PAI-2. The uPA receptor (uPAR) is a uPA-binding, cell-membrane-anchored protein that focuses plasminogen activation activity on cell surfaces (105). The uPA system plays a critical role in cell migration and invasion via mechanisms involving multiple interactions between uPA, uPAR, PAI-1, extracellular matrix proteins, integrins, endocytosis receptors, and growth factors. These processes seem to temporally permit spatial reorganizations of the plasmin system during cell migration and selective degradation of ECM proteins during invasion (106).

Several studies suggest an important role for plasmin catalyzed by the urokinase type plasminogen activator (uPA) in cancer invasion through the ECM and cancer-cell directed tissue remodelling, e.g. angiogenesis and desmoplasia (induction of fibroblast proliferation and ECM protein synthesis) (105, 107).

The serine protease plasmin (M₉=90,000) contains two disulfide bond-linked polypeptide chains (108, 109). The N terminal A chain contains five domains namely the kringle domains, while the C terminal B chain contains the typical serine protease domain that is responsible for the catalytic activity (110). Plasmin hydrolyzes the peptide bonds on the C-terminal of the Lys and Arg residues (111, 112).
The classical role of plasminogen activation is the synthesis of pericellular plasmin to counteract the cell-substratum and cell-cell adhesion by degradation of adhesion receptors and their ECM ligands (105, 113). Quite interestingly, under certain conditions the binding of uPA to its receptor uPAR, may actually promote adhesion, e.g. the integrin-independent adhesion of several human cell lines to vitronectin is stimulated by bonds of uPA or uPA fragments and uPAR (114, 115). These results indicate that uPAR can act as an adhesion receptor since uPA-uPAR may initiate signaling cascades affecting cell adhesion.

Several studies concluded that uPA stimulates cell migration by catalyzing the synthesis of plasmin (116) supporting the theory that plasmin-induced degradation of ECM proteins and adhesion receptors facilitates release of the trailing cell edge. The limited proteolysis of the uPAR and the subsequent initiation of signaling cascades may be another possible proteolytic mechanism that contributes to the uPA stimulation of cell migration (117). Additionally, non-proteolytic stimulation of cell migration was attributed to uPA-uPAR bond probably by enhancing adhesion on the leading edge by stimulation of uPAR-vitronectin binding (118, 119). Furthermore, non-proteolytic uPA and uPAR effects on cell migration may be associated with integrin binding activity at both the leading and the trailing edge (115).

*In vitro* invasion assays for various cell types and a range of substrata showed that synthesis of plasmin after uPA-uPAR bond formation on the cell surface, is a rate-limiting factor for invasion (116). However, an inhibitory effect on invasion was associated with excessive plasminogen activation (120, 121). High levels of PAI-1 were shown to inhibit the synthesis of plasmin and protect the ECM against proteolysis and
therefore invasion though low levels of PAI-1 may be needed to protect ECM for traction.

E. The Biology of Cell Locomotion in 3D Substrata: A 3-Step Concept

As mentioned earlier, cell migration plays critical role in several physiological processes and health disorders. Cells inside the body interact with the three dimensional connective tissue primarily via cell adhesion receptors. These interactions modulate the phenotype and a spectrum of cellular functions such as cell attachment, polarity, adhesion, migration, growth and differentiation for both normal and tumor cells (15, 122, 123). The ECM produces complex signals that are not available to cells simply attached on a 2D ligand-coated surface (124). For example, growth arrest was reported for tumor cells in 3D cultures (14) and upregulation of the integrin α2β1 for fibroblasts and melanoma cells cultured in 3D collagen gels compared to collagen coated surfaces (125).

Cell migration through 3D substrata requires the ability of the cells to adhere to and detach from the matrix via periodical formation and destruction of receptor-mediated bonds and additionally, the ability to proteolytically degrade the matrix components to overcome the biophysical barrier imposed by the matrix. Currently, cell migration is viewed as haptokinesis, or translating from Greek, a touch-dependent motion. Several cell types migrate by attachment of their leading edge, contraction of the main cell body, and detachment of the rear end (2, 3, 123).
1. Receptor-Mediated Attachment

At the leading edge of a moving cell membrane-bound adhesion receptors bind on the underlying substratum. Such contacts initiate and maintain actin polymerization that gives rise to ruffling of filopodia, pseudopod protrusion and eventually formation of new contacts with the substrate. The main receptors mediating cell migration are integrins. Additionally, cell surface proteoglycans (syndecans, phosphacan), the CD44, the CD36, the CD47/integrin-associated protein, and the dystroglycan complex may be also involved (126).

During integrin-mediated migration and upon or just before firm attachment on the substrate, the integrins cluster in the cell membrane (127) and their cytoplasmic domains initiate a cascade involving: (i) a range of structural proteins (α-actinin, talin, vinculin, zyxin, F-actin), (ii) several adapter proteins (Grb2, Sos), and (iii) various signaling molecules (focal adhesion kinase (FAK), C-terminal Src kinase (Csk), PI-3-kinase, phospholipase Cγ (PLCγ), Rho, Ras, Raf, mitogen-activated protein (MAP) kinase/extracellular signal-regulated (ESR) kinase (or MAP/ESR), Jun, N-terminal kinase) (122, 128). This highly structured cascade leads to the formation of complexes of cytoskeleton-binding, densely packed transmembrane receptors known as focal adhesions (128). The focal adhesions anchor the cell to the ECM and trigger recruitment and polymerization of actin monomers for stress fiber formation (123, 129).

2. Cell Contraction

After the receptor-mediated attachment, a gradient of binding and traction forces is generated across the cell body from the leading edge to the rear that enables the cell to
contract and move forward (130). Although the formation of the focal contacts is triggered from the extracellular environment, the maturation and maintenance of these contacts depends interactively on the forces transmitted via the integrin clusters. It was observed that substrate rigidity and hence stronger traction, strengthen the integrin-cytoskeleton interactions (123). This additionally indicates that cell migration via low adhesive and contractile forces, as in the case of 3D substrata, may be less dependent on focal adhesions (16).

3. Detachment

Obviously, for a cell to migrate, the focal contacts that attach its leading edge to the substrate need to be able to resolve periodically. At least two mechanisms are known to mediate the cell detachment. One mechanism lowers the integrin-ligand affinity causing gradual weakening of the bond until cell detachment. Then the integrins are endocytosed and recycled to the new leading edge of the cell (131). Alternatively some integrins dissociate from the membrane and remain attached to the substrate via a tyrosine phosphorylation-dependent event (2, 132).

F. Three-Dimensional Cell Migration Models

Haptokinetic migration studies on 2D substrata defined the dynamics of the cell-substrate interactions, the extra/intra-cellular signaling and the cytoskeletal re-organization. However, the 3D architecture of the natural migration substrata the cells encounter in vivo may involve different mechanisms. Cell migration requires extension and binding of integrins and other adhesion receptors to their ECM ligands at the leading edge of the
cell. The cell-ECM bonds at the leading edge and the forces generated by contraction of the cytoskeleton offer guidance and traction for pulling the cell forward. Invasion involves further complexity compared to migration, since it comprises not only cell locomotion but also active penetration of the ECM.

The three-step migration model as developed for the 2D cell migration seems generally applicable for the 3D migration of various cell types such as fibroblasts, leukocytes, and tumor cells (133-136). However, additional migration mechanisms or strategies may be used by cells for overcoming the biophysical resistance of the 3D matrix since such obstacle is not involved in the 2D migration.

1. **Overcoming the Resistance of the 3D Matrix**

Cell locomotion in 3D requires a means of overcoming the matrix resistance. To overcome the matrix barrier the cells temporarily (i) change their morphology, or (ii) deform the structural elements of the ECM, or permanently compromise their environment by (iii) secreted matrix degrading enzymes, or with (iv) contact-dependent matrix remodeling.

a. **Morphology**

Migrating cells on 2D substrates acquire a stretched, flat morphology. The leading edge consists of at least one flat pseudopod with ruffling membrane formations due to integrin clusters at the substrate-contact site, while the detaching rear end is narrower (135). Periodically, the ruffling filopodia establish new contacts with the substrate and a flat
lamellipod expands at the cell front. Such flat, well-spread morphologies do not occur in 3D environments. Rather than a flat ruffling leading edge, in 3D substrata cells develop cylindrical pseudopodia that extend through the matrix and contain surface microspikes and small lamellipodia (ruffles) interacting with the 3D infrastructure (134, 137). These cylindrical formations expose smaller contact area and may overcome easier the resistance of the matrix.

Migrating cells adapt their morphology to their microenvironment and follow the path of least resistance through the meshwork structure of the matrix. If the available openings in the matrix are large enough or deformable enough for a specific cell to slip through, then proteolysis may be not necessary for its migration (16, 138).

b. **Permanent Compromise of the Matrix with Proteolysis**

Proteolytic degradation of the matrix, as described above, is a common tactic employed by migrating cells. Membrane bound or secretory enzymes enlarge the matrix openings upon contact, or destroy the contacts with the ECM to facilitate the passage of the cell body. Several MMPs including MMP-2, MMP-9, and MT-MMPs have been located on normal fibroblasts and endothelial cells and on melanoma, breast epithelial cancer cells and neoplastic keratinocytes (139-141). These enzymes are either self-expressed transmembrane proteins or extracellular proteins that bind on transmembrane receptors.

c. **Substrate Remodeling During Cell Migration**

Remodeling of the substrate has also been associated with cell migration in the form of matrix contraction or formation of migration trails.
i. Matrix Contraction

Dermal fibroblasts migrating in collagen gels can contract the matrix into fibril bundles that are different from the microfibrils formed during collagen gelation at neutral pH without cells (142, 143). Considering standard conditions of culture media, cell type, and collagen origin, the gel contraction depends on the cell concentration and the quantity and quality of the collagen. Thus, collagen gel contraction can be employed as a quality-testing model for cell populated gels intended for tissue equivalent grafts or models for pharmacological studies (144).

ii. Migration Trails

Another case of substrate remodeling is the creation of trailing paths behind migrating cells. Such paths have been observed both in 3D collagen gels (134) and on fibronectin-coating 2D migration assays (145, 146). The trails seem to be the result of contact-specific proteolysis rather than a diffuse degradation of the matrix and often maintain shed cell membrane receptors (i.e. α2β1 integrin), CD44, MMPs, and cytoplasmic portions (126, 134). A carved trail is a path of least resistance in the matrix and may be a mechanism employed to guide following cells or facilitate their confrontation with the matrix barrier.

G. Factors Affecting Motility and Invasiveness

Cell motility and invasiveness are influenced by several molecules including components of the extracellular matrix, e.g. laminin, FN, collagen, thrombospondin and elastin. Motogenic cytokines such as epidermal growth factor (EGF) (147, 148), platelet-derived
growth factor (149) insulin-like growth factor-1 (150), hepatocyte growth factor (HGF), or scatter factor, SF, (149, 151), basic fibroblast growth factor (bFGF) (152, 153), transforming growth factor-β (TGF-β) (154, 153), the macrophage stimulating protein (155, 156), and the fibroblast derived motility factor epitaxin that affects epithelial cells (157). HGF/SF a heterodimeric protein with Mr 57,000 and 30,000 subunits has motogenic, angiogenic, and morphogenic effects on epithelial and endothelial cells. The migration-stimulating factor, Mr 70,000, enhances the motility of normal and breast cancer fibroblasts (158, 159). Epitaxin, Mr 36,000, enhances the motility of normal epithelial and epithelial carcinomas (157). Soluble thrombospondin is a trimeric protein with Mr 145,000 subunits that enhances motility of breast adenocarcinoma and melanoma cells (160-162). In addition, the tumor cells are capable of synthesizing growth factors and autocrine motility factors (AMFs), such as AMF/neuroleukin (163) (164), migration-stimulating factor (158), and autotaxin (165).

H. Motility and Invasion Assays

A spectrum of assays has been developed for studying the migration characteristics and the invasive potential of different cell types, e.g. bacteria, leukocytes, fibroblasts and tumor cells. These assays vary in characteristics such as: the dimensionality of the substrate (2D vs. 3D), the composition of the substrate, the experimental time range, the data collection technique, the supporting instrumentation, the degree of automation, the type of the acquired data, and the data analysis. The cell migration and invasion techniques have been classified with respect to the type of acquired data into two major
categories: (i) cell-population assays that monitor the response of large numbers (thousands) of cells for a single property, and (ii) individual-cell assays.

1. Cell Population Assays

Cell population assays provide a fast method to acquire some rough information on the migratory potential or even compare motility among cell lines when detail of the cell locomotion is not the main target. They measure a bulk property, usually the number of cells, to quantify a specific migratory response or invasion under certain experimental conditions. Non-visual techniques such as fluorometry, quantification of radioactivity and the Coulter counter have been employed for measuring large number of cells during gel infiltration assays and the transmigrating cells at the Boyden chamber. Radiolabeled cells, e.g. $^{51}$Cr labeled neutrophil granulocytes (166) or $[^3]$Hthymidine labeled tumor cells (167), were seeded on top of collagen gels. At the end of the incubation period the surface of the gel was washed and the bulk of the gel was measured with liquid (beta) scintillation counting for residual emission corresponding to the percentage of the cells that invaded the gel. Additionally, amnion membranes denuded from the endothelium have been used to evaluate the effectiveness of radiolabeled cells $[^{14}]$C-thymidine to penetrate the matrix (168). The infiltrating cells were also measured with a Coulter counter, after removal of the surface cells and digestion of the collagen matrix with collagenase (169).
a. The Boyden Chamber

The Boyden chamber (170) has been the most popular cell-population assay for quantifying cell motility of several leukocytes and tumor cell lines. For the Boyden assay, a micropore filter is coated at the upper side with an ECM component. Then a suspension of the desired cells is placed on the filter while the bottom side contacts a solution of the desired stimulus. After assembly, the system is incubated for a sufficient amount of time to allow the cells to migrate to the lower surface of the filter, or fall into the lower compartment. Various types of collagen, laminin, fibronectin, vitronectin (171) have been used as coatings, however the most popular filter treatment for the Boyden haptokinetic assay is reconstituted basement membrane, sold commercially as Matrigel (172). The number of cells that traversed the filter can be quantified using a Coulter counter, staining and manual counting under microscope, or by measuring their fluorescence. Another measurable quantity is the distance traveled into the filter by the fastest moving cells (173). These metrics provide a measure of the haptotactic motility or the invasive potential among various cell lines under certain experimental conditions. Additionally, the number of cells that passed through the filter versus the needed incubation time reveals if a test substance is chemotactic for the cells under study.

i. Limitations of the Boyden Chamber Assay

The Boyden chamber has been the most popular invasion and migration assay for being simple, fast, easily repeatable and relatively cheap. However, this method could be characterized as a "blind" assay that produces cell population data but may have small in vivo resemblance. The cells encounter a very thin, low-density coating of ECM
components and a polymeric filter as a barrier against invasion. The number of transmigrating cells, which is practically the only quantifiable parameter in this assay, is significantly subjected to the interaction with the polymeric filter, a non-tissue material. Additionally, the size and tortuosity of the filter pores and cell adhesion on the filter may also influence the results.

There is significant evidence that tumors contain cell sub-populations expressing different phenotypes associated with the malignant potential, growth rate, drug sensitivity, migration, or response to chemotactic factors (174). Results derived from the Boyden chamber reflect the behavior of large cell populations (rather than single cells) and could be strongly biased by the relative proportion of the responding, or non-responding cells in a heterogeneous population (175). Due to the small in vivo resemblance the output of the Boyden assay has been found in some cases, inconsistent with the in vivo metastatic potential. More specifically, the ability of tumor cells to invade normal tissue, while necessary for the generation of metastases, is not in itself sufficient. Therefore results from the Boyden chamber have not always matched well the in vivo behavior (i.e. brain tissue microsphere invasion assay) (176).

2. **The Need for 3D Migration and Invasion Substrates**

The 3D natural ECM produces complex signals necessary for tissue-specific gene expression and for a cell to achieve a differentiated phenotype (177, 178). Such interactions are not available to cells simply attached on a 2D ligand-coated surface (124). As a result, the behavior or the phenotype of the cells in a 3D environment may vary significantly from the corresponding cases in 2D. For example growth arrest was
reported for tumor cells in 3D cultures (14). Also the integrin α2β1 was upregulated for fibroblasts and melanoma cells cultured in 3D collagen gels compared to collagen coated surfaces (125). Additionally, 3D collagen gels upregulated production of IL-1 from lymphocytes while factor inducing cytokine production were more effective in the collagen gels rather than the tissue-culture plate control (179). Additionally, there is evidence that migration and invasion of 3D matrices involves different mechanisms, or different receptors compared to the 2D case (180).

A reliable in vitro model for quantifying the invasion and motility of tumor cells is critical for defining the mechanisms by which tumors invade in situ, and for designing anti-metastatic therapies by modifying the invasive, or motile behavior of the malignant cells. For such a model to be realistic, it should simulate the architecture and the composition of the natural extracellular matrix that the cells encounter in vivo.

a. Collagen Matrices: Resemblance to the Natural Tissue

Hydrated collagen lattices were the first in vitro 3D substrate for cell culture (181) since collagen is the major component of the connective tissue that several cell types encounter and migrate through in vivo. Thereafter, it was shown that histological and functional differentiation is induced or maintained by three-dimensional culture of cells in collagen gels (181, 182). Hydrated collagen gels have shown resemblance to the natural tissue that the cancer cells invade and migrate through, while tumor tissue cultured in collagen gels responded to drugs in a manner similar to that observed in vivo (183). Additionally, fibroblasts maintained the polarized morphology (181, 135) of their natural phenotype and their in vivo alignment e.g. in parallel for tendon fibroblasts, or the perpendicular
network orientation for dermal fibroblasts (184). Normal thyroid cells organized into follicle-like structures and follicular adenoma cells obtained in vivo formations (185).

Upon gelation of collagen solutions at natural pH, a random architecture is obtained that simulates mainly the interstitial soft tissues (186). The properties of an in vitro collagen matrix depend on the type of the collagen, the concentration and the gelation temperature. Such parameters are involved in the fibrillogenesis process affecting the filament morphology and texture. Collagen solutions of 0.6 to 3.5 mg/ml can polymerize to form fibers of 50-300 nm. These fibers join in fibrillar bundles of 100-1000 nm in diameter and interconnect constructing a 3D network (181, 187). The density of the gels has a major impact on the ability of cells to migrate. Too low or too high fiber densities impair the cell migration while maximum migration was observed for 1.5-2 mg/ml (188, 189). Luckily, this concentration range produces gels of good optical clarity allowing observation of the fibrils (at high magnification) or monitoring of the cells with phase contrast microscopy.

b. Multicomponent Matrices

The natural ECM is an architectonic spectrum of multiple components and an interactive source of complex signals for the embedded cells. In order to account for this diversity various natural and artificial multicomponent matrices have been used as in vitro invasion substrates to evaluate the effect of the matrix composition for several tumor cell types.

Several mixtures of ECM proteins have been prepared to simulate the ECM and used as in vitro models of tumor invasion. Some of these combinations contained: (i) laminin and collagen (190), (ii) collagen I and collagen IV or fibronectin (191), (iii) collagen,
fibronectin, glycosaminoglycans and link proteins (187), or (iv) various amounts of laminin, collagen IV and heparan sulfated proteoglycan (192). The main issue with these in vitro models is the architecture of the self-assembled multicomponent matrix. Ultrastructural studies of the multicomponent matrix revealed various structural patterns in the resulting matrix. Some structures contained irregular strands of various proteins forming a network of randomly arranged cord-like elements. However, convoluted sheets similar to the lamina densa of the natural basement membranes were also observed (192).

The commercially available reconstituted basement membrane Matrigel, prepared from the Engelbreth-Holm-Swarm tumor, superseded the mixtures of ECM proteins to become the most popular filter treatment for the Boyden chamber haptokinetic assay. Another artificial basement membrane model is Amgel (167), an all-human matrix derived from non-neoplastic sources. Human amniotic membranes were denuded from the endothelium and imposed to pepsin digestion and homogenization. Natural tissue invasion models have been mainly intact basement membranes as found in the bladder wall (193), amnion membranes denuded from their endothelium (194), lens capsule (195), and the chick chorioallantoic membrane (196).

c. Collagen gels as in vitro Migration and Invasion Substrates

A more realistic invasion assay was presented by Schor et al. (169, 197) and followed by several others. During this first study, various tumor cell lines were tested for their proliferation rate in collagen gels and their ability to invade the 3D substrates during a 10-14 day period. The number of cells that infiltrated the gel was counted with a Coulter
counter, after removal of the surface cells and digestion of the matrix with collagenase. Additionally, the invaded cells were counted manually by focusing at a series of Z sections in the gel body.

Subsequently, collagen gels were used as 3D substrates for migration, invasion, matrix contraction (125), as well as cell growth and differentiation (14). Cells were incorporated in the collagen solution before gelation, or they were seeded on the gel for infiltration studies or they were sandwiched between identical gel layers (198). Cell migration has been studied in randomly gelated collagen matrices or in oriented lattices prepared with exposure in magnetic field during gelation (199). Moreover, in vitro models of cluster invasion were developed with tumor explants placed on collagen gels. The cells at the contact interface became motile and invasive while the cells inner in the cluster mass were more passive (10).

3. **Individual Cell Assays**

Individual cell assays were developed to overcome the limitations of the cell population techniques. In reality, the phenomenon of cell migration or invasion, is the combined output of several cells whose behavior depends on their individual characteristics and on the way they perceive and interact with their surroundings. Description of cell migration and invasion at the cellular level is indispensable to evaluate the effect of the microenvironment composition and structure or the influence of external stimuli. For this goal, parameters such as individual cell speed, duration of motion, directionality of motion, and turning behavior need to be extracted experimentally. Such detailed information can only be gathered via continuous monitoring and positioning of several
individual cells over a sufficient period of time. The experimental embodiment of this concept can have various degrees of difficulty depending on data to be acquired. Individual-cell assays are based on two main aspects: the development of a data acquisition technique for individual cell tracking, and the implementation of a data analysis method that can quantify the migratory behavior at the cellular level.

a. Cell Tracking Data Acquisition

Tracking at the cellular level requires a visual or microscopy assay, contrasting with the "blind" cell population assays. Simply introducing the dimension of time in the experimental protocol, even for 2D migration, was enough to complicate the data acquisition procedures. Cell monitoring over time demanded experimental platform with more sophisticated equipment than common tissue culture. At that point, the solution to the data acquisition problem came from time-lapse microscopy initially combined with serial acquisition of photographs (200) and reconstruction of the cell trajectories by hand (201). Later, videomicroscopy developed into the most popular technique for monitoring cells over time (see following sections).

The first individual cell assays in 3D matrices maintained characteristics of the cell population methods. Although effort was taken to focus manually with a calibrated fine focus of the microscope at successive z sections throughout the gel and extract the leading front of the invasive cells and their distribution at each section, still the number of cells was practically the only quantitative piece of data (166, 169). Hereafter, tracking over time of individual cells in 3D matrices was the obvious next step.
b. Computer-Assisted Cell Tracking

i. The Need For Computer-Assisted Cell Tracking

Extracting experimental data for motility at the cellular level during long periods of time is a complex task with a degree of complexity increasing with the number of the tracked cells and the dimensionality of the substratum, e.g. large number of cells moving randomly in a 3D substrate for several days. Larger numbers of tracked cells have better chance to be statistically representative of the total cell population. Another issue is the kind of data that should be acquired during a tracking experiment to assess effectively the cellular behavior. Any approach not restricted to an “on/off” analysis (i.e. moving/non-moving cell) can get quite complicated in an attempt to acquire large amounts of data and then analyze it to get useful information. Studies focusing on invasion and migration of individual cells give a more representative approach of the *in vivo* reality but the cell positioning, i.e. the extraction of the X, Y, Z positional coordinates, in 3D matrices can be very tedious when performed manually.

ii. The History of 3D Cell Tracking

The pyramid of development for the 3D tracking rises from a basis of several simple video recording applications and qualitative observations in gels, to reach over a period of about twenty years towards its peak containing less than a handful of fully automated computer-assisted 3D cell tracking systems.

Videomicroscopy based semi-automated cell tracking systems in 3D gels were a huge improvement from the on-slide (2D) cell migration or the Boyden chamber. The very first methods used video recording to capture the cell motion while the cell trajectories
were reconstructed manually. Most of the previous cell migration studies in 3D matrices extracted the 2D (XY) projections of the actual 3D trajectories in the 3D matrix, rather than finding the exact (X,Y,Z) positions of the invading or migrating cells (10, 134, 202-204).

An upgrade in cell tracking was the computer-controlled XY stage positioning for image acquisition at multiple XY fields of view. Using this method gel infiltration by tumor cells was studied with time-lapse image acquisition performed automatically at various XY fields on the top of the gel. Then the image sequence was observed manually to estimate the beginning of infiltration and calculate the residence time on the gel surface (191).

The next advance was automatic focusing in different Z positions in the gel. 3D images were acquired with time-lapse optical sectioning, or acquisition of images at successive Z position across the axis of the gel, at multiple XY fields of view (205). This method was used in algorithms for individual cell tracking in 3D as described by Dickinson et al. (205), i.e. for single cell at high magnification and for multiple cells at low magnification. They used image analysis routines from the Kontron IBAS image processing and analysis system incorporated into structured macros for automatic execution. However, the initial position of the cells was extracted manually. The system was applied in tracking contact guided fibroblasts in oriented collagen gels with phase contrast optics. In this application, the motion of the cells was two dimensional along the axially oriented collagen gels. The extracted cell trajectories were practically the 2D projections of the cell trajectories since the motion at the third dimension was comparatively insignificant.
To date, very few systems have been reported capable of tracking cells in three dimensions with relatively little manual involvement. Quite interestingly, the first serious attempt for automated 3D tracking was reported quite early. In 1987 Noble (206) (207) developed a computer-driven optical sectioning system that extracted the X, Y, Z coordinates of migrating cells in collagen gels using a time lapse optical sectioning. The cells to be tracked were selected manually at the beginning of the tracking with a light pen. Subsequently the (X, Y, Z) positions of individual cells were obtained over time by a three-dimensional converging squares algorithm in a cubic nearest-neighbor tracking window. The 3D cube was big enough to include the cell positions between two successive time points. After identifying the new position of the cell, the cube was automatically recentered around the new position and the same procedure is repeated for each individual cell.

A fully automated 3D cell tracking system based on parallel computing was presented by Awasthi et al. (208) for tracking of fluorescently-tagged dictyostelium. Their technique used thresholding to identify automatically the cells at time zero then a template-matching algorithm to locate the cells in the 3D specimen over time. A volume surrounded each tracked cell that contained the location of best match at the next timepoint, which becomes eventually the new template for searching at the next timepoint. The size of the volume is determined from manual tracking data and trial and error in actual tracking. Four different template-matching statistics, which used as criterion a correlation between distance from the origin and the identified fluorescent intensities in the tracking volume, were evaluated. The maximum efficiency of the automated tracking was 70% compared to the manual tracking. Fluorescent tags may
facilitate cell identification, however computationally demanding deconvolution for removal of the out of focus signals may be needed. Additionally dyes may affect the cell phenotype and behavior or may impair the tracking accuracy and restrict the tracking period due to decreased fluorescence associated with photobleaching or cell division.

Another application of videomicroscopy and optical sectioning has been the study of cell morphology over time, such as evolution of pseudopods and cellular shape during migration in 3D matrices (209).

Computers and appropriate software can expedite tasks that normally would not be performed due to time constraints. Advanced computer-assisted cell tracking systems are still evolving and they are not commercially available in a user-friendly version that is in parallel flexible enough to ensure applicability in a range of experimental cell tracking bioassays.

c. **Data Analysis and Quantification of Cell Migration**

Quantification of the cell migration was performed by using the tracking data to extract simple locomotory characteristics or implement mathematical models.

Gel infiltration and cell migration in 3D gels has been quantified with: (i) the average speed of motion, (ii) the persistence (directionality) defined as the length of the traveled path over the distance between the start and ending point (1.0 for moving on a straight line) (10, 203, 204), (iii) the duration of motion during the tracking period, and the percentage of moving cells over time (134).
Moreover, gel infiltration data has been modeled as a diffusion process analogous to Fickian diffusion (191) across the direction z, parallel to the gel axis. The invading cells resembled a random motion flux (J) down the concentration (C) gradient between the cell populated gel surface and the initially cell-void inner gel. A diffusion coefficient (μ) was calculated and considered to reflect the cell speed and the frequency of directional changes.

\[ J = -\mu \frac{\partial C}{\partial z} \]  

[1]

In combination with the random diffusion, the conservation equation was used to describe the time evolution of the cell concentration on the gel surface and the gel interior.

In general, mathematical models for cell migration were developed for: (i) random motion on isotropic substrata that are homogeneous in texture and composition, and (ii) in the presence of taxis (ordered or directional locomotion). Directional motion is imposed by a chemical gradient of a diffusible substance (chemotaxis), or by a texture gradient due to geometrical orientation of the substrate (haptotaxis or contact guidance), or by substrate-bound stimuli. The motility of randomly moving cells was modeled with the unbiased and persistent random walk while the taxis was described by the biased random walk (see following sections). Random motility is applicable in macroscopically uniform concentrations of stimuli and the cells follow an unbiased random walk. Temporal polarization of the cell morphology or uneven distribution of the cell receptors allows for a persistent random walk on a short time scale. Finally, in the presence of macroscopic gradients of stimuli the cells follow a biased random walk. The preferential
directionality of motion has been described by: (i) partial differential equations for the cell density and the mean flux of the cell population (210) and (ii) stochastic processes for the direction of motion (211, 212).

i. **Unbiased Random Walk**

The random motion of particles, atoms, molecules or even animals has been described by Einstein as a diffusion process (213). This behavior resembles Brownian motion so that the average distance traveled by a cell is proportional to the square root of the time of motion and was quantified by:

\[ <D^2> = 2\eta \mu t \]  \[ [2] \]

\( <D^2> \) is the mean square displacement of the cell, \( \mu \) is the random motility coefficient (equivalent to the diffusion coefficient), and \( n \) indicates the dimensionality of the motion (\( n=2 \) (or 3) for 2D (or 3D) motion). According to the above description randomly moving cells cover short distances rapidly but travel long distances much more slowly, e.g. it takes 100 times longer time to reach only 10 times farther.

ii. **Persistent Random Walk**

In a migrating cell, traction forces, generated at the leading edge (leading lamella), overcome the resistance imposed at the rear part (uropod), which is anchored to the substrate. Thus, a cell follows temporarily a persistent motion towards the direction of the leading lamella. However, when observed for longer time-periods, cells do not move
in a constant direction but rather engage in a meandering path, depending on the distribution of stimuli or available receptors in the pericellular environment. Cell migration in isotropic environments is a non-directional long-term motion with frequent directional described as persistent random walk. By monitoring experimental cell trajectories and using certain assumptions Dunn et al., described the persistent random walk as a model with two major parameters. The root-mean-square speed of cell locomotion (S), defined as the displacement of the cell centroid per unit time, and the persistence time (P), which is a measure of the average time between "significant" directional changes. According to the persistent random walk model the expected distance (or displacement) \(<D>\) of the cell centroid from its original position varies with time according to the formula:

\[
<D^2> = nS^2[Pt-P^2(1-e^{-P})]
\]

[3]

\(<D^2>\) is the mean square displacement of the cell, S is the root-mean square cell speed, and P is the persistence time. The constant n describes the dimensionality of the random walk (n=2 for 2D and n=3 for 3D motion). Practically, after reconstruction of the trajectories of the individual cells, the mean square displacement of the cell centroid can be calculated and plotted versus time. By fitting the random walk model to these data the speed (S) and persistence (P) can be extracted (214). The directional persistence time has been characterized as a function of four cell parameters: the receptor sampling index (equal to the dissociation constant for the receptor-chemoattractant complex), the total number of receptors with affinity for each of the two compartments, a system response
time constant, and a signal decay time constant. For long times (t >> P), or very small persistence time, the cells change their direction of motion very frequently and equation (3) reduces to a random walk:

\[ <D^2> = nS^2Pt \]  \hspace{1cm} [4]

In this case, the mean square displacement is proportional to the time interval with the random motility coefficient \( \mu \) being the constant of proportionality.

\[ \mu = \frac{S^2P}{2} \]  \hspace{1cm} [5]

iii. Biased Random Walk

A major application of the biased walk model is for the description of chemotaxis (the directional motion towards a concentration gradient of a chemical substance). The most common mathematical description of chemotaxis is the Keller and Segel model (210), which defines the flux of the cell population \( J_c \) as a function of the local cell density \( c \), the local concentration of the chemical stimulus \( s \), a random motility coefficient \( \mu \), and a coefficient of chemotaxis \( \chi \):

\[ J_c = -\mu \nabla c + \chi c \nabla s \]  \hspace{1cm} [6]
Additionally, Othmer et al. derived various cell population models to predict statistics of migration such as mean square displacement, expected displacement at the direction of the chemoattractant gradient etc., for motion in one, two, or three dimensions (215).

Some later models expressed the cell migration parameters as functions of individual characteristics of the cells such as morphology, the distribution of the cellular receptors, the contact area with the substrate, receptor-ligand association-dissociation properties (216).

Another example of biased motion is the contact guidance. Matthes and Gruler (217) described contact guidance as a direction-dependent random walk using a formulation of the equation 3 (see above) considering a direction-independent diffusion coefficient D and the directional persistence time P for a biased walk in n dimensions:

\[
<D^2> = 2nD[t-P(1-e^{-tP})]
\]  

[7]

Later, Dickinson et al. (205) used tracking data of individual cells in oriented collagen gels to compare the diffusion coefficients of the correlated walk at the direction of contact guidance and the lateral direction.

iv. **Stochastic Approaches: Markov analysis**

Most of the cell tracking data has been modeled as a random walk or it was analyzed to extract diffusion coefficients, persistence, or the average speed of the cell population. Although all of these parameters have been successful in comparing the motility of cells, they fail to provide a detailed characterization of the migration process. A large body of
evidence suggests that the cellular motion is not constant but rather variable in time and space. Velocity cycles have been identified in a variety of cell types such as *Dictyostelium* amoebae (209), human T-cell lines (218) and even giant HIV-induced T-cell syncytia (218). In addition, experimental observations revealed that cells slow down or cease their motion during cell divisions, intercellular collisions or before performing directional changes. Therefore, a stochastic approach may be more realistic in describing the dynamic character of cell locomotion.

Factor analysis was one of the first statistical means for identifying independent locomotory phenotypes in 3D gels within a cell population (207). The angle distribution obtained from the individual cell trajectories, was grouped into 15-degrees sectors (between 1 and 180 degrees). This analysis revealed two different turning behaviors: the cells with relatively small and relatively large angular changes in direction between steps. Additionally categorization according to the number of time that each cell stopped (waiting time) and the cell speed also revealed two major cell subpopulations, although the distributions overlapped partially.

An adequate description of the cell motion requires parameters such as: (1) the percentage of the motile (or stationary) cell population, (2) the speed of the individual cells, (3) a measure for the directionality of motion among the cell population and (4) for the duration of motion at a certain direction, (5) the frequency and (6) duration of cell stops; the turning frequency, and (7) a prediction of preferred directions of motion in case of chemotaxis or spacial heterogeneities (219). The Markov chain model, as
implemented on cell tracking by Boyarski (220), can provide such information and was a radical innovation to the quantitative analysis of cell trajectories. One of the first applications was the 4-state Markov analysis for 2D cell migration (201). Subsequently the first Markov application for the 3D migration analyzed the cell trajectories with a nine-state continuous Markov chain model featuring the stationary and eight directional states. Transformed 3T3 cells (MOS-11) were tracked in 3D gels and the trajectories were analyzed with Markov analysis (206, 221).

The implementation of the Markov theory is described in more detail in the Data Analysis section of this thesis. Assuming the position of a cell is identified at fixed time intervals then the cell trajectory can be reconstructed by joining the sequential time points with linear segments. This zig-zag line approximates the actual trajectory for sufficiently small time intervals. The direction of motion between successive timepoints t-1 and t define the direction of motion at time t. In contrast to the random walk model, this implementation is applicable both in random and biased cell motion in anisotropic substrata. Additionally, the Markov chain approach gives a more detailed description of cell motion compared to the random walk approaches. It can discriminate between motility and stops and provides a more detailed representation of the directionality of motion, employing more than one descriptor (e.g., transition state probabilities and average waiting times) to define persistence. A detailed comparison (219) of the random walk and the Markov chain models based on 2D migration data for endothelial cells indicated that all the Markov locomotory parameters influence the speed S and the persistence P estimated by the persistent random walk model. The persistent random
walk model can provide a measure of the speed of locomotion $S$, appropriately weighted to account for the frequency and duration of cell stops. The persistence time $P$, however, is a composite descriptor of all the aforementioned Markov locomotory parameters [3-7] (222).

Tumor cell invasion and migration have been the subject of several reports but the dynamic migratory behavior of individual tumor cells and its modulation by the 3D microenvironment are still open fields for investigation. The present work focused on two major objectives: the development of a fully automated system that allowed quantification of the 3D migration and invasion at the cellular level, and the implementation of a novel Markov chain model that assessed the effect of ECM proteins on cancer cell motility and invasive potential. An innovative thirteen-state Markov chain modeled the gel infiltration by tumor cells and quantified the potential for invasive or lateral motion and cell stops, the duration of motion towards certain directions, the frequency of turns to specific directions and the ultimate probabilities for directional motion. The analysis was completed with the calculation of the speed of the individual cells over time and the dynamic development of the invasion profile in the three dimensional matrix. The current study described the tumor infiltration of 3D matrices with a dynamic set of descriptors for speed, directionality, and infiltration depth at the cellular level, and showed that tumor invasion and migration depend on both the cell type and the tumor microenvironment.
II. DEVELOPMENT OF AN IN VITRO MODEL FOR

ASSESSMENT OF TUMOR CELL INVASION AND MIGRATION

AT THE CELLULAR LEVEL

1. Design of a 3D Tumor Infiltration Assay
   a. Extracellular Matrix Analogs

Three-dimensional extracellular matrix analogs were prepared by gelation of extracellular matrix components. Gels 2-3 mm thick were formed in polycarbonate filter wells of 2.5 cm diameter and 8 μm pore size, accommodated in 6-well plates (Figure 3). Two different gel compositions were used to evaluate tumor cell invasion and migration: plain collagen type I gels and “alloy” gels made by mixing collagen type I and Matrigel®. The collagen gels were prepared by further modifying the protocol of Faassen et al. (223).

The plain gels contained 1.8 mg/ml collagen type I, Vitrogen® (Cohesion, Palo Alto, CA). Vitrogen® is a solution of purified pepsin-solubilized bovine dermal collagen type I. Overall the composition of the collagen type I gels was 61.7%v/v Vitrogen®, 29% Dulbecco’s Modified Eagle Medium (1x), 9.1% Dulbecco’s Phosphate Buffered Saline (DPBS) (1x) with calcium chloride and magnesium chloride, 0.2% Penicillin-Streptomycin (P/S).

The alloy gels contained 1.7 mg/ml collagen type I and 0.8 mg/ml Matrigel® (Collaborative Biomedical, Bedford, MA). Matrigel® is a basement membrane extract from the Engelbreth-Holm-Swarm (EHS) mouse sarcoma. Its major component is laminin, but also includes collagen type IV, heparan sulfate proteoglycans, entactin, nidogen and growth factors that naturally occur in the EHS tumor, such as TGF-β,
fibroblast growth factor, and tissue plasminogen activator. The alloy gels were a mixture of 57%v/v Vitrogen®, 8% Matrigel®, 27% DMEM, 7.8% PBS, 0.2% P/S.

The gel components for both gel types were mixed aseptically while kept on ice to prevent gelation. 1.1 to 1.5 ml of the collagen mixture was poured on each filter and the filters were placed in a humidified CO₂ incubator to gelate. After one hour, 1 ml of DMEM was placed on top of the gels and 1.5 ml DMEM in the lower compartment of the multiwell plate, and the gels were cured in the incubator. After twelve hours, the media on top of the gels and at the lower compartments was replaced with the same quantity of invasion medium (DMEM containing 0.5% FBS and 1% P/S). Four hours later, the invasion medium was renewed and the tumor cells were seeded on top of the gels.

b. Tumor Cell Culture

Human fibrosarcoma HT-1080, passage number (P) 19, and human breast adenocarcinoma MDA-MB-231 P27, were acquired from the American Type Culture Collection (ATCC). The cells were expanded in 75 cm² tissue culture flasks using as culture medium Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% Fetal Bovine Serum (FBS), and 1% Penicillin-Streptomycin (P/S). Upon 90% confluence the P20 HT1080 and the P28 MDA-MB-231 cells were detached with short exposure to trypsin, suspended in 95% cell culture medium and 5% DMSO and stored in liquid nitrogen in the ratio of (T-75 flask) : (6 cryogenic vials). Thereafter, proliferating cells were maintained in 25 cm² surface area tissue culture flasks. All proliferating cells were maintained in a 5% CO₂ humidified incubator.
c. Cell Seeding

The passage number of the tumor cells was kept constant for all the tracking experiments. At 90% confluence, HT1080 P21 cells or MDA-MB-231 P29 cells, were harvested with short exposure (1-2 min) to 5 mM EDTA in Ca$^{2+}$ and Mg$^{2+}$ free PBS. After detachment, the cells were washed three times with invasion medium (DMEM containing 0.5% FBS and 1% P/S) by successive centrifugation at 1500 rpm for 5 min and re-suspension in invasion medium. Then the cells were suspended in invasion medium and seeded on the top of the gels in a density of $3 \times 10^3$ cells/cm$^2$. Finally, the gels were placed in a humidified 5% CO$_2$ incubator until the automated tracking was initiated, 15 hours post seeding for the HT-1080 and 24 hours post seeding for the MDA-MB-231.
Figure 3. The extracellular matrix analogs are extracellular matrix proteins that form a gel on a polycarbonate filter. The filters are accommodated in multi-well plates for simultaneous tracking of several specimens. The gels are immersed in media that may contain factors stimulating or inhibiting invasion or migration.
2. Development of a Fully Automated System for Three-Dimensional Cell Tracking

a. Experimental Equipment

The experimental equipment for the automated cell tracking is comprised of individual subunits devoted to environmental control, optics, automation of time-lapse image acquisition/analysis and data analysis.

A Plexiglas chamber surrounded the microscope stage and optics to help maintain environmental control. A heater-controller (Nicholson Precision Instruments Inc., Beheads, MD) thermostated the chamber at 37 °C. A CO₂ controller (Forma Scientific Inc., Marietta, OH) automatically buffered the air in the chamber at 5% CO₂ to keep the gels at physiologic pH. 2-4 wells of the 6-well plate that contained the gels were filled with sterile water to provide humidification at the plate microenvironment. Under these conditions of the stage environment, the gels maintained their original dimensions for at least 3 days, as found by monitoring the top of the gel. In addition, the pH was maintained at 7.2 as verified by measurements before and after the tracking experiments.

During the automated cell tracking the 6-well plate containing the gels was mounted on an inverted microscope, a Nikon Diaphot TMD, equipped with Hoffman Modulation Contrast Optics (Modulation Optics, Greenvale, NY). The 10X objective was used for all the tracking experiments. The microscope stage was motorized and connected to a MAC2002 stage controller (Ludl Electronic Products Ltd, Hawthorne, NY) equipped with X, Y and focus (Z) control modules. The repeatability of the positioning was 1 µm assessed with repeated positioning of a calibrated specimen.
The heart of the automated-cell-tracking system was an IBM Intellistation M Pro Pentium II (266 MHz, 512 MB RAM) and Optimas® 6.2 image analysis software (Media Cybernetics L.P., MD). Optimas contains several standard image analysis functions that can be integrated into custom structural macros. All the programs for Stage Positioning, Image Acquisition, Image Analysis, Cell Identification and Trajectory Reconstruction were written in the interpreter Analytical Language of Images (ALI) supported by Optimas® software. The Optimas® image analysis software package contains standard image acquisition and image analysis functions that were embedded in the designed macros.

Computer programs automatically controlled the XYZ positions of the stage via an RS-232 interface. During the performance of the image acquisition routine (see also Image Acquisition section), the gel sample was placed at desired XYZ positions where images of the corresponding field of view (0.28 mm²) were acquired with a Panasonic WV-BL200 video camera. The images were digitized with the FlashPoint 128 capture board (Integral Technologies, IN) and then either fed to the Image Analysis routine for on-line analysis, or saved to the hard disk for off-line analysis. Compact disks (CDs) were used for long term data storage. The dynamic link exchange feature of the Optimas 6.2® software additionally allows export of the data directly to Microsoft Excel spreadsheets were the data were plotted or analyzed. The Data Analysis was mainly a shared task between ALI and Matlab programs.
Figure 4. The experimental equipment.
b. **Infrastructure of the 3D Cell Tracking Program**

Cell tracking is the extraction of the relative position of individual cells as they migrate over time. Therefore tracking requires a means of scanning the migration substrate at frequent time intervals and subsequently identifying the cells and quantifying their translocation. The tracking program developed and used for this thesis is a composition of four subprograms. Three of these routines are executed repeatedly at each time-point to perform: (i) stage positioning and 3D image acquisition, (ii) image analysis and (iii) cell identification. Finally, the output of the three routines over the total tracking period, is fed to a (iv) trajectory reconstruction subprogram, which builds the cell trajectories in a time ordered fashion. Figure 5 shows a simplified overview of the tracking program. More detail is given in Appendix A.
Initialize XYZ origin

For \( Z = 0 \ldots (\text{total Z sections}) \)
Move to position "Z"
Acquire Image ♦ Identify Objects ♦ Extract X, Y, GV

For Reference Section = 0..(total reference Z sections)
For obj = 0..(total obj in the Z reference section)
♦ Construct window Wxy(obj)
♦ \( Z(\text{cell}) = \max(\text{variation GVi}), \ i \in \text{Wxy(obj)} \)
♦ Append Position Vector \( P(t, X(\text{cell}), Y(\text{cell}), Z(\text{cell})) \)

Figure 5. Simplified overview of the 3D tracking algorithm.
i. **Stage-Positioning and 3D Image Acquisition**

A 3D gel provides a field for cellular motion in all three dimensions. With time the tumor cells initially seeded on the top of the gel are subjected to a continuous positional change as they migrate on the gel surface, or invade the matrix. 3D cell tracking requires a means of scanning the three-dimensional substrate over time, and a technique for "visualizing" the cells contained in the scanned volume so as to extract their positions. Images were acquired over time via a 3D scanning pattern constructed of a mosaic of meandering XY fields of view combined with optical sectioning at each field. More specifically, the "stage positioning and image acquisition" program at each time point gradually moves the stage (and therefore the gel specimen) at a series of X and Y positions forming a pattern of meandering XY fields. At each individual XY field of the XY mosaic, optical sectioning is performed by automatically moving the objective (i.e. the focal plane) at gradually decreasing (or increasing) Z positions across the axis of the gel, with simultaneous image acquisition at each section (Figure 6).

For all of the experiments, a nine-field mosaic was formed by three fields at the X direction and three fields at the Y direction. Each frame grabber captured field is 630 x 473 μm, resulting in a XY projection of the tracked volume of 1890 μm x 1420 μm (0.28 mm²) (Figure 7). The step for the optical sectioning, or the distance between sequential Z-fields, was 20 μm (Figure 8). The 3x3 XY mosaic and a maximum Z depth of about 300 μm defined a tracking volume large enough to contain the entire paths of a statistically sufficient number of HT1080 and MDA-MB-231 cells during a tracking period of about 2 days. For a cell seeding density of 3x10³ cells/cm² an average of 8 cells was tracked per field of view or around 70 cells per mosaic. The same 3D Image
acquisition pattern can be replicated over time at either all or selected wells of a multi-well plate.
Figure 6. 3D image acquisition with optical sectioning at meandering XY fields of view.
Figure 7. 3x3 xy mosaic of 630x473 μm fields of view.
Figure 8. Optical sectioning in a at a 630 x 473 μm field of view.
ii. Image Analysis

The Image Analysis was performed at the individual 8 bit (256 gray levels) 640 x 480 pixels, 300 kByte frame grabber captured images. The images were subjected to average 3x3 filter operations for noise removal. This treatment prepared the images for gray level thresholding whereby the outline of each cell was defined. Then the variation of gray level in the outlined area and then the positional coordinates (X, Y) of the area centroid (which defines the cell location) were extracted. Morphometric features of the area such as the size, length, perimeter and orientation could be also extracted at this point. Objects with areas much smaller or much larger than the average cell area were discarded from the image analysis output.

iii. Cell Identification

Due to light scattering throughout the 3D gel, traces of a certain cell exist not only at the optical sections that physically dissect the cellular body but also at the sections acquired above and below its body limits. Therefore, multiple sets of (X, Y, Z, area size, gray level variation, etc.) in the image analysis data corresponded to the same cell. The role of the cell identification routine of the tracking program was to find the (X, Y, Z) triplet that best represents an individual cell. The triplet (X,Y,Z) assigned as the "position of the cell" is a point of the Cartesian space selected to represent a three dimensional cellular body. This point fitted best the selection criteria imposed by the cell identification program.

All traces of a certain cell have quite similar X, Y positional coordinates, however the out-of-focus traces of the cell are more blurred than the sharp trace of an in focus cell.
Therefore, an XY-window is placed around each object identified by the image analysis routine and the Z position of an actual cell was calculated as the Z section where the cellular trace in the corresponding XY-window had the maximum variation in gray level. The extracted Z-position represents either the "actual" Z-coordinate of a cell or the Z-coordinate that is nearest to the "actual", since the few acquired optical sections do not necessarily include the in-focus traces of all the cells contained in the 3D matrix.

iv. Trajectory Reconstruction

Every series of image acquisition, image analysis and cell identification resulted in an update of the positional coordinates (as well as morphological features) of all cells throughout the gel sample at each time-point. However, there is no indication as to what the migration path of the individual cells is during the tracking period. During this time cells have migrated away from their starting positions, some cells might have exited, or entered the tracking volume, or they might have divided. The "trajectory reconstruction" program assembled the (t, X, Y, Z) data from the "cell identification" program in a time ordered fashion to form the path of migration for each individual cell.

The basis of the trajectory reconstruction is a nearest neighbor selection modified to maintain the individuality of cells with crossing paths, and to sense cells that move in or out of the 3D-mosaic area tracked. The major criterion for a nearest-neighbor selection is that the translocation of a cell between two sequential time points should be less than a certain distance determined by the speed of cellular locomotion. This distance defines a spherical window around each individual cell that is following the motion of the cell over
time and contains the positions of the cell's neighbors at the next time-point (nearest neighbor window). More than one neighboring cell could exist in the nearest neighbor window of a certain cell. Thus starting with the known position of a cell at time t ([t, X, Y, Z]), the most probable new position at time t+1 for this cell is the position occupied by a cell identified at t+1 ([t+1, X', Y', Z']) that falls in the nearest neighbor window of the cell at time t and has the minimum Euclidean distance from this cell \( ((X-X')^2 + (Y-Y')^2 + (Z-Z')^2)^{1/2} \).

Comparisons between automated and manual tracking, revealed that the calculation of the nearest neighbor is more accurate during a "two-stage selection" in a cylindrical window rather than a spherical one. Thus for a cell with position vector [t, X, Y, Z] its neighbors at the next time point t+1 fall in the cylindrical window with axis defined by the XY-position of the cell at time t ([t, X, Y]) and radius a distance depending on the cell speed. The most probable position of the cell at t+1 is the neighbor that belongs in the cylindrical window and has minimum XY Euclidean distance.
3. ANALYSIS OF THE 3D CELL TRAJECTORIES

The trajectories of cells migrating through a 3D matrix can be analyzed to extract the characteristics of motion and quantify the interaction between the cells and their surrounding microenvironment.

The tracking program extracts the 4D position vector \([t, X, Y, Z]\) (Figure 9) of all the cells that existed in the 3D tracking field during the tracking period. Only cells residing the tracking field for 95% of the total time were fed to the Data Analysis program since the goal was to study long-term behavior of the cells. An average of 60 HT1080 and 80 MDA-MB-231 cells per gel met this criterion. The efficiency of the automated tracking increased with lower cell motility and seeding density and reached a maximum of 93% compared to the manual approach. The Data Analysis was a shared task between ALI and Matlab programs. The descriptors for cell migration and invasion, that have been calculated from the 3D cell trajectories, are described at the following sections.

a. Speed of Cell Locomotion

The instant speed of locomotion at time \(t\), \(v_i(t)\), is the displacement between the successive time-points \(t\) and \(t-1\) over the time lapse \(\Delta t\):

\[ v_i(t) = \frac{d_i(t)}{\Delta t} \quad [8] \]

The average speed of all the individual cells was calculated considering only the time points when the cell was in motion or displacements larger than the average half-cell diameter (Equation 1). \(d_i(t)\) is the cell displacement of the cell \(i\) between time \(t\) and the
previous time-point \((t-1)\), \(\Delta t\) is the time lapse for image acquisition, and \(N\) is the number of times that the cell has a non-zero displacement during the tracking period.

\[
\vec{v}_i = \frac{\sum_{t=1}^{N} d_i(t)}{N\Delta t}
\]  

[9]
Figure 9. Typical trajectory of a tracked HT1080 cell in a collagen type I gel. The points represent the sequential positions of the cell centroid every 30 min as extracted from the cell tracking program throughout the tracking period.
b. The Markov Chain Theory

Let us consider a random variable $X_t$ which at any time $t$ can take any value from the set \( \{i_1, i_2, \ldots, i_n\} \), which contains all the possible values, or states, of the variable $X$. The stochastic process $[X_t, t \geq 0]$ is a Markov chain if for all times $0 \leq t_1 < t_2 < \ldots < t_n < t$ and $\tau \geq 0$, and for any states $i_1, i_2, \ldots, i_n$, $i, j$ holds (224):

\[
P(X_{t+\tau} = j \mid X_t = i, X_{t_1} = i_1, \ldots, X_{t_n} = i_n) = P(X_{t+\tau} = j \mid X_t = i)
\]

[10]

The above equation implies that if the process is in state $i$ at time $t$, the probability of being in state $j$ at time $(t+\tau)$ depends only on the state of time $t$ and is independent of the states that the process entered before that time. A set of parameters can be calculated from the Markov model to quantify the duration of maintaining a certain state, the probability of transition among the different states and to check if there is a preference for obtaining a certain state. These parameters have been used to model 2D and 3D cell migration (201, 211, 219-221).

Let the random variable $T_i$ be the time that a cell spends at state $i$ before shifting to another state. For a Markov process the waiting time at a state $i$ ($T_i$) has an exponential probability distribution function with a parameter $Q(i)$:

\[
p(T_i > t) = \int_0^t Q(i)e^{-Q(i)\tau}d\tau
\]

[11]
From the properties of the exponential distribution, the parameter \( Q(i) \) is the inverse of the expected value of the \( T_i \):

\[
Q(i) = \frac{1}{E(T_i)} \tag{12}
\]

In general \( p(j/i) \) is the probability of the process currently at state \( i \) to shift into state \( j \). Let us assume that as \( t \) goes theoretically to infinity (\( t \to \infty \)), there exist an ultimate set of probabilities so that the probability of the process to obtain the state \( j \) at time \( t \) does not depend on the initial state \( i \):

\[
p_i(j/i) \to \tilde{p}(j) \quad \text{as} \quad t \to \infty \quad \text{for any initial state } i \tag{13}
\]

The values of the set: \( \tilde{p}(j), \quad j = 0, 1, ..., n \) are the steady-state probabilities.

It can be shown that the set of steady-state transition probabilities is the unique solution of the following algebraic system of \( n \) linear equations (224, 225):

\[
Q(j)\tilde{p}(j) = \sum_{k \neq j} p(j/k)Q(k)\tilde{p}(k) \quad j = 0, 1, ..., n \tag{14}
\]

The applicability of the Markov Model is generally based on two assumptions (220-223). First that the probability that a cell will move to another state depends only on the present state and not on the past history. That can mathematically expressed as:

\[
p(X_{t=\text{future}} = i \mid X_{t=\text{past}} = j, X_{t=\text{present}} = k) = p(X_{t=\text{future}} = i \mid X_{t=\text{present}} = k) \tag{15}
\]
The second assumption states that the waiting times for each of the 13 Markov states, are exponentially distributed (see also at the Results section).

i. **Continuous Markov Model for 3D Cell Trajectories**

The Markov Chain model can quantify invasion and migration at the cellular level. A cell trajectory is practically a series of points in the Cartesian space that represent the cell centroid over time. Let us assume that the Cartesian space, in which the cells move, is divided into directional subsets, i.e. the Markov states. When the cell centroid at time \( t \) is the point \( A \) and at time \( t+1 \) the point \( B \), the linear segment \( AB \) defines the direction of motion between \( t \) and \( t+1 \). By applying the Markov theory, the trajectory of each cell is described as a stochastic process so that the direction of motion between successive time points is mapped to one of the Markov states. Therefore, every change in the direction of motion will be a change of state. Assuming random directional changes, independent of (i) the previous turning history and (ii) the duration of a certain directional motion preceding a directional change, the cell trajectory is a Markov Chain. Practically, an infinite number of possible directions are achieved during cellular motion. However, the Markov model is applied to a finite number of states, which for the migration case will be partitions of the Cartesian space (198, 201, 211, 219, 220).

The first Markov model for 3D cell migration was presented by Noble and Levine (225) and modeled the cell migration in 3D using eight directional and one stationary Markov state. We expanded this approach by defining a novel Markov chain with twelve directional and one stationary state in order to distinguish between lateral and invasive motion during gel infiltration. More specifically, the three-dimensional Cartesian space
is partitioned in twelve subspaces containing all the possible directions of cellular motion as shown in Figure 10. Therefore, every possible direction of motion is mapped to one of the twelve directional subspaces. States 1, 2, 3 and 4 represent lateral motion on the XY plane, which is parallel to the gel surface. For example, a cell is at state 1 between two successive time points if it is moving towards the X+ Y+ directions. States 5-8 describe cells moving towards the top of the gel at various X and Y directional combinations, while states 9-12 describe invading cells. Practically, the direction of motion of a cell is calculated from the positional differences $\Delta x$, $\Delta y$, and $\Delta z$ between successive time-points and then the appropriate Markov state is assigned to a cell. If the displacement of a cell between two successive time-points is less than half-cell diameter the cell is assigned the stationary state (state 0).
Figure 10. Definition of the 13 states of the Continuous Markov Model. The Cartesian 3D space is divided into thirteen subspaces (states). Each possible direction of motion is mapped to one of the twelve directional subspaces. States 1-4 represent cells moving on the XY plane (parallel to the gel-medium interface). States 5-8 describe cells moving towards the top of the gel (positive Z direction) and states 9-12 represent cells invading the gel (negative Z direction). For example a cell is at state 1 if it moves on the Z=0 plane in positive X and positive Y direction. Similarly, a cell is at state 12 if it is invading the gel (negative Z direction) heading in a positive X and negative Y direction. Cells at state 0 (stationary state) move less than half-cell diameter (HCD) between two successive time points.
ii.  **Cell Migration Parameters from the Continuous Markov Chain Model**

a) **Waiting Times** \((T_i)\) which give the average time that cells spend at each state, or the persistence for a certain direction. The expected value of the waiting time at state \(i\) is:

\[
E(T_i) = \frac{\text{(total time that all cells spent in state } i\text{)}}{\text{(total number of transitions out of state } i\text{)}}
\]  \[16\]

b) **Transition Probabilities** \(P(i/j)\) which give the probability of a cell currently at state \(j\) to transit at state \(i\) (Equation 3). They quantify the probability of individual cells or cell subpopulations to shift from one state to another. They represent changes in the direction of motion, and thus the turning behavior of the cells.

\[
p(i/j) = \frac{\text{(number of transitions from state } j\text{ to state } i\text{)}}{\text{(number of transitions from state } j\text{ to any other state)}}
\]  \[17\]

c) **Steady-State Probabilities** \(P(j)\) quantify the probability of being at state \(j\) as \(t \rightarrow \infty\).

Apart from quantifying the transient migratory behavior of the cells, it is useful to predict if there is an ultimate trend, or a preferential directionality among the cell population. The steady-state probabilities quantify the ultimate directionality of motion, therefore disproportionately high values reveal such trends.

c.  **Invasion Profiles**

The distribution of the cell population at the various \(Z\)-sections throughout the gel was plotted over time during the tracking period, i.e. the cell population percentage at each \(Z\)-section from the gel surface up to the maximum depth of image acquisition, and at each time-point from time zero until the end of the tracking period.
4. Statistical Analysis of the Data

Three separate sets of experiments (n=3) have been performed for each cell line on each matrix and the one-way ANOVA was performed to test the significance of the data. Significance was assumed for P-value of the test <0.05. The data is presented and plotted as average ± standard deviation.
III. TUMOR CELL TRACKING IN EXTRACELLULAR MATRIX ANALOGS

4. Results

a. Efficiency of the Automated Cell Tracking

The efficiency of the cell tracking is subjected to cell identification and trajectory reconstruction limitations. Comparisons between the manual and automated cell identification and trajectory reconstruction showed 80-90% efficiency for the optical tracking depending mainly on the cell type and seeding density. Even though the efficiency of image analysis and cell identification was similar for both cell types, the efficiency of trajectory reconstruction was higher for the MDA-MB-231 cells due to their lower motility and lower probability for transitions among the Z sections, as shown in detail at the following sections. Figure 11 analyzes the error sources affecting the efficiency of the tracking in the case of the lowest efficiency. "Negative positive" errors are cells lost during cell identification. "Positive negative" errors identified cells that are practically noise. "Out of NN window", are cells lost from the trajectory reconstruction due to large jumps in their speed that shift them out of the nearest neighbor identification window. "Crossing mismatch" are mismatches at the trajectory reconstruction due to path crossing of different cells. For MDA-MB-231 cells the efficiency was practically limited only by the cell identification errors and reached the maximum of 90% since crossing mismatch and exiting from the nearest neighbor window was minimal. The minimum efficiency of 80% was for HT1080 cells in alloy gels.
Figure 11. Factors determining the minimum efficiency of the automated tracking.
b. **Validity of the Continuous Markov Model Criteria**

Figure 12 and Tables 4(a) and 4(b) summarize the results for the validation of the Markov criteria for the trajectories of HT1080 cells in collagen type I gels. The distribution of the waiting times satisfies the first assumption of the continuous Markov model (see also the implementation of the Markov model at previous section).
Figure 12. Distribution of the waiting times for the stationary (state 0) and the directional Markov states (state=1,2,...12). At all the plots the abscissa is the number of time intervals (frames) spent by the cell at the certain state. The ordinate gives the number of times a cell spent that number of frames at the corresponding state.
Table 4a and Table 4b include the evaluation for the validity of the second Markov criterion for HT1080 cells in collagen I gels. To validate the criterion, the transition probabilities were calculated between the stationary and each one of the generalized cumulative directional states, i.e. the planar (sum of the probabilities corresponding to states 1-4), the Z+ (sum of probabilities for states 5-8), and the Z- (sum of probabilities of states 9-12), for 70 HT1080 cells in a tracking experiment. The Table 4a gives the probabilities of cells that exist at state 0 at the time-point 20, to be at each one of the general directional states at time-point 25. Table and 4b summarize the probabilities for serial transitions from a past state ($X_{\text{past}}$) at $t=10$, to the present state ($X_{\text{present}}$) at $t=20$, and from the present to a future state ($X_{\text{future}}$) at $t=25$. It is obvious that the serial transition probabilities (memory) (Table 4b) do not match the non-memory transitions (Table 4a). In other words there seem to be a bias in the turning behavior of the cells. This is expected from the preferential transition probabilities of the HT1080 cells to the planar state and the oscillatory transitions among the Z+ and Z- states. The second criterion is similarly not satisfied for the biased transitions of the MDA-MB-231 cells due to preference for the stationary state and Z+/Z- transitions. The non-memory criterion is best satisfied for the HT1080 in alloy gels due to higher freedom of directional motion (see description of transition probabilities).

Despite the fact that the non-memory assumption is not fully satisfied for the gel infiltration bioassay, the Markov analysis has been applied for the analysis of the 3D cell trajectories since the only Markov output depending on the mathematical assumption of the model is the steady-state probabilities.
Table 4

a. State transitions between current ($t=20$) and future ($t=25$) time points for HT1080 cells in collagen I gels.

<table>
<thead>
<tr>
<th>State transition</th>
<th>$p(X_{t=25} = i / X_{t=20}=0)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$0 \rightarrow \text{planar}$</td>
<td>0.52</td>
</tr>
<tr>
<td>$0 \rightarrow Z^+$</td>
<td>0.13</td>
</tr>
<tr>
<td>$0 \rightarrow Z^-$</td>
<td>0.35</td>
</tr>
</tbody>
</table>

b. State transitions between past ($t=10$), current ($t=20$) and future ($t=25$) time points for HT1080 cells in collagen I gels.

<table>
<thead>
<tr>
<th>State transition</th>
<th>$p(X_{t=25} = i / X_{t=10}=0, X_{t=10}=Z^+)$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$Z^+ \rightarrow 0 \rightarrow \text{planar}$</td>
<td>0.25</td>
</tr>
<tr>
<td>$Z^+ \rightarrow 0 \rightarrow Z^+$</td>
<td>0.10</td>
</tr>
<tr>
<td>$Z^+ \rightarrow 0 \rightarrow Z^-$</td>
<td>0.65</td>
</tr>
<tr>
<td>planar $\rightarrow 0 \rightarrow \text{planar}$</td>
<td>0.71</td>
</tr>
<tr>
<td>planar $\rightarrow 0 \rightarrow Z^+$</td>
<td>0.08</td>
</tr>
<tr>
<td>planar $\rightarrow 0 \rightarrow Z^-$</td>
<td>0.21</td>
</tr>
</tbody>
</table>
The data analysis revealed distinct migratory and invasive phenotypes among the two cell lines and gel types, characterized by significantly different patterns in the calculated parameters.

c. **Speed of Cell Locomotion**

   i. **Instant and Average Speed of Cell Locomotion**

   The speed of locomotion was calculated for each individual cell for all the successive time-points during the tracking period. Then the average speed was extracted over the total time that the cell was in motion. These average speed values for all the cells in a typical tracking experiment are shown at Figure 13 for HT1080 cells on collagen type I gels.
Figure 13. Average speed values for all the HTi080 cells of a typical tracking experiment in alloy gels. In the current experiment, 78 cells were tracked for 35 hours.
ii. Distribution of the Cell Population with the Average Speed of Locomotion

The distribution of the cell population with the average speed of locomotion is approximately normal. Figure 14 shows the distribution of the HT1080 and the MDA-MB-231 cells respectively with the average speed of locomotion in alloy gels.
Figure 14. Distribution of the HT1080 and MDA-MB-231 cell populations with the average speed of locomotion in alloy gels.
For easier comparison, the distribution of the cell populations was plotted versus grouped ranges of the average speed (Figure 15). Three speed ranges were considered with respect to the average speed of the total cell population, i.e. 0.5±0.03 μm/min for MDA-MB-231 cells in alloy, 0.6±0.06 μm/min for HT1080 cells in plain collagen, and 0.7±0.06 μm/min for HT1080 cells in alloy. More specifically the range 0-0.5 μm/min contains speed values lower than all the three average speeds, the range 0.5-0.7 μm/min contains the average speeds, and the range 0.7-2.0 μm/min includes speeds higher than all the average values. The data show an almost normal distribution for all the cases. Matrigel increased the average speed of fibrosarcoma cells and shifted the cell distribution at higher speeds. However, the change was not significant (p=0.11). Furthermore, statistical comparison (one way ANOVA) between the fibrosarcoma subpopulations at low (0-0.5 μm/min), average (0.5-0.7 μm/min) and high (0.7-2.0 μm/min) speed ranges revealed no significant differences between collagen type I and alloy gels.

Speed comparison at the cell type level showed significant differences between the HT1080 and the MDA-MB-231 cells. The cell subpopulations at the lower speed range of less than 0.5 μm/min, were similar for both cell lines and matrices and approximately 37% of the total cell population. However, the subpopulations at the average and high speeds were characteristic of the tumor cell type. The majority of HT1080 cells maintained speeds higher than 0.7 μm/min, both in collagen I (38.0±4.3%) and in alloy (46.6±9.1%), while only 24.8±2.9% of the adenocarcinoma reached this speed level.
Figure 15. Cumulative distribution of the cell population according to the average speed of cell locomotion for HT1080 cells in collagen I gels, HT1080 cells in alloy gels, and MDA-MB-231 cells in alloy gels. The error bars show the calculated standard deviation. The asterisks indicate significant differences.
d. Parameters From The Continuous Markov Model

At the following presentation of the Markov model parameters some data are combined into sums or the averages among geometrically equivalent groups of the Markov states (see Figure 10) in order to facilitate the description. Therefore the waiting times, the transition probabilities and the steady-state probabilities are compared among: the stationary state (state 0), the state of lateral motion, which contains states 1-4, the state of anti-invasive (Z+) motion, states 5-8, and the state of invasive (Z-) motion, states 9-12.

i. Waiting Times

The waiting times in the Markov states followed significantly different trends depending on the cell type and the gel composition as depicted in Figure 16. The times that the HT1080 cells spent in each of the thirteen Markov states were comparable between the collagen type I and the alloy gels. Subsequently the geometrically equivalent states had similar waiting times in both matrices. There was no significant difference between the average waiting time at the stationary state of 34.3±2.0 min in collagen I gels and 29.7±4.4 min in alloy gels, or 34.9±3.1 and 31.4±1.6 min respectively at a state of lateral motion, or 26.6±0.4 and 26.8 ±1.4 min at anti-invasive (Z+) motion, or 27.1±0.6 and 27.5 ±1.3 at invasive (Z-) motion.

Significant differences appeared in the waiting times among different directionalities of motion. The lateral (XY) motion and the duration of the stationary state of HT1080 cells at both matrices were significantly longer than the Z-motion. Fibrosarcoma cells stayed stationary, or maintained lateral motion for significantly 5-10 min longer than the average duration of invasive or anti-invasive motion. On the other
hand, the waiting times of fibrosarcoma cells in both gel types were not significantly
different comparing the stationary state versus the lateral (XY) motion, or the duration of
invasive (Z-) versus the anti-invasive (Z+) motion.

Matrigel decreased both the persistence for the stationary state (29.7±4.4 min),
and for lateral motion (31.4±1.6 min), but it did not affect much the duration of the anti-
invasive (26.8 ±1.4 min) or invasive (27.5 ±1.3 min) motion, compared to the collagen I
gels. The reduction of the stationary time brought the difference between stationary state
and Z motion in alloy gels, to insignificant levels. However, the duration of lateral
motion of HT1080 cells on alloy gels was still significantly 4 min longer (compare with
5-10 min in collagen I) than their Z motion.

The waiting times of the MDA-MB-231 cells on alloy gels followed a totally
different trend. These cells showed a well-maintained stationary phenotype. Their
stationary time of 55.9±7.5 min was dramatically 30-35 min longer than the average
duration of planar (23.7±1.0 min), anti-invasive (21.6±0.8 min), or invasive (21.6±0.9
min) motion while the lateral, Z+ and Z- motion were comparable. The stationary time
of the MDA-MB-231 cells was significantly about 20 min longer than the stationary time
of the HT1080 cells, while in contrast to the HT1080 there was no preference for any of
the directional states since their planar motion was only slightly (p=0.08) longer than the
Z motion.
Figure 16. The average waiting time at each Markov state for HT1080 cells in collagen I gels, HT1080 cells in alloy gels, and MDA-MB-231 cells in alloy gels. The error bars represent the calculated standard deviation. The planar motion of the HT1080 cells was significantly longer than their Z motion. For the MDA-MB-231 cells, the duration of the stationary state was significantly longer than the duration of all the directional states. In all the three cases no significant differences were observed among geometrically equivalent states (see also text).
ii. **Transition Probabilities**

For a 13-state Markov model there exist $12 \times 13 = 156$ possible transitions among different states. Figure 17 shows the average transition probabilities for HT1080 cells in collagen type I gels, HT1080 cells in alloy gels and MDA-MB-231 cells in alloy gels.

The transition probabilities revealed an interestingly distinct turning behavior among the two cell lines and gel compositions. HT1080 cells in collagen type I gels exhibited active turning behavior with high preference to transit to and maintain a state of planar motion and eminent oscillatory $Z^+$ to $Z^-$ transitions. The addition of Matrigel to the matrix enhanced the transition profile for fibrosarcoma, with turns that were more frequent among the directional states and less probable transitions to the stationary state. In the alloy the transition profile was "sharper" revealing more probable turning behavior.

MDA-MB-231 cells in alloy gels gave an even smoother transition profile revealing little turning activity. Their relatively flat transition profile contrasted with unproportionally high probabilities of motile cells to become inert. The oscillatory $Z^+$ to $Z^-$ transition pattern was still well represented.

To facilitate the comparison of the turning behavior among the two cell lines and the two matrices, Figure 18 summarizes the transition probabilities among groups of geometrically equivalent states.
Figure 17. Average transition probabilities for:
(a) HT1080 cells in collagen I gels, (b) HT1080 cells in alloy gels,
and (c) MDA-MB-231 cells in alloy gels.
Figure 18. Cumulative transition probabilities for: (a) HT1080 cells in collagen I gels, (b) HT1080 cells in alloy gels, and (c) MDA-MB-231 cells in alloy gels. Transitions are considered out of the stationary state, the state of planar motion (states 1-4), the Z+ state (states 5-8), and the Z- state (states 9-12). The new states are shown with solid bars for the stationary state, horizontal line pattern for lateral motion, with lines going up (///) for the anti-invasive motion (Z+), and with lines going down (\\\) for the invasive motion (Z-).
Cumulative Transition Probabilities for HT1080 cells in Collagen type I gels

Fibrosarcoma cells on collagen type I had high probability to shift into and maintain lateral motion. Stationary fibrosarcoma cells that become motile on collagen I gels had a highly favored 54.0±4.9% probability to enter a state of planar motion rather than an invasive (25.2±2.2%) or anti-invasive (20.8±2.6%) motion (p<<0.01). Transitions from the stationary to the Z+ or Z- states were not significantly different (p=0.15) despite the higher probability to enter an invasive state of motion.

HT1080 cells in lateral (XY) motion had 36.5±1.5% probability to maintain their planar motion. This probability was significantly higher than the probability to halt their motion (27.5±0.7%, p=0.0015), or start moving at the Z direction (p=0.0005). When entering a Z motion, planarly moving cells had a significantly higher probability to enter an invasive state (20.8±1.7%) rather an anti-invasive (15.2±2.3%) since the majority of the planarly moving cells were located on the top of the gel (P(Z-|planar)>P(Z+/planar), p=0.047).

The transitions from the Z+ and Z- states were of great interest. Invading cells had a strikingly high probability to transit into a state of anti-invasive motion and the opposite the most probable transition of cells moving towards the top of the gel was to start invading the gel. The next most favorable transition from the Z motion states was to the states of planar motion (about 30%). Overall, invading cells had high probability to maintain a state of motion and significantly smaller probability (about 19%) to halt their motion.
Cumulative Transition Probabilities for HT1080 cells in Alloy gels

The incorporation of Matrigel in the matrix abated significantly the characteristic feature of the collagen type I gels for preferential transitions to the states of lateral motion. Alloy gels increased significantly the invasiveness of the stationary and planar moving HT1080 sub-populations. Invasion was still the most probable Z transition from the stationary and planar states, while the transitions from the invasive and anti-invasive states were similar to the collagen I case with the oscillatory Z+ to Z- transition pattern still well represented.

Matrigel caused a significant 35% decrease in the probability of stationary cells to shift into a lateral motion accompanied with a significant 39.1% increase in the probability to become invasive (34.6±2.8%) and a 44.2% increase in the probability for anti-invasive motion (29.6±2.2%). However, the probability of stationary cells to transit into an invasive state was still significantly higher than the Z+ motion.

Additionally, Matrigel significantly increased by an extra 30.2% the probability of planar cells to become invasive (27.1±3.5%) and produced a 40.4% increase for anti-invasive (21.3±2.0%). Despite that, the planarly moving fibrosarcoma cells had 21% lower probability to maintain their two-dimensional motion (28.7±1.0%) compared to the plain collagen case, and this motion was still more probable than shifting into the stationary (22.9±3.2%) or the anti-invasive state.
Cumulative Transition Probabilities for MDA-MB-231 Cells in Alloy gels

The MDA-MB-231 cells alloy gels formed a very different transition profile. Although HT1080 cells maintained efficiently a state of motion, the MDA-MB-231 cells had a flatter transition profile, contrasting with high probabilities for immobility.

Stationary adenocarcinoma showed no preferential transition among the directional states. Transitions from the stationary to invasive states (39.8±8.8%) had higher probability than transitions to anti-invasive (29.1±5.8%), or planar states (30.9±14.5%) but the difference was not significant.

Laterally moving cells had a strikingly high probability to become stationary (47.2±5.0%). The probability of planar cells to continue their planar motion (20.2±1.9%) was higher (though not significantly) than entering invasive (18.4±4.9%) or anti-invasive (14.1±2.3%) motion. As in the HT1080 case, transitions from lateral to invasive states (planar→Z+) were more probable than the opposite transitions (planar→Z-) but the difference was not significant.

The invasive behavior of MDA-MB-231 cells deviated partially from the well-maintained oscillatory Z+, Z- transitions, a distinguished feature of HT1080 cells in both matrices. Invasive MDA-MB-231 cells were preferably (p<0.01) shifting to an anti-invasive motion towards the top of the gel (51.9±1.9%), compared to all the other transitions out of the invasive state. In parallel, their transition to the stationary state was also a very probable event (34.1±3.0%).

Quite interestingly, the transition from the anti-invasive states to the invasive states was not as specific as for the HT1080 cells since transitions to immobility were also comparably probable (P(Z-/Z+) = 43.7±3.4% > 39.0±1.6% = P(stationary/Z+), p=0.097).
iii. Steady-State Probabilities

There exist 13 steady-state probabilities describing the ultimate preference of motion as time goes to infinity, or a time independent directionality. The steady-state probabilities predict the likelihood, or the population percentage, of cells being stationary, motile, moving laterally or invading the matrix, based on information gathered throughout the whole tracking period. The steady-state probabilities for all the examined conditions are presented in Figure 19. Overall the stationary or the motile subpopulation (% motile=100-% stationary) and the subpopulations engaged into lateral and invasive or anti-invasive motion were very distinctive among the cell and gel types, yet a common feature was the similar probabilities for the X, Y directional states.

Matrigel altered the preference of fibrosarcoma for planar motion resulting to similar probabilities for planar, invasive and anti-invasive motion.
Figure 19. The average steady-state probabilities for HT1080 cells in collagen type I gels, HT1080 cells in alloy gels, and MDA-MB-231 cells in alloy gels. The error bars show the calculated standard deviation.
As for the transition probabilities, the sum of geometrically equivalent steady-state probabilities helped the visualization of the trends (Figure 20). Quite different motile behavior and directionality of motion were predicted for the two cell lines and the two gel types.

The matrix composition (plain collagen I versus alloy) did not affect significantly the motility of HT1080 cells. Fibrosarcoma cells were predicted to be highly motile with a subpopulation of 79.7±0.5% in motion in collagen I gels. The incorporation of Matrigel increased the motile fibrosarcoma sub-population to 82.9±3.2% but the effect was not significant (p=0.23). The major effect of matrix composition was expressed in the directionality of motion.

In collagen type I gels the majority (52.6%) of the motile fibrosarcoma population (41.9±0.7% of the total population) was involved in planar motion (Markov states 1-4), and a significantly smaller percentage 21.8% to anti-invasive (states 5-8), and 25.5% to invasive motion (states 9-12) (17.4±0.4% and 20.3±0.2% of the total population respectively). A unique result was that the invasive motion of HT1080 cells in alloy was significantly 16.7% more probable than the anti-invasive motion.

Even though Matrigel did not affect considerably the motility of fibrosarcoma, this basal lamina extract caused a very significant enhancement to the invasiveness, shifting cells from lateral to Z motion. The laterally moving sub-population of fibrosarcoma decreased significantly (p<<0.01) to 30.4±1.6% in alloy gels with parallel significant increase of the probability for Z motion from 37.7±0.5% to 52.4±2.8% (p<<0.01). Overall, the addition of Matrigel reduced the preference of fibrosarcoma for
planar motion to similar values of steady-state probabilities for planar, invasive and anti-invasive motion.

The sub-populations of stationary and motile cells were a very distinct feature among the studied cell-lines. About 80% of HT1080 cells were motile in both matrices while only 50.28±4.3% of the MDA-MB-231 cells were motile in alloy gels. Additionally, the migratory and invasive behavior of the MDA-MB-231 was strikingly different. Although relatively small, the motile subpopulation of adenocarcinoma cells was preferentially engaged in a Z motion (36.6±3.3%) rather than lateral motion (13.7±5.4%). As in the case of HT1080 cells in alloy, the steady-state probabilities for invasive and anti-invasive motion were similar. Quite interestingly, this Z motion potential of about 18% was comparable between the fibrosarcoma on plain collagen and the adenocarcinoma on alloy.

In all cases, there was no preferential X or Y directionality reflecting random planar motion.
Figure 20. Cummulative steady-state probabilities for HT1080 cells in collagen I gels, HT1080 cells in alloy gels, and MDA-MB-231 cells in alloy gels. The error bars show the calculated standard deviation. Matrigel did not enhance significantly the motility of HT1080 cells. However, it increased significantly the probability of Z-motion compared to the plain collagen case. Motile MDA-MB-231 cells had significantly higher probability for Z-motion rather than planar motion. The X, Y, Z directional states had similar probabilities for all the three cases (see also text).
e. **Infiltration Profiles over Time**

The distribution of the cell population with the invasion depth in the gel over time showed different characteristics for the two gel and cell types (Figure 21).

The Z distribution for the HT1080 cells at both gel types followed an invasion pattern reflecting advanced invasion of the 3D matrix and high transition frequency among the Z sections. With the lapse of time, the top of the gel was depleted from the invading cells that enriched the inner gel body. About 90% of the initially seeded fibrosarcoma cells are located from 20 to 260 μm inside the gel (12+36) 48 hours post seeding (end of the tracking period). At the same time, cells already in the gel were moving to all the directions including towards the top of the gel. Thus at a certain depth inside the alloy gel body, around 200 μm deep, a second peak in the cell concentration appeared shortly after the beginning of the tracking experiment. This behavior is expected from cells expressing a high motility and highly invasive phenotype. The cell concentration at the secondary peak and the Z location depend on the cell type, the matrix and the time. The invasion profile of fibrosarcoma on collagen I had similar trend, however the maximum invasion depth reached up to 150 μm while only 75% of the cell population was located from 20 to 150 μm during the same time.

HT1080 cells at both matrices invaded more vigorously comparing to the MDA-MB-231 cells forming a more robust invasion profile and a deeper invasion front. The invasion front (maximum invasion depth) was about 120 μm (24+36) 60 hours post seeding, less than half of the front of fibrosarcoma yet 9 hours later. Fibrosarcoma on collagen I gave
only a slightly deeper invasion front comparing to adenocarcinoma on alloy. However, MDA-MB-231 cells gave a significantly less efficient invasion since only 70% of the initial cells left the gel surface while more than 50% were located 20-40 μm deep.

The adenocarcinoma cells showed an invasion characterized by a decaying cell concentration along the negative Z dimension throughout the tracking period. This pattern is explained from the weak turning behavior and low motility such that the slow infiltration depleted the top of the gel from its cell count to increase the cell count at the neighboring Z sections. This pattern also represents initial stages of gel infiltration and could represent the invasion profile of fibrosarcoma shortly after seeding. Another distinct feature of MDA-MB-231 cells was the ability to invade in clusters and survive up to several months in culture without renewal of the culture medium (Figure 22).
Figure 21. Distribution of the (a) HT1080 and (b) MDA-MB-231 cell population with the invasion depth in alloy gels over time. The data represent 35 hours of automated cell tracking that started 12 and 24 hours post-seeding for the HT1080 and MDA-MB-231 cells respectively.
Figure 22. Invasion pattern of MDA-MB-231 cells in alloy gel after 10 months without medium renewal. (a) $Z=0$ (top of the gel), (b) $Z=-100 \ \mu m$, (c) $Z=-250 \ \mu m$. 
6. Discussion

Cancer invasion during metastasis has been characterized as a temporary phenomenon where the tumor cells do not need to be continuously invasive but rather express an unstable phenotype depending on the stimuli generated from the surroundings (226, 227). It is possible that the various biochemical and biophysical stimuli will not affect identically the whole tumor cell population due to differences in the signal intensity or due to intracellular heterogeneities among the individual cells. These observations emphasize the need of an experimental model that follows the cancer invasion or the cell migration in general, as a dynamic and multi-faceted action of individual cells, whose characteristics evolve over time and throughout the three-dimensional tissue microenvironment.

This study quantified the 3D locomotion of cells during gel infiltration, as a set of time dependent variables and at the cellular level. This work was built on two main foundations: a computer-based system for automated cell tracking in 3D, and a statistical analysis of the individual cell trajectories, that computed distinct migratory and invasive patterns associated with certain tumor cell lines and the ECM composition.

The 3D cell migration and invasion substrates were prepared to comply with two criteria: simulate the ECM and contain good optical clarity for contrast microscopy. The gel matrices were plain collagen type I gels or alloy gels made of collagen type I and Matrigel. The collagen type I is the major component of the tissue stroma that the tumor cells encounter during metastasis. Thus infiltration of collagen I gels by tumor cells was
used as a model for stromal invasion during metastasis. Matrigel was chosen to simulate the basement membrane. During the first attempts to develop a basement membrane invasion model suitable for tracking, bilayer gels were prepared with collagen type I gels overlaid with a thinner layer of Matrigel. These structures were proven very unstable due to low affinity between the different layers and low integrity of the Matrigel layer. Hydrated layers of pure Matrigel are known for their low density and lack of mechanical integrity that permit passive penetration rather than active cell-gel interaction (228). Therefore, Matrigel was mixed with collagen I to form thick gel structures of relatively high density that allowed evaluation of basement membrane components on invasion and migration.

Automation is necessary for tracking large number of cells over long periods. It would be unrealistic to try to focus manually on about 90 different cells that move randomly in a 3D gel in order to quantify their (X,Y,Z) position every 30 min for a two day period. The automated system composed of the current experimental equipment and the developed software made the task of long-term individual cell-tracking, reality. Maximizing the accuracy of the automated cell tracking is a matter of optimization involving several parameters such as: the cell-seeding density, the time-lapse for image acquisition, the speed of cell locomotion, the external noise that interferes with image analysis, the efficiency of the cell identification criteria, and the efficiency of the cell trajectory reconstruction. Small probability for cell mismatches during requires low seeding density and relatively small time-lapse for image acquisition depending on the cell speed, noise elimination treatments. However, there exists a minimum in the
experimental interval $\Delta t$ for time-lapse image acquisition defined by the time required for stage positioning and image acquisition. The time-lapse $\Delta t$ is also bound by an upper limit. The speed values as calculated from equation [9] and all the Markov parameters are more representative of the cell behavior when calculated on partitions (or steps) of the cell trajectory corresponding to homogenous cell behavior. Such partially stable cell behavior in terms of locomotion speed, stops, and directionality cannot be assured over large time intervals. Therefore, the "optimum" $\Delta t$ is selected empirically as described above, or with the help of the Richardson plots (219, 229).

The total tracking period, the frequency of image acquisition (i.e. time interval), the total tracked volume (i.e. number of X,Y fields and number of Z sections) during the experimental tracking are all depended on the availability of hard disk space for digital data storage and whether the image analysis is performed on- or off-line. For a typical experiment with: tracking in 9 fields of view, optical sectioning with a step of 20 $\mu$m up and 15 total Z-sections in the gel, and 35 hours tracking period with time interval for image acquisition 20 min, the memory space needed for saving all the images is:

\[
(9 \text{ fields}) \times \left( 15 \frac{\text{sections}}{\text{field}} \right) \times \left( 300 \frac{\text{kB}}{\text{section}} \right) \times \left( \frac{35\times60\text{min}}{20\text{min}} \right) = 4252500\text{kB} \approx 4.3\text{GB}
\]

A solution to storage limitations could be the on-line image analysis, object identification and deletion of the raw images. This obviously eliminates the applicability of manual evaluation of the tracking process. Additionally, on-line analysis of the raw images will demand a certain amount of time (0.6 sec/image) which depending on the number of
acquired images at each time-point, will raise accordingly the lower available limit for the time-lapse interval. For the accuracy of the cell tracking may need to be compromised over larger tracking volume/larger cell population and longer tracking periods. Another limiting factor for the length of the tracking period is the integrity of the gel. Overexposure to the partially humidified environment of the microscope will eventually dehydrate the gels.

The analysis of the 3D cell trajectories produced several quantitative descriptors for cell infiltration and migration, such as speed distributions of individual cells, the Markov model parameters, and profiles of the invasion depth over time. The continuous Markov chain model is a probabilistic approach that can represent the individualistic character of the cell migration. This character is represented and quantified as probability of subpopulations defined by the Markov states, which in our case are subsets of the 3D Cartesian space. A novel Markov chain was designed with 13-states for the statistical analysis of the 3D trajectories of individual cells. This implementation identified cells moving laterally during gel infiltration as well as stationary, invasive and anti-invasive cells. The present analysis revealed distinct migratory and invasive phenotypes among human HT1080 fibrosarcoma and human MDA-MB-231 adenocarcinoma depending on the composition of the 3D substrate. These phenotypes were quantified and compared via a set of descriptors calculated over time at the cellular level such as the cell speed over time, the turning frequency, the directionality and duration of lateral and invasive motion, the frequency of occurrence and duration of cell stops, the ultimate probabilities of motion towards certain directions, and the invasion depth over time. The used Markov
chain is composed of groups of geometrically not equivalent groups, i.e. stationary state (with a sphere with diameter the average half-cell diameter), the planar state (a rectangular parallelepiped with height the cell diameter), and the Z motion states (each making up for 1/8 of the 3D space). These states were chosen to describe the different substrate geometry the cells encounter during invasion of the 3D matrix. The cells on the top of the gel are the major contributors to the planar motion. In other words, exclusion of the gel-top cells from the Markov analysis, would probably decrease significantly the steady-state probabilities and the preferential transitions to the planar state with respect to the eight states of Z motion.

**Migratory and Invasive Phenotypes at the Cell Type Level**

Cell speed and stability of the motile phenotype were distinct characteristics among the two cell types. HT1080 cells in either matrix showed a significantly more motile behavior compared to MDA-MB-231 cells in terms of both the speed and the percentage of the motile subpopulation. The majority of HT1080 cells stretched their speed distribution up to 2 μm/min while MDA-MB-231 cells obtained speed in the average range. The calculated average speeds for HT1080 and MDA-MB-231 cells belong in the range of 0.6-0.7 μm/min and are comparable to values found for other tumor cells in collagen gels, e.g. 0.82 μm/min for mouse M4 K1735 melanoma in Vitrogen gels (230), or 0.1±0.01 μm/min for MDA-MB-361 in collagen gels (204). The 361 is a non-tumorigenic and non-metastatic member of the MDA breast adenocarcinoma cell lines.

In general, the speed of locomotion can be very different among different cell types, i.e. speeds of 4-18 μm/min were calculated for lymphocytes in 3D collagen gels.
(231). In vivo migration data for HT1080 and MDA-MB-231 have not been found. Some studies reported in vivo cell speeds quite higher than the in vitro “norm”, i.e. 3-10 μm/min in vivo migration rates for V2 carcinoma cells (232). The natural matrix imposes a denser and more structured barrier against invasion and migration compared to the collagen gels that might be harder to penetrate. However, the in vitro ECM analogs lack the multicomponent chemical and architectural structure and the multicellular environment of the natural ECM that may practically provide a more accessible migration substrate and trigger the initiation of migration signaling cascades.

Another dissimilarity between the two tumor cell types was their morphology in the ECM analogs. Fibrosarcoma cells appeared mostly elongated, with the characteristic pseudopod formation reflecting good adhesion to the matrix and high motility. The MDA-MB-231 cells though well attached on the alloy gels, were more rounded and less motile than the HT1080 indicating smaller affinity for the gel components. The HT1080 fibrosarcoma is a malignant fibroblast cell line. Its stromal origin is expected to promote adhesion and stretching on stromal components such as collagen type I. The MDA-MB-231 adenocarcinoma cells derive from epithelial origin and their affinity for the basal lamina components such as Matrigel, is anticipated. These cells stretch and invade less in plain collagen type I gels (data not shown).

A very interesting infiltration pattern was observed in long-term 3D cultures of MDA-MB-231 cells in ECM analogues. In some cases, MDA-MB-231 cells seeded on collagen gels formed colonies of 5-20 cells with round morphology as soon as after five days in culture or after several weeks. Such colonies exhibited a distinct gel infiltration
strategy by forming "invasion wells". The colony members appeared to engage into a synergistic matrix proteolysis that degraded the matrix and created depressions or potholes starting from the gel surface up to a depth of up to 400 μm or more inside the gel body. A tumor colony of slowly dividing cells was always located at the bottom of the pothole. Such pothole formations were found to be stable and the involved cells viable for more than 10 months in 3D cultures maintained in 5% CO₂ humidified incubator and without media renewal (see Figure 22). Such invasion scheme or this extent of homotypic affinity was not observed in HT1080 cells. The fibrosarcoma cells were less resilient in long-term 3D cultures without media renewal.

Apart from the affinity for the substrate, the ability of tumor cells to degrade their environment is also required for efficient invasion and migration (see also Introduction). The interplay among the extracellular matrix, the autocrine products and the surface molecules of the cells determines the phenotype of the cells and the impact on their microenvironment. The HT1080 and MDA-MB-231 cells produce several matrix degrading enzymes and proenzymes among which are the gelatinases MMP-2 and MMP-9 that may contribute to the high invasiveness of both cell lines (233-236). The 92 kDa MMP-9 (gelatinase B) has been extensively characterized at the structural level and shown to degrade specifically the gelatins derived from basement membrane collagen type IV and V (85, 237, 238). The closely related 72 kDa MMP-2 (gelatinase A) has a similar enzymatic spectrum (see also Introduction).

HT-1080 cells secrete MMP-9 and partially activate the endogenous MMP-2 proenzyme (239). An antiserum specific to MMP-2 was shown to inhibit the in vitro
invasiveness of HT1080 fibrosarcoma (240). Additionally, synthetic peptides from the autoinhibitor sequence of the 72 kDa collagenase-IV pro-peptide region (241, 242) reduced the Boyden chamber invasiveness of the HT1080 cell line (242). In addition to the MMPs, HT1080 cells secrete urokinase-type plasminogen activator (uPA) (243), scatter factor (SF) and an autocrine motility factor (AMF) (244) as identified at serum free conditioned medium of HT-1080 cells. These factors are important mediators of cell invasion and metastasis (245-248). The Met receptor tyrosine kinase and its ligand, hepatocyte growth factor/scatter factor (HGF/SF), are known to play important roles in tumor cell migration, invasion, and metastasis (245-247). HGF/SF induces the expression of urokinase plasminogen activator (uPA) as well as the uPA receptor (uPAR), which are important mediators of cell invasion and metastasis (248). It has been reported that serine proteases of the uPA system are involved in collagen degradation by HT1080 cells (239, 249, 250). The urokinase-type plasminogen activator receptors were shown to associate with β1 and β3 integrins of fibrosarcoma cells (251). The carboxyl-terminal propeptide of procollagen type I (CPP-I) was found to support the attachment and spreading of HT1080 cells, demonstrating that it can function as an adhesion protein. Two other approaches supported the identification of α2β1 as the CPP-I receptor (252).

Several studies attributed to the MDA-MB-231 high relative invasiveness and metastatic potential among the human breast cancer cell lines. Breast tumor invasiveness has been associated with loss of the estrogen receptor (ER), increased vimentin expression and E-cadherin under-expression and expression matrix digesting enzymes (253). MDA-MB-231 is an estrogen receptor negative (ER-), uvomorulin-negative (UVO-), vimentin-positive (VIM+) (the intermediate filament glycoprotein), cell line, expressing high levels
of cathepsin-D (253, 254) and low levels of E-cadherin. The vimentin-positive subgroup of human breast cancer cell lines, which are highly invasive of basement membranes and more metastatic in nude mice, show activated MMP-2 when cultured on interstitial collagen gels (Vitrogen®), similarly to human fibroblasts (255, 256). In these cell lines the Vitrogen-induced activation of MMP-2 is independent of their MMP-2 production (256). MDA cells express MMP-9, MMP-2 and MMP-2 activator, TIMP-1 and TIMP-2. ELISA and Western analysis of TIMP-1 and TIMP-2 showed no clear association with the expression of MMP-2 and MMP-9 respectively with which they show differential and specific association when the enzyme is in the latent form (257). Similarly to the HT1080 case, synthetic peptides from the autoinhibitor sequence of the 72 kDa collagenase-IV pro-peptide region (241, 242) inhibited the in vitro invasiveness of the MDA-MB-231 cell line in both the Boyden chamber chemoinvasion assay and the Matrigel outgrowth assay. The MDA-MB-231 cell line express high levels of the β1, α2, α3, α5, and α6 integrin subunits along with moderate levels of the αvβ3 integrin (236). Treatment of MDA-MB-231 cells with a function-blocking anti-α3 antibody strongly inhibited migration and invasion. This correlated with a marked reduction in MMP-9 activity produced by MDA-MB-231 cells, suggesting a role for α3β1 ligand binding in cell signaling and regulation of extracellular matrix degradation. Additionally, recent inhibition studies using function-blocking antibodies showed that adhesion and invasion of MDA-MB-231 cells is mediated by multiple integrins including β1, β3, β4, and β5 (258).
MDA-MB-231 cells might have used an additional mechanism for overcoming the matrix barriers, the phagocytosis of their extracellular matrix. Previous studies showed that these cells contained large acidic vesicles that homed high concentrations of mature cathepsin-D (254, 259). Characterization of the vesicles revealed that their presence was more frequent in cells that had migrated through Matrigel and that the overexpressed cathepsin-D could be activated in these vesicles. Furthermore, the vesicles were shown to contain phagocytosed extracellular matrix (heterophagosomes).

The degradation of the extracellular matrix is suggested to occur at the cell-matrix contacts (260). Several studies reported that the gelatinases are localized on the plasma membrane of tumor cells (261-263). More specifically the latent form of MMP-9 (pro-MMP-9) was found to bind on the surface of HT1080 and MDA-MB-231 cells among other malignant breast epithelial (141, 264), endothelial (265), fibrosarcoma (141, 263, 266), and non malignant cells (267). Toth et al. (234) identified a surface-bound α2(IV) chain (~190 kDa) of collagen IV that formed high-affinity complex with the pro-MMP-9 enzyme. Their pulse-chase experiments revealed that α2(IV) was secreted into the culture media of HT1080 and MDA-MB-231 cells both as a single chain and as a part of a >400 kDa protein containing α1(IV). The secreted nondisulfide-bonded α2(IV) chains were stable in the media for at least 10 h. Additionally, the MDA-MB-231 cells were found to contain surface-bound α2(IV) chains. These data (234) may also explain the faster and more efficient invasion of HT1080 versus the localized invasion of MDA-MB-231 at the cell-matrix contact points.
Higher proteolytic action combined with a variety of motility factors may contribute to the more active migration and robust invasion of HT1080 cells compared to MDA-MB-231. Fibrosarcoma cells both in collagen type I and alloy gels invaded more efficiently than adenocarcinoma. Their invasion was faster and denser giving more cells per gel unit volume and reached deeper invasion front. The invasion profile of the HT1080 cells formed a secondary peak in the gel body characteristic of their high motility and high transition frequency among the Z sections. The Z location and the cell concentration at the secondary peak depend on time and the matrix composition. More peaks may appear at deeper Z sections as the infiltration time increases. The exponentially decreasing profile of adenocarcinoma is explained from the weak turning behavior and low motility. A slow infiltration depleted the top of the gel from its cell count to increase the cells at the neighboring Z sections. This pattern also represents initial stages of gel infiltration and could represent the invasion profile of fibrosarcoma cells shortly after seeding. The duration of the stationary state of MDA-MB-231 cells was twice as long than the directional motion. When moving they were more likely to invade, but their motion was short and often interrupted by long inert periods. Instead of maintaining a state of motion, the adenocarcinoma cells exhibited high transition probabilities from Z+ or Z-motion to the stationary state. This staggered motility of adenocarcinoma gave their invasiveness a localized character, where cells had low tendency for lateral motion and infiltrated the gels slowly probably by degrading or phagocytosing their immediate surroundings rather than actively push their way through the matrix. This resembles initial stages of a mass (cell) diffusion process towards the inner gel that creates an exponential cell concentration profile. Similar profiles during gel infiltration have been
reported by other investigators (191, 203). Additionally, a case of secondary peak formation in the invasion profile was reported for lymphocyte infiltration in collagen gels (268).

**Migratory and Invasive Phenotypes at the Matrix Composition Level**

Matrigel did not alter significantly the kinetic characteristics of fibrosarcoma such as the motile subpopulation, the speed distribution or the average speed of locomotion and their preference to maintain a state of motion and especially with lateral directionality. However, Matrigel amplified the turning frequency and invasiveness by shifting a significant cell percentage from planar to invasive motion and reducing the persistence of fibrosarcoma for lateral motion. The effect of Matrigel may be attributed in part to the collagen type IV content that provided substrate to the gelatinase A and B enzymes of the HT1080 cells and made the alloy gel more susceptible to degradation leading to enhanced enzymatic proteolysis of the gel body. A previous study is consistent with this result, for collagen IV in collagen I gels increased invasiveness of melanoma cells (191). Additionally, Matrigel contains several ECM components and growth factors that might have induced the activation of tumor-secreted enzymes, that ultimately enhanced the enzymatic proteolysis of the gel body and facilitated both invasion and cell migration.

Certain behaviors were common in both gel and cell types. The values of the Markov parameters corresponding to $X\pm$ and $Y\pm$ directional motion were similar revealing random motion on the XY plain. This behavior is expected from the geometrical equivalency of the X and Y directions in the randomly oriented gels. Another very
interesting similarity was the solid pattern of significantly preferred transitions from the
Z+ to Z- and from Z- to Z+ directional states for both HT1080 and MDA-MB-231 in
either collagen I or Matrigel containing gels. About 20% of the transitions from a Z
motion state occur at the exactly opposite direction (e.g. from state 8 to 10 or from state
11 to 5). The exactly opposite transitions, with opposite X and Y and Z, summed up to
almost 50% of all the transitions to the opposite Z direction. This indicates that both
tumor cell lines degrade their immediate surroundings and invade their way in the
collagen matrix by means of an oscillatory motion in the path of least resistance.
HT1080 cells are known to leave well-defined tracks (depressions or channels) when
migrating (269). A similar behavior was observed before for MOS-11 (transformed 3T3
cells) dispersed and migrating in collagen type I gels. The Markov analysis of the cell
trajectories showed that frequent cell turns occurred at 150° on either side of the previous
step. In the same study the waiting times at the directional states were alike while cells
stayed at the stationary state for about three times longer time (206).

To summarize, the HT1080 cell line embodies an efficiently migrating entity. It
combines a highly motile phenotype supported by autocrine factors, good adhesion to the
ECM molecules and enhanced invasion sustained by a spectrum of proteolytic enzymes.
These cells gave a robust invasion and high turning frequency that were further enhanced
by Matrigel as expected. MDA-MB-231 cells tended to form multi-cell clumps or star-
like clusters, showing higher homotypic adhesion and lower motility comparing to the
HT-1080 cells. Despite MDA-MB-231 being not as efficient invaders as the HT1080
cells, all the Markov output such as residence times, steady-state probabilities, transition
probabilities and of course the Z invasion profiles attributed to the MDA-MB-231 cells a significant ability to degrade their in vitro ECM. Their staggered motility attributed to their invasive phenotype a localized character where cells degraded or phagocytosed their immediate surrounding engaging themselves to more a quiescent invasion of their microenvironment.

In general, the regulation of the migratory phenotype under developmental, physiological and pathological conditions is the output of continuous cell-tissue interactions. The complexity underlying cancer has already made anti-cancer research a time and money-digesting effort, while several questions remain unresolved. What is the role of the tumor microenvironment during metastasis? How tumor proteinases promote progression of certain types of cancer? What are the corresponding substrates? Which proteinases should be inhibited for optimal therapeutic effect? The resolution of these issues calls for a reliable experimental model that can evaluate how the cross talk between multiple receptors and ECM molecules influences quantitatively and/or qualitatively the expressed phenotype of the metastasizing cells. The designed fully automated 3D tracking system combined with the novel Markov analysis provides a technique for the quantitative assessment of the migratory and invasive phenotype at the cellular level. The present technique based on extracellular matrix analogs enables the design of an experimental model that can isolate a set of well-defined parameters, i.e. cell type, ECM component, motility enhancing or inhibitory factors, and assess quantitatively their combined effect on migration and invasion over time. Implementation of this method can help elucidate how ECM components modulate the characteristics of tumor invasion, and quantify the
effect of motility enhancing or inhibitory factors. Information at this level has not been collected yet from three-dimensional tissue resembling experimental models.

The gold standard for evaluating the metastatic potential of various cancer types or the effectiveness of therapeutic treatments is yet to be found. *In vitro* systems though indispensable sources of basic information may be unable to relate efficiently to the *in vivo* complexity. The versatile synergism of the malignant components with the sufferer host is what makes cancer a villain menace. Intravital tumor tracking at the cellular level is the ultimate evolution of the present system that will contribute to a sturdier bridge of understanding over the gap between Science and the Cancer Enigma.
7. **Suggestions for Future Studies**

A range of experimental studies can be based on the present instrumentation and developed software by maintaining or modifying the parameters of the current system such as the extracellular matrix analogs, the tracking programs, or the data analysis.

Future experimental work could focus on quantifying the effect of various other extracellular matrix components on invasion and migration of various cell types. Maintaining the current tracking software, the dynamic migratory and invasive phenotypes of various cell types in association with various ECM proteins and the effect of motility enhancing or inhibitory factors are easily applicable. Intracellular interaction among different cell types could be assessed either with the use of conditioned media or co-culture in the ECM analogs. The application of the tracking in co-culture systems could require modifications at the level of cell identification criteria and trajectory reconstruction. Another approach could maintain the current tracking and employ development of a different data analysis model such as 3D persistent random walk. Improvement of the tracking technique is also field for further exploitation. The current work was based on a cell positioning axiom mapping the three-dimensional cellular body to a point \((X,Y,Z)\) in the 3D space based on morphological and optical features for object identification. Modifications of the current tracking program could help perform dynamic analysis at the cellular level emphasizing in the cell morphology, cellular volume and pseudopod formation over time during invasion and migration. That would require higher magnification and more elaborate edge detection or boundary identification routines to outline the cell shape.
Studies to dissect the invasion mechanisms could be performed in synthetic 3D matrices, engineered for desired controlled structure. The target in this approach would be to evaluate the role of specific peptides from ECM proteins in migration and invasion, using a system exhibiting solely the properties of these peptides isolating their effect from the other ECM components. This effect could be accomplished theoretically by embedding the bioactive peptides in a neutral, bioinert matrix with low interaction with the cells or their products. The incorporated peptides could provide adhesion substrates, or degradation substrates for specific cell secreted proteases. Such properties have been reported for hydrogels that provide additionally a three-dimensional structural network. Hydrogels can be derivitized with bioactive peptides to engineer desired adhesion or substrate specificity properties (270). Bioinert networks such as hydrogel matrices (271, 272) could be useful in determining the effect of specific cell attachment in the 3D substrate and specific proteolytic degradation of the matrix could be correlated with invasiveness and ability to migrate (273). The basic hypothesis underlying the semi-synthetic invasion model is that cells would invade or migrate if they can collectively adhere on and topically compromise the matrix. In a system designed with such properties questions at the level of receptors required for migration and invasion could be addressed. Additionally, the proteases involved in migration and invasion could be identified for specific cell types and schemes for their inhibition could be evaluated.
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APPENDIX A

A1. Cell Tracking Algorithm

Define

- total_time_points
- total_XY_FOV (fields of view)
- total Z sections
- min_cell_size (~100 μm²)
- max_cell_size (~550 μm² for HT1080, ~610 for MDA-MB-231 μm²)
- cell_identification_window

Initialize the origin (X=0, Y=0, Z=0)

For t=0..(total time points)
   For FOV=0..(total FOV)
      For Z=0..(total Z sections)
         Focus at Z
         Acquire image
         Average 3x3 filter
         Object Identification Routine
            Impose thresholding
            Select cellular traces
            Keep only min_cell_size < cell_trace < max_cell_size
            Extract centroid and gray value roughness
            for each cellular trace
      end
   end
Export cell_trace(X,Y,Z) to Microsoft Excel®

Cell Identification Routine
For Z_section=0..(total Z sections)
   For cell_trace=0..total_cell_trace
      common_cell_trace=trace:
      distance{centroid(cell_trace)-centroid(all_cells, Z≠Z_section)}<0
      cell(Z)=cell_trace(Z)[max(gray_value_variation)]
      position vector \( P \) [cell, t, X, Y, Z].
   end
end
Export \( P \) to Microsoft Excel®
Nearest-Neighbor Reconstruction
Define NN_window

For t=0..total_time_points
    For cell=0..total_cells
        For distance \{P(t, cell, X, Y)- P(t+1, 0..total_cells)\} < NN_window
            cell(t+1,XY,Z)=
            cell(t+1)|| min(distance \{P(t, cell, X, Y)- P(t+1, 0..total_cells)\})
        end
    end
end

Export P to Microsoft Excel®

A2. Implementation of the Continuous Markov Chain Model
As mentioned before three cell migration descriptors can be extracted from the Markov modes: (i) the waiting times \( T_i \) (i=0..12), (ii) the transition probabilities among the Markov states \( P(j/k) \) (j,k=0..12, i\neq j), and (iii) the steady-state probabilities \( \bar{p}(j) \) (j=0..12).
The input for the Markov analysis program is the vector \( P \) of the positional coordinates \( (X,Y,Z) \) of all the tracked cells for all the time points of the tracking period: \( P \{cell, t, X, Y, Z\} \). To calculate the Markov parameters a state counter \( SC(i) \) (i=0..12) and a transition counter \( TC(k,i) \) (k,i=0..12, k\neq i) are created.

Define state counter \( SC(cell,i)=0, \ i=0..12 \)
Define transition counter \( TC(cell, k/i)=0, \ i,k=0..12 \)
Define HMC (half_cell_diameter)
Define \( \Delta t \) (time lapse)

For cell=0..total_cells
    For t=1..total_time_points
        \( \{\Delta X, \Delta Y, \Delta Z\}_{cell,t} = \)
        \( \{[X_{cell}(t)-X_{cell}(t-1)], [Y_{cell}(t)-Y_{cell}(t-1)], [Z_{cell}(t)-Z_{cell}(t-1)]\} \)
        if \( \{\Delta X, \Delta Y, \Delta Z\}_{cell,t} < (HMC,HMC,HMC) \) then \( SC(0)=SC(0)+1 \)
        continue for the other Markov states (see also Figure 10)
        SC(cell, i)
        TC(cell, k/j)
    end
end
\[ E(T_i) = \frac{\text{total time that all cells spent in state } i}{\text{total number of transitions out of state } i} = \frac{\Delta t \cdot SC(i)}{\sum_{k=0}^{12} TC(k/i)} = \frac{1}{Q(i)} \]

\[ p(j/k) = \frac{\text{number of transitions from state } k \text{ to state } j}{\text{number of transitions from state } k \text{ to any other state}} = \frac{TC(j,k)}{\sum_{i=0}^{12} TC(i,k)} \]

The 13 steady-state probabilities can be calculated from the non-trivial solution of the homogeneous 13x13 system defined from the above equation for \( j = 0..12 \). The system is solved easily at Matlab.

\[ Q(j)\overline{p}(j) = \sum_{k=0}^{12} p(j/k)Q(k)\overline{p}(k) \quad j = 0,1..n \]