Review

In vitro cell migration and invasion assays

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A B S T R A C T

Determining the migratory and invasive capacity of tumor and stromal cells and clarifying the underlying mechanisms is most relevant for novel strategies in cancer diagnosis, prognosis, drug development and treatment. Here we shortly summarize the different modes of cell travelling and review in vitro methods, which can be used to evaluate migration and invasion. We provide a concise summary of established migration/invasion assays described in the literature, list advantages, limitations and drawbacks, give a tabular overview for convenience and depict the basic principles of the assays graphically. In many cases particular research problems and specific cell types do not leave a choice for a broad variety of usable assays. However, for most standard applications using adherent cells, based on our experience we suggest to use exclusion zone assays to evaluate migration/invasion. We substantiate our choice by demonstrating that the advantages outbalance the drawbacks e.g. the simple setup, the easy readout, the kinetic analysis, the evaluation of cell morphology and the feasibility to perform the assay with standard laboratory equipment. Finally, innovative 3D migration and invasion models including heterotypic cell interactions are discussed. These methods recapitulate the in vivo situation most closely. Results obtained with these assays have already shed new light on cancer cell spreading and potentially will uncover unknown mechanisms.

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Abbreviations: ECM, extracellular matrix; BME, basal membrane extract; EMT, epithelial mesenchymal transition; PET, polyethylene terephthalate; PC, polycarbonat; ECIS, electric cell-substrate impedance sensing; EHS, Engelbreth-Holm-Swarm tumor; HTS, high throughput screening; MMP, matrix metalloproteinase; CAF, cancer associated fibroblast; LMAT, leukocyte migration agarose technique; FFPE, formalin fixed paraffin embedded; EC, endothelial cell; SEM, scanning electron microscopy.

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1. Introduction

Normal processes of cell migration/invasion include gastrulation [1] embryonic morphogenesis, branching morphogenesis (e.g. breast ducts), development of the nervous system [2], vascular sprouting [3], placental development, wound healing or immune-cell trafficking [4]. There are also pathological situations in which deregulated cell movement is highly significant. Tumor cell migration and invasion is for example clinically most relevant. Cancer is the second leading cause of death and accounted for 7.6 million deaths worldwide by 2008 [5]. Metastasis represents the major problem in the treatment of cancer, is indicative for poor prognosis and has dramatic effects on the survival of patients. Several models of tumor invasion and metastasis have been proposed. These are for example based on multi-step progression [6], “intrinsic” metastasis [7] or metastatic dissemination [8] although no clear mechanisms were defined. Metastasis involves multiple processes such as infiltrative growth through the extracellular matrix (ECM), cell migration through blood or lymph vessels and rise of distant colonies [9]. It is strictly associated with tissue invasion of the primary tumor. Matrix metalloproteases (MMPs) have been identified to break down the ECM. Consequently, small molecular weight compounds, which block MMP function, have been developed. Unfortunately, these therapy approaches failed in clinical trials. Since then novel insight into the mechanisms of cancer cell spreading has been gained, showing that tumor cells could escape the MMP inhibition by using protease independent mechanisms to travel through the ECM [10,11]. Hence, therapeutically targeting proteases in combination with additional features of tumor cell invasion and migration remain potent concepts for anti-cancer strategies.

Therefore, it is of great interest and of potential therapeutic importance to understand the basic principles and molecular pathways, which are altered in cancer cells to drive the first step of metastatic dissemination.

1.1. Definition of migration and invasion

Migration is often used as umbrella term in biology to describe any directed cell movement within the body. The ability to migrate allows cells to change their position within tissues or between different organs.

In pathology, invasion of carcinomas is defined as the penetration of tissue barriers, such as passing the basement membrane and infiltration (invasion) into the underlying interstitial tissues by malignant tumor cells. Intestinal cancers for example are classified as invasive, when the tumor mass has crossed the basal membrane and entered the submucosal muscle layer. Defining invasiveness in non-epithelial cancers is more difficult, since there is no clear-cut separation of for example mesenchymal cells by a basement membrane. An explicit discrimination of migration from invasion is not made in many fields of biology dealing with the in vivo description of directed cell movement.

Migration and invasion are clearly separated terms in experimental cell biology. Migration is defined as the directed movement of cells on a substrate such as basal membranes, ECM fibers or plastic plates. Therefore, migration is occurring on 2D surfaces without any obstructive fiber network (an example for a collagen I network is shown in Fig. 1A). Invasion is defined as cell movement through a 3D matrix, which is accompanied by a restructuring of the 3D environment. In order to travel through the matrix, a cell must modify its shape and interact with the ECM, which on the one hand provides the cell attachment substrate, on the other hand represents a barrier toward the moving cell body (Fig. 1B). Invasion requires adhesion, proteolysis of extracellular-matrix components and migration [12], therefore invading cells remodel the ECM (Fig. 1C). However, passenger leukocytes can migrate through 3D tissues and do not require proteolytic action and tissue remodeling [13]. Thus, the term “invasion” solely describes the restructuring or destructive movement of cells through a 3D barrier, whereas “3D migration” is used herein to describe non-destructive, non-proteolytic movement in 3D tissues or matrices.

Despite the difficulties in defining different modes of migration it is a fact that the ability to migrate is a prerequisite to invade; a cell cannot invade without migration but can move without invasion. In analogy, migration can be seen as walking on a pave, while invasion would correspond to moving through a thorn hedge (requiring all the tools needed to bushwhack including the ability to walk).

1.2. Different modes of cell motility

The basic features occurring in a migrating cell have already been clarified. These cells display directional polarity, with a leading edge at the front and a lagging edge at the back of the cell body [14]. Common to all migration modes is the actomyosin cytoskeleton-mediated change of the cell body shape [15]. However, external clues such as the physical and molecular characteristics of the environment and intrinsic cellular determinants influence the mode of migration. The two main types are collective migration of multicellular compartments and single cell movement [12].

The migration of single cells can be subdivided into amoeboid- and mesenchymal types of movement.

Amoeboid movement is characterized by cells moving as rounded, ellipsoid bodies without the involvement of focal adhesions and cell attachment but with the aid of propulsing blebs. This is called blebbly amoeboid migration [13,16,17] and is used for example by embryonic cells, leukocytes migrating through the ECM (3D migration) or by certain cancer cells. The second form of amoeboid migration is characterized by outgrowth of actin-rich filopodia and weak substrate interaction [18,19] for example in moving neutrophils and dendritic cells or tumor cells [20].
Mesenchymal migration involves strong focal attachment to the extracellular matrix, cytoskeletal contractility and elongated spindle-like cell bodies [21]. Fibroblasts, sarcoma cells and highly dedifferentiated tumor cells, which have undergone epithelial to mesenchymal transition (EMT) [22], use this mode of motility. EMT has not only been proposed to play a key role in the acquisition of a migratory or invasive phenotype of many carcinoma cells, but is also discussed to increase the proportion of cancer stem cells [23,24]. Epithelial cells could therefore dedifferentiate via EMT to cancer stem cells. This might have important clinical consequences for the curative treatment of cancers as the increased plasticity within tumors will require a combination of cancer stem cell and non-cancer stem cell targeting [25].

Collective migration is characterized by the movement of a cellular cohort through the ECM with the preservation of functional cell–cell junctions. This type of group movement of cells, which are physically attached to each other, occurs either on 2D surfaces, when epithelial sheets travel collectively across basal membranes (wound healing [26,27]). Alternatively, collective migration happens in 3D as branches (mammary gland [28]), tubes (vascular sprouting [29]), strands or clusters (lateral line migration in fish [30]) or cancer cell invasion [31,32]. Here, leading cells predominantly travel invasively and the following cells move on pre-paved routes through the ECM.

1.3. Importance of migration/invasion in cancer

Metastasis is by far the main cause of cancer lethality; 90% of deaths from solid tumors can be ascribed to metastatic dissemination. It is obvious that there is need for novel therapeutic strategies in the clinic to avoid metastatic spreading. New anti-metastatic drugs can only be developed upon knowledge of the basic principles of metastasis. To date the major challenge to clinically monitor inhibition of migration, invasion and metastasis is far from being solved. As metastasis is a multistage process and takes place over months if not years, a clear-cut clinical readout is extremely difficult to obtain and will represent a major challenge for clinically testing anti-metastatic drugs.

As already described above, metastasis involves multiple processes; however, it is strictly connected to initial tissue invasion at the primary tumor site. Therefore, it is essential to know, how cancer cells acquire an invasive phenotype and to understand the respective molecular mechanisms. Functional interferences with different modes of invasion are necessary to counteract the different strategies cancer cells have evolved to move through tissues and organs. These interfering treatment strategies need to be established, which can either be achieved by the use of in vivo and/or in vitro models. Here we will focus on in vitro assays that measure the migratory potential and/or the invasive property of cells and give an overview of commonly used experimental setups. The main advantages of in vitro assays are their relatively easy handling and high reproducibility. Moreover, they often allow the examination and phenotypic analysis during the assay. Furthermore, in vitro assays are suitable for high throughput drug testing (high throughput screening, HTS) and are less expensive than in vivo assays. In addition, animal experiments raise ethical concerns, which can be reduced by the use of in vitro models. A summary of the features of these assays is given in Tables 1 and 2. In vivo migration/invasion assays will not be summarized at this point as these assays were excellently reviewed recently [33]. However, it should be noted that so far none of the described assays could fairly recapitulate all essential steps in metastasis, but only address parts of it. It seems that a comprehensive in vitro metastasis assay is far from being technically achievable in the near future.

2. Migration assays

2.1. Transwell migration assay (Boyden chamber assay)

The transwell assay was originally introduced by Boyden (and is therefore often called Boyden chamber assay) to analyze the chemotactic responses of leukocytes [34]. Improved, simplified and disposable versions of the original chambers were developed. The principle of this assay is based on two medium containing chambers separated by a porous membrane through which cells transmigrate (Fig. 2A). The size of the cells to be analyzed, determines the required pore size of the membranes. It is essential to choose a pore diameter, which allows an active transmigration i.e. being smaller than the cell diameter to avoid unspecific dropping of the cells. Membranes are available with pore diameters between 3 and 12 μm. Generally, cells are seeded in medium in the upper part and can migrate in vertical direction through the pores of the membrane into the lower compartment, in which medium containing an attractant or simply higher serum content is present. Of note, a major determinant is the phase of horizontal migration of the cells until a pore is being actually reached. There are two possibilities to detect and quantify the migrated cells: first, the cells that passed the membrane can be fixed on the membrane, stained and quantified. It is important to individually determine the proper incubation time, until the motile cells appear at the other side of the filter, since it significantly varies between different cell types. Thereafter, the membrane is fixed and non-migrated cells remaining on the topside of the filter are removed with a cotton swab. The migrated cells are stained with cytological dyes (such as hematoxylin, toluidin blue or crystal
Fig. 2. Schemes of commonly used migration assays. An overview of the technical setup is schematically drawn for each assay and a close-up view is given right to it (inside the big circles). Arrows indicate the direction of cell movement. Hatched areas symbolize ECM. (A) Transwell migration assay (Boyden chamber assay). Migration of a cell through a pore in the membrane is depicted. (B) Wound healing assay. Scratching off cells from a dense monolayer produces a cell-free area. This is often performed with a vertically held pipette tip. The close up view depicts that cells can be seeded on plastic or glass surfaces or on ECM coated surfaces (hatched areas). (C) Cell exclusion zone assay (Platypus migration assay). The cell-free area is produced by cell exclusion upon seeding by using silicone stoppers, which are removed before starting the experiment. (D) Fence assay. Cells are seeded inside a ring-shaped plastic device, which is placed on a cell culture dish. After cell attachment the ring is removed and the cells migrate from the circular area to the non-covered surrounding. Migration is measured as the increase of the area covered by moving cells. (E) Microcarrier bead assay. Microcarrier beads are coated with cells to confluence and subsequently the cell-coated beads are placed onto cell culture dishes and incubated. Cells from the microcarrier attach to the plate surface and perform radial movement, the area of which can be measured. (F) Spheroid migration assay. Multicellular spheroids of a certain cell type are produced and put onto conventional tissue culture dishes. The spheroids attach to the surface and cells start moving concentrically outward. The increase in the spreading area can be measured over time. (G) Horizontal capillary assay (Dunn chamber, Zigmund chamber). The cells migrate along a stable gradient of a chemoattractant within a thin bridging capillary. None or special surface coating can be used (hatched areas, as in B and C). (H) Capillary tube migration assay. In small capillaries, leukocytes migrate out of the "bully coat area" into serum. The migration capacity of the cells can be directly measured by determining the position of the migration front in correlation to the starting line. (I) Leukocyte migration agarose technique assay. Holes of a defined diameter and distance are punched out of agarose gels; in one of these holes leukocytes are seeded, which migrate underneath the agarose layer toward a medium reservoir containing a chemoattractant (or repellent), whereas migration toward medium alone serves as control. The area of migration (dotted line) is visible and the difference in migration distance is determined. (J) Single cell motility assay. Normal tissue culture plates are preincubated with colloidal gold particles and thereafter cells are seeded at low density above. On their way across the plate, the cells clear the gold particles on their routes leaving bright tracks behind, which can be measured.

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through dissociation assay tracks over with migrate surface coated circular area gold migration horizontal line) can be measured. Alternatively, dark colored porous membranes are available, which block light transmission (FluoroBlok, Becton Dickinson) from unseeded cells. The detection of the migrated labeled cells is therefore simplified and there is no need to remove the remaining cells from the top-side of the membrane. The advantages are the availability of different cell culture inserts and sizes, the relative easiness of the experimental setup and a short lasting medium/cytokine/chemokine gradient between the upper and lower culture medium reservoir. This assay is most frequently used to assess cell migration. Except the cell culture inserts no special equipment is needed. The disadvantages are that it is an endpoint assay; the optimal time of analysis has to be determined individually for each cell type tested. If histological cell staining procedures are used there are several precautions to make: non-invaded cells, which stayed on the upper side of the transwell insert, have to be removed prior to staining of the invasive cells at the bottom of the membrane. This is commonly done by getting rid of the cells with a cotton swab, which often turns out difficult, non-quantitative and of variable success. We strongly recommend using fluorescent dyes, lysing the cells and quantifying them in a plate reader or to rely on the light blocking membranes and quantify migrated cells without lysis. The transwell migration assay is suitable for many different cell types including epithelial [35], mesenchymal [36] and brain [37] cancer cell lines as well as many primary cells from all three germ layers. Conventional cell culture inserts or whole transwell migration kits are commercially

violet), and the number of cells that have migrated is determined. For the second method, the migrated cells are stained fluorescently, removed from the membrane by dissociation (using cell dissociation agents such as trypsin) and quantified using a fluorescent reader. Alternatively, dark colored porous membranes are available, which block light transmission (FluoroBlok, Becton Dickinson) from unseeded cells. The detection of the migrated labeled cells is therefore simplified and there is no need to remove the remaining cells from the top-side of the membrane. The advantages are the availability of different cell culture inserts and sizes, the relative easiness of the experimental setup and a short lasting medium/cytokine/chemokine gradient between the upper and lower culture medium reservoir. This assay is most frequently used to assess cell migration. Except the cell culture inserts no special equipment is needed. The disadvantages are that it is an endpoint assay; the optimal time of analysis has to be determined individually for each cell type tested. If histological cell staining procedures are used there are several precautions to make: non-invaded cells, which stayed on the upper side of the transwell insert, have to be removed prior to staining of the invasive cells at the bottom of the membrane. This is commonly done by getting rid of the cells with a cotton swab, which often turns out difficult, non-quantitative and of variable success. We strongly recommend using fluorescent dyes, lysing the cells and quantifying them in a plate reader or to rely on the light blocking membranes and quantify migrated cells without lysis. The transwell migration assay is suitable for many different cell types including epithelial [35], mesenchymal [36] and brain [37] cancer cell lines as well as many primary cells from all three germ layers. Conventional cell culture inserts or whole transwell migration kits are commercially
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**Abbreviations:** 3D, three-dimensional; –, not suitable; *, suitable; PC, polycarbonate; PET, polyethylenterephthalat; BME, basal membrane extract; IF, immunofluorescence; IHC, immunohistochemistry; HTS, high throughput screening; n.d., not done so far.

a For adherent and suspension cells, no special equipment needed, technically non-demanding.
b For adherent cells only, any plate can be used, movement in defined direction, no special equipment needed, variation in wound area, damaged cells, damaged ECM.
c For adherent cells only, pre-defined assay formats, simple technical setup, no expensive equipment needed, exactly defined cell free area, no damaged cells.
d For adherent cells only, custom-made rings needed, technically non-demanding, directed movement.
e For adherent cells only, microbeads needed, technically medium-demanding.
f For adherent cells only, spheroid formation capacity required, technically medium-demanding.
g For adherent cells only, chemotaxis assays, standard chambers available, directed movement.
h For suspension cells only, technically non-demanding.
i Only for suspension cells tested, any plate can be used, movement in defined direction, no special equipment needed, directed movement.
j Simple, technically non-demanding, individual cell paths, simple readout, time consuming if non-automated analysis.
k For adherent and suspension cells, individual cell movement measurable, path detection, expensive equipment, advanced technical setup, non-directed movement.
available from many providers (e.g. Life Technologies, Becton Dickinson, Merck Millipore and many others). Multwell transwell assays for high throughput screening of migration were developed in a 96-well format (e.g. the disposable ChemoTx system from Neuro Probe Inc., Gaithersburg, USA; www.neuroprobe.com).

2.2. In vitro wound-healing assay (scratch assay)

This popular, technically non-demanding and cheap assay can be used to study migration of cells on 2D surfaces. A confluent plate of any type of attached cells is “wounded” by scraping off an area of cells, which is most easily done using a plastic pipette tip (Fig. 2B) [38,39]. Cell migration can subsequently be monitored microscopically, as cells travel from the intact zones into the scratched region. Cell movement can be calculated by measuring the decrease of the uncovered region at different time points until the “wound” is closed. Coating of the plates with e.g. collagen I, collagen IV, fibronectin, or basal membrane extract (BME, often also called Matrigel) prior to cell seeding offers the possibility to study migration on different substrates. A long term wound-healing assay (>24 h) cannot distinguish cell proliferation and changes in cell survival from cell motility [40]. Cells can either migrate as single cells, as loosely connected population (mesenchymal cells) or collectively as sheets of cells (epithelial cells). A wide variety of cells have been analyzed for migration with this assay e.g. epithelial and mesenchymal cancer cells [41], keratinocytes [42], normal epithelial cells [43], endothelial cells [44] and fibroblasts [45,46]. The advantages of the assay are its simple and rapid setup, easy readout and analysis and of course its cheapness. One drawback of the assay is that the scratch is often unevenly thick. The migration speed of cells just prior to wound closure is often increased; therefore variations in gap width prior to the migration start of the cells are critical. In addition, some cells keep attached to the border of the scratch after wounding. These cells often reattach to the plate and move into the wounded area, which leads to adulterated results. Third, the plastic surface or the ECM substrate is scraped off in an uncontrolled manner [47] and artifacts might be induced by mechanical cell damage. To circumvent these problems new techniques have been developed to ensure invariable wound sizes with defined edges. One way to generate wound areas of defined size without significant variation is electric cell–substrate impedance sensing (ECIS) [48,49] as for example provided by Applied BioPhysics (New York, USA; www.biophysics.com). Instead of mechanically disrupting the cell layer and following the migration microscopically, electric signals are employed to both, wound the cell monolayer and to monitor the re-population of the cell-free area. Electrical wounding is applied to cells in contact with a small ECIS electrode by a pulse of high current, which leads to cell death. Thus a well-defined 250 µm wound is created. The insulating property of living cells is lost in the treated region and the impedance drops. Thereafter, the migration of cells into the cleared area is measured by increased impedance in real time as the wound proceeds to close [50]. The advantage of the system is that it can be automated and is feasible for medium to high throughput assays. Alternatively, wounding can be achieved by laser ablation of the cells in a defined area [51]. One drawback of both systems might be cell remnants, which are left after the electric ablation in the wounding area [52] and possibly influence wound closure. To our knowledge simple scratch assays, which are based on mechanical wounding by hand, are not commercially offered. Electric cell ablation assays can be bought from Applied Biophysics as described in more detail above. Of note, a free software tool called “TScratch” (www.chaton.ethz.ch/software) is available, which allows simple automated image analysis of any wound healing and cell exclusion zone assay [53].

2.3. Cell exclusion zone assay

A possibility to circumvent the above described cell remnants is to create cell exclusion zones at the time of cell seeding with e.g. microstencils [52] or by applying an electrical fence (Applied BioPhysics, New York, USA; www.biophysics.com). Platypus Technologies Inc. (Madison, USA; www.platypustech.com) designed small silicone stoppers that fit into each well of a 96-well plate. These stoppers are positioned prior to seeding of the cells and create an exclusion zone with the tip of the stopper (OriS™ cell migration assay). The cell density is adjusted that cells are fully confluent. After cell adhesion, the stoppers are removed creating a 2 mm diameter circular cell-free area, wherein the cells then will migrate (Fig. 2C). The advantages of this setup are wound zones of reproducibly similar sizes and sharp borders. In addition, there is no damage to the cells from mechanical scraping or electric ablation (see previous section). Moreover, the assay is well-standardized, easy to set up and does not need special equipment for analysis. This assay is only suited for adherent cells. Care must be taken that the stoppers are tightly attached; otherwise cells enter the cell exclusion zone beneath the silicone device. After removal of the stoppers, the wells have to be washed carefully with medium in order to prevent attachment of floating cells in the cell exclusion area, which would compromise the results. A derivative of the assay was recently developed and employed as fast dissolvable biocompatible gels instead of the silicone stoppers (OriS™ Pro cell migration assay). Of note, this assay is available in a 96-well and 384-well setting. The standard silicone stopper assay has been widely used e.g. for assessing epithelial [54,55] or smooth muscle cell migration [56]. Results generated with the OriS™ Pro migration assay have not been published so far. Similar replacement devices are also available from other companies (e.g. Cell Biolabs, Inc., San Diego, USA; www.cellbiolabs.com). One of these devices (Ibidi GmbH, Martinsried, Germany; www.ibidi.com) provides two cell culture reservoirs when placed on a cell culture dish surface. The reservoirs are separated by a 500 µm thick wall. Seeding cells in both reservoirs and removing the silicone insert from the surface results in two defined cell patches separated by a cell free gap of 500 µm width. This setup also allows investigating the migratory behavior and interaction of two different cell populations seeded separately into the two different wells. As already described above, migration can be measured by photomicrography or quantified using microplate readers if fluorescently labeled cells are used. For this a mask is used to cover the areas, which are populated by the cells before starting the experiment. As cells move, they leave the masked area and can be detected and quantified in fluorescence microplate readers. The detected fluorescence signal is proportional to the amount of migrated cells.

2.4. Fence assay (ring assay)

The fence assay is in principle a reversal of the cell exclusion zone assay described above. Here, cells are seeded into the inner area of a Teflon, glass or metal fence (ring) placed on a standard cell culture dish (Fig. 2D). The cell attachment area is restricted to the inner part encircled by the ring device: After the ring is detached, non-attached cells are removed by gently washing and the cells are incubated and allowed to migrate from the circular area in a radial way outward. The rate of movement is measured as the increase of the newly area covered by migrating cells, which is often done digitally by automated image analysis [57,58]. Using this assay the migratory potential of human endothelial cells was quantified [59,60]. In a slightly different assay cell aggregates of defined size formed by centrifugation can be placed onto microscopic slides and the subsequent migration evading out of the structure can be measured [61]. Due to the same principles the advantages and
2.5. Microcarrier bead assay

This assay measures cell motility based on migration of the cells from microcarrier beads onto 2D cell culture vessel surfaces [62] (Fig. 2E). Therefore microcarrier beads (e.g. DEAE Dextran beads; e.g. Cytodex beads from GE Healthcare, Chalfont St. Giles, UK) are coated with cells. These cells can be grown to confluence on the surface of the beads. Thereafter the cell-coated beads are placed onto cell culture dishes and incubated for a defined period. Subsequently the beads are removed by suction. The cells, which moved to the cell culture vessel surface area, are fixed, stained and microscopically evaluated or densitometrically measured. Here the advantage is that cells established close cell–cell interactions on the beads, which is more closely mimicking the tight contact of cells in vivo. In addition, due to the limited space and little cell size variation a fairly constant amount of cells are present on the bead surface (e.g. approximately 400 HUVECs on collagen coated Cytodex beads), when confluence is reached. Cell coating can be easily monitored with conventional light microscopy. There are always some beads, which are not or insufficiently covered by cells. These beads should be identified and not used for analysis. Another drawback of the assay is that the Cytodex beads are rather expensive. The initially described assay is an endpoint assay; however, we have used live cell microscopy to monitor cell migration off the beads in kinetic experiments without fixation and staining of the cells. The model was rarely used as a simple migration assay in the literature. For example it was deployed to demonstrate the negative effects of PAI-1 on cell migration [63]. The cell coated beads are more often embedded into ECM and invasion is monitored (see below). There are no commercially available kits on the market.

2.6. Spheroid migration assay

This assay combines 3D with 2D technologies by placing multicellular tumor cell spheroids of a certain cell type on top of a conventional cell culture dish. The assay principle closely resembles that of the above mentioned microcarrier bead assay. After attachment of the spheroid to the plastic surface, the cells start to migrate and the area of attachment is increased concentrically as the cells move out. Cell movement can be measured microscopically (Fig. 2F) over time. One major advantage compared to the above described assays is that the 3D structure of the spheroids represents a more physiologic tissue-like morphology with close cell–cell contacts and different cellular statuses as nutrient and oxygen supply is concerned. In respect to tumor biology for example, the migration of cells out of small cancer clusters can be closely mimicked. Of note, this assay is only possible if the cells investigated are able to form spheroids. Some pre-experience with spheroid formation is necessary, but this can be easily achieved. The method was used to demonstrate that tissue factor pathway inhibitor-2 (TFPI-2) expression inhibited cell migration and invasion in prostate cancer cells [64]. In a different study spheroid migration was used to prove that valproic acid has either pro- or anti-migratory effects on malignant gliomas, dependent on different cell lines analyzed [65].

We currently use a modified spheroid migration assay, which reflects tumor–stroma interaction. Spheroids from fluorescein labeled tumor cells (genetically modified to express fluorescent proteins e.g. GFP or labeled with fluorescent cell tracker dyes like 5-chloromethylfluorescein diacetate, CMFDA) are formed and applied to confluent fibroblasts (e.g. cancer associated fibroblasts, CAFs), which are grown on a conventional plastic dish. This setup can be used to analyze the migratory changes upon interaction between the two cell types. Upon attachment, the tumor cells start to interact with the monolayer cells. The close interaction ensures paracrine crosstalk and direct cell–cell interaction at the migration front of the tumor cells. Migration can again be measured as increase of the attached tumor cell area over time. Fluorescent labeling helps to distinguish tumor cells from the CAFs (Dolznic et al., unpublished results). The general feasibility of the assay to analyze the interaction of cancer cell spheroids with different stromal cell types was demonstrated using colon carcinoma cell spheroids co-cultured with monocytes and fibroblasts [66]. However the authors did not specifically address migration. To our knowledge there is no commercial assay available on the market.

2.7. Capillary chamber migration assays (microfluidic chamber assays)

In a horizontal setting two chambers are linked side-by-side by a narrow connecting bridge (e.g. Zigmund chamber [67], Dunn chamber [68,69]). One of the chambers is loaded with cells resuspended in medium, whereas the other is filled with medium containing a chemoattractant. The system is covered by a glass slide. A stable concentration gradient develops between the two reservoir chambers in the connecting capillary area and the number of migrating cells is counted on the surface of the connection by light microscopy (Fig. 2G). These assays are often used for leukocyte migration studies. The advantage lies in the small assay volumes, making these assays well suitable for studies with rare cell types and to test expensive compounds. The use of small media volumes demands daily changes and careful humidification of the incubator. In addition, automation processes are difficult to set up with the available systems. The horizontal capillary migration chamber was further advanced to the mSlide Chemotaxis assay (Iibi, Martinsried, Germany, www.iibi.com). It consists of two medium reservoirs and a perpendicular channel, where cells can be seeded. A gradient can be applied via the two reservoir chambers and the movement of cells can be monitored microscopically. The main advantage to the above-mentioned methods is the easy handling and the assessment of directed chemotaxis along a gradient. Recently, the capillary chamber assay was used to prove that the Src family kinase Fyn is involved in HGF-mediated chemotaxis of prostate cancer cells [70]. For HTS, 96-well and 192-well formats have been developed [71] and are commercially available (BellBrook Labs, WI, USA, www.bellbrooklabs.com).

3. Capillary tube migration assays

The capillary tube migration assay was developed many decades ago to address leukocyte migration [72]. For this assay, glass capillaries are filled with blood, sealed and centrifuged. From the leukocyte “buffy coat”, which is located above the red blood cell pellet, leukocytes start to move out to the plasma layer (Fig. 2H). The glass capillaries are placed onto a microscopic slide and the distance of migration can be measured by microscopy with ocular micrometer devices. This assay only needs small sample volumes and the use of multiple capillaries leads to proper statistical analyses. In an advancement of the assay, the capillaries are cut at the buffycoat/erythrocyte interface and the leukocyte containing part is placed onto plastic culture dishes. Subsequently it is covered with test medium and cells start to migrate. The size of the area of cells that migrated out of the capillary onto the plastic surface is a measure of cell migration [73]. Neutrophil migration in response to an anti-inflammatory response was reported using this
assay [74]. This specialized migration test for white blood cells seems not to be used any more, maybe because it vanished into oblivion. It is described here for reasons of completeness and to demonstrate its potential. Companies do not offer this assay type.

3.1. Leukocyte migration agarose technique assay (LMAT assay)

Another assay to analyze white blood cell migratory potential was introduced in 1968 [75]. This method involves a pre-cast thin agarose layer on a tissue culture dish into which circular wells were cut in defined distances. Leukocytes are loaded into one of these wells, whereas the adjacent holes are filled with different media containing either chemotactic factors, or inhibitors or controls [76]. The leukocytes migrate below the agarose layer toward the different media reservoirs (Fig. 2I) and the distance of movement toward the test substance (divided by the migration distance toward the control medium) defines the migratory index. 

The advantages for this assay are the easy and cheap setup as well as the simple detection method. The assay seems to be outdated; only a very limited number of citations still using this assay can be found. In addition, this method is restricted to analyze the migration of white blood cells. The LMAT assay was employed to show that monocytes and macrophages increase the migration of neutrophils in the presence of glucocorticoid [74] and CXCL8 might be involved in reduced neutrophil migration [77]. We could not find any commercially available LMAT assay.

3.2. Single cell motility assay (colloidal particle assay, colloidal gold single cell migration assay)

A simple way to measure migration on the single cell level is to use colloidal gold particle coated surfaces [78–80]. Tissue culture plates are coated with colloidal gold particles and cells are seeded onto these plates at low density (1 × 10^3 cells/ml). The gold colloidal particles are seen as a homogenous layer of small dark dots under the microscope. The migrating single cells phagocytose the gold particles and thereby remove them from the plastic surface, resulting in white tracks, which can be photographed and the cleared areas can be evaluated quantitatively (Fig. 2J). This single cell motility assay was used to track the migratory paths of e.g. keratinocytes [81], fibroblasts [82], epithelial cancer cells [83,84]. This assay has also been used to demonstrate that rapamycin reduced motility of tumor cells at the single cell level [85]. The method is suitable for automation and high throughput assays and the gold particles can be replaced by quantum dots [86].

The advantages of this assay are: single cell tracking; undirected movement (chemokinesis) can be monitored; real-time path detection is possible and therefore the absolute speed of migration can be determined. Disadvantages are: intense analysis labor or automated system needed; rather small sample sizes (one cell–one track). We are not aware of a currently available assay sold by life science companies.

3.3. Time-lapse/cell tracking

Another possibility to analyze single cell migration is the tracking of individual cells with videomicroscopy in time-lapse experiments. The selection and tracking of the cells can either be done manually, semi-automatic or fully automatic. For automated cell tracking many algorithms have been developed which can cope with the recording of the migration path of several dozens of cells simultaneously. The automated analysis can also cope with cell division of the moving cells occurring within the recording time interval [87,88]. A full description of the novel automated tracking systems goes far beyond the scope of this review and is given elsewhere [89]. Essentially, the individual movement of many cells can be analyzed at once and the recorded migration paths provides information about the total movement length as well as the direction and velocity at a given time point. Time lapse cell tracking has been used for example to analyze myogenic cell motility [90] or to show that collective migration is responsible for pattern formation in co-cultures of different keratocytes [91]. As we are no specialists in this field we do not comment on pros and cons of these systems and again refer to specialized review articles (for example [89]).

4. Invasion assays

4.1. Transwell invasion assay

The principal technical setup of the transwell invasion assay equals the transwell migration assay (see above). In addition, the porous filter is overlaid by a thin layer of ECM, before seeding the cells into the top chamber [92–94]. The ECM occludes the membrane pores, blocking non-invasive cells from migrating through. By contrast, invasive cells can degrade the matrix and move through the ECM layer and adhere to the bottom of the filter (Fig. 3A). Depending on the detection method, the invasive cells, which crossed the membrane pores, are either stained and counted with a light microscope, or detached, stained and lysed using fluorimetric detection. The ECM can be variable and often BME obtained from Engelbreth–Helm–Swarm (EHS) mouse sarcomas or collagen I is used. It is recommended to calculate the ratio of invaded (passed through the ECM coated filters) against the migrated cells (non-coated filters, see migration assay), the so-called “invasive index". This determines the relative contribution of invasion to the overall motility speed [93]. The advantages of the method are the broad availability of different cell culture inserts and sizes; the relative ease of the experimental setup and the – albeit only short – medium or cytokine/chemokine gradient between the upper (cell culture insert) and lower (culture vessel) growth medium. This method is an endpoint assay and there are some further drawbacks, if simple cell staining procedures are used. Non-invaded cells, which stayed on the upper side of the transwell insert, have to be eliminated prior to staining of the invasive cells at the bottom of the membrane. This is generally done by removing the cells with a cotton swab, which often turns out difficult, non-quantitative and of variable success. We strongly recommend to use fluorescent dyes, lyse the cells and quantify them in a plate reader, for reliable results. The transwell invasion assay is the most frequently used invasion assay and has for example been employed to analyze human trophoblast [95], melanoma [96], or colon cancer invasion [97]. Transwell invasion assay kits provided by many cell biology companies (e.g. Becton Dickinson, Merck-Millipore, Corning) are widely used, due to their well-standardized assay conditions and protocols.

4.2. Platypus invasion assay

The Platypus invasion assay (Oris™ cell invasion assay, Platypus Technologies, Madison, USA; www.platypustech.com) uses the same equipment as the migration assay described previously in the cell exclusion zone assay section: small silicone stoppers fitting into 96-well plates. However, the setup of the method is quite different. First, the bottom of individual wells is covered by a thin layer of BME. Subsequently the silicone stoppers are positioned and create an exclusion zone when cells are seeded. After cell adhesion on top of the first layer of BME, the stoppers are removed and the cells as well as the cell-free circular center region are overlaid by a thicker second layer of BME. Thereby a layer of cells embedded between two sheets of ECM is made and a central cell-free area filled with ECM is generated (Fig. 3B). Invasive cells
Fig. 3. Schemes of commonly used invasion assays. An overview of the technical setup is schematically drawn for each assay and a close up view is given right to it (inside the circles). Arrows indicate the direction of cell movement. Hatched areas symbolize ECM. (A) Transwell invasion assay (Boyden chamber assay). The thin ECM coating (hatched areas), which occludes the pores of the filter is indicated. (B) Platypus invasion assay. Cells are seeded on top of a thin ECM coated surface and are overlaid by a second thicker layer of ECM. In the center, a silicone plug creates a cell-free exclusion zone. Therefore, invasive cells are embedded in ECM and migrate from an outer ring into the center. (C) 3D cell tracking assay. Cells are tracked on their route through the ECM via automated microscopy and state of the art image analysis software. Their routes through 3D space can be recorded and measured. (D) Gelatin degradation assay. Cells are seeded onto a thin fluorescently labeled gelatin layer. At sites of invadopodia mediated matrix degradation a loss of fluorescence occurs, which can be documented and quantified by microscopic imaging. (E) Vertical gel invasion assay (organotypic skin model). Squamous epithelial cells are cultivated at the liquid-air interface on top of a collagen gel. Cells invade vertically into the collagen matrix, which can harbor stromal fibroblasts as indicated. (F) Spheroid/monodispersed cell invasion assay. A single cell suspension of a certain cell type is incubated with preformed spheroids of a different cell type, and cells attach to and eventually invade into the spheroid. Fluorescent labeling of the cells and subsequent flow cytometry after trypsinization or confocal imaging can reveal the invasive capacity of one cell type moving into tissue like structures composed of the other cells. (G) Spheroid confrontation assay. Spheroids from different cell types are forced to attach to each other and fuse. An invasive cell type will invade into the cell clusters of non-invasive cells, which can be visualized using fluorescently labeled cells and optical or mechanical sectioning. (H) Spheroid gel invasion assay. Multicellular spheroids are embedded into ECM gels, which are submerged in growth medium. Invasive cells emerge from the cell clusters and give rise to astral outgrowing structures.

migrate into the middle and can be followed over time with a microscope and quantified. In addition confocal analysis of the invading cells after fixation and immunofluorescent staining is possible (Fig. 4A). This commercially available assay only provides BME as ECM substrate. However, in our experience it is possible to substitute BME with substrates such as collagen I as the ECM component (Dolznig, unpublished data). Of note, the silicone stoppers can be washed, autoclaved and reused provided that a fitting 96-well plate is used (e.g. Nunc 96 well optical bottom plates, Cat. Nr. 165305). Main advantages of this invasion assay are: a well standardized assay format and a rather easy setup; no specialized equipment is needed; kinetic analysis is possible; live-imaging is feasible and invasion takes place horizontally in a thin ECM layer which facilitates evaluation with a standard microscope; invading cell morphology can be studied and at the end of the assay the gels can be further processed e.g. fixed and processed for immunofluorescence analysis. Disadvantages are: no growth factor/cytokine/chemokine gradient can be formed; the cells are embedded as single cell suspension, therefore no real cell–cell interaction is established prior to the assay. A recent publication used this assay to demonstrate that p53 inhibits tumor cell invasion via degradation of Snail in liver cancer [98]. There is also an Oris™ Pro cell invasion assay in a 96-well setting available, with pre-applied disposable biocompatible gel drops, which act as cell exclusion devices. This kit is better suited for cellular screening assays because of less assay-handling time, automation suitable design and start to end live imaging possibility.

4.3. 3D cell tracking

As discussed above for 2D cell tracking, 3D cell tracking uses computer-aided time-lapse videomicroscopy for automated image analysis to track the path of moving cells. However, there is the surplus difficulty to follow individual cells in 3D environments over time (Fig. 3C). Many different strategies have been developed to track either labeled or unlabeled cells in 3D matrices. These techniques include for example wide field fluorescence, confocal or multiphoton microscopy as well as contrast enhancing or digital holography microscopy. A detailed description of these techniques can be found elsewhere [99]. The major advantage of this method is that individual paths of cells travelling through 3D structures can be monitored in real time. Therefore, direction changes and the actual length of the invading cell paths can be exactly determined. However, only a limited set of cells can be analyzed simultaneously,
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<td>*</td>
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<td>Invasion area</td>
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Abbreviations: 3D, three-dimensional; –, not suitable; *, suitable; PC, polycarbonate; PET, polyethyleneterephthalat; BME, basal membrane extract; IF, immunofluorescence; IHC, immunohistochemistry; HTS, high throughput screening; n.d., not done so far; n.a., not applicable.

a For adherent and suspension cells, no special equipment needed, technically non-demanding, commercially available.
b For adherent cells, no special equipment needed, technically non-demanding, commercially available.
c Special equipment needed, technically highly demanding, individual cell paths detectable, non-directed cell movement.
d Subcellular invasion areas detectable, no long-term movement detection, commercially available, fluorescence microscope needed.
e Used for leukocyte and epithelial cell invasion, quantification using radioactive labeled cells or microscopy, heterotypic cell interaction possible, quantification needs confocal microscope or fixation and sectioning.
f Heterotypic cell interaction, in vivo like condition, invasion through tissue-like structures with cell–cell contacts, difficult to distinguish attachment and invasion, quantification by FACS.
g Heterotypic cell interaction, in vivo like condition, invasion through tissue-like structures with cell–cell contacts, confocal microscopy or fixation and sectioning needed, time consuming.
h Heterotypic cell interaction possible but not required, in vivo like condition, invasion through ECM, confocal microscopy or fixation and sectioning needed, time consuming, technically demanding.
a specialized microscope for live imaging is necessary as well as there is need for advanced knowledge in data processing. The 3D cell tracking was essential to demonstrate that migration of tumor cells is directed by the stiffness of the 3D matrix in addition to cell adhesion properties to and proteolysis of the matrix [100]. Extensive 3D cell tracking analysis was also used to prove that rapid amoeboid migration of leukocytes is integrin independent and relies on F-actin mediated protrusions and a Myosin II reliant squeezing of the nucleus in narrow pores [101]. This study used ImarisTrack (Bitplane AG, Zurich, Switzerland; www.bitplane.com), a commercially available software for 3D cell tracking.

4.4. Gelatin degradation assay

This assay allows visualizing and quantifying invasion at the subcellular level rather than analyzing the invasive behavior of whole cells. The higher resolution of this method led to the discovery of cellular protrusions called invadopodia and podosomes [102], which degrade the ECM. The principle of the assay is to seed cells on top of a thin layer of fluorescently labeled matrix and to record and measure regions where the cells degraded the matrix leaving behind areas that lack fluorescence (Fig. 3D) [103,104]. The plus of this method is that subcellular high resolution data of invading structures can be made visible. On the other hand, cells cannot be followed as a whole during their movement. In addition, the assay is not a real 3D invasion assay, since cells are attached to a thin layer of ECM and therefore adapt a 2D cell shape. Therefore invadopodia structures might be not exactly reflecting the 3D reality. The gelatin degradation assay was used in a study, which demonstrated that the Src substrate Tks5 is implicated in podosome mediated macrophage invasion [105]. In another report the method was employed to demonstrate that bioactive laminin-derived peptides increased invadopodia activity in adenoid cystic carcinoma [106]. This assay is also commercially available, providing either red or green fluorescent labeled gelatin as extracellular matrix (QCM™ Gelatin Invadopodia Assay, Millipore).

4.5. Vertical gel 3D migration/invasion assays

3D migration into collagen gels were first described using leukocytes, which were placed on top of few millimeter thick collagen gels [107]. The vertical 3D migration was monitored by optical sectioning and counting the cells or by radioactive labeling of the cells and scintillation counting [108]. Vertical invasion from epithelial cell layers can be followed by plating carcinoma cells on top of a collagen gel layer (Fig. 3E). Therefore, thick collagen plugs are prepared and cells are seeded on top of the gel surface (Fig 3E). A variant of the vertical invasion assay is the upward movement of cells from a monolayer, onto which a layer of ECM has been poured.

The first mentioned assay is often used as organotypic skin model (skin equivalents) to study skin cancer cell invasion [109–111]. Here, most studies are performed in the presence of stromal subcutaneous fibroblasts embedded in the collagen gel (Fig. 3E). Invasion can be quantified by (immuno)histochemical staining [112] and quantification of the invasive areas using image analysis software. The advantage is that the organotypic skin cancer model most faithfully mimics the in vivo situation for invasion. It combines 3D invasion into ECM with heterotypic cell–cell interaction of epithelial cancer cells and stromal fibroblasts. Best analysis of invasion is possible with formalin fixed paraffin embedded (FFPE) samples, which are cut perpendicular to the surface. This sample processing also provides information about histological properties and cellular morphology. On the other hand, there is the drawback that sample preparation is quite labor intense and needs special equipment including embedding stations and microtomes as well as FFPE cutting experience. Using the organotypic skin cancer model it was shown that stromal fibroblasts lead to collective invasion of epithelial cells [113] in squamous cell carcinomas. Recently, based on the vertical invasion assay it was shown that inhibition of autophagy resulted in impaired invasion in a glioma cell line, whereas cell viability, proliferation and migration were unaffected [114]. One impressive organotypic 3D model investigates invasion of squamous cell carcinoma cell lines seeded on top of primary human myoma tissue instead of the collagen gel [115]. Here the in vivo situation is recapitulated even more closely. So far there is no vertical invasion assay available on the market.

4.6. Spheroid/monodispersed cell invasion assay

In vivo tumor cells invade the surrounding tissue from cancer cell clusters. These tumor clusters are 3D structures, which can be mimicked by small aggregates of cells formed in vitro, called multicellular spheroids [116]. Cells grown as multicellular spheroids closely recapitulate the in vivo situation of solid cancers [117–119]. The single cell/spheroid invasion model is a model to study the invasive property of a certain cell type (cell type A, which in most cases is a non-malignant cell) into a tissue like structure composed of a different cell type (cell type B). However, it is important to note that this assay might also be used to monitor the invasion of malignant cells into spheroid structures made of normal or non-malignant cells. In principle, spheroids of cell type B are co-cultivated with a single cell suspension of cell type A, which attach to the spheroid surface and eventually start to invade into the spheroid (Fig. 3F). The use of fluorescently labeled cells provides a tool to analyze 3D migration or invasion by (confocal) fluorescence microscopy. The quantification of cells attached to or invading into the spheroids can be done by flow cytometry after trypsinization. Another possibility is to fix the cells, make slices...
and perform immunofluorescence or immunohistochemical analysis. The main advantage of this method is that the barrier to be invaded is composed of tightly arranged multicellular 3D structures with established cell–cell interactions as it is the case in vivo. It is ideally suited to study immune cell infiltration in vitro. However, a prerequisite must be fulfilled: the cell type B has to be capable of forming spheroids, which some cell lines do not. The quantification of invading cells is not straightforward and requires either deep confocal imaging or sample preparation for immunohistochemistry including paraffin embedding and tissue sectioning equipment as well as technical expertise. If using flow cytometry for quantitative detection a prior trypsinization step is required to remove the outmost cells prior to single cell dissemination. This is important to distinguish cells attached to the spheroid surface from truly invading cells. A standard protocol for this has not been developed yet. For example, the spheroid/monodispersed cell invasion assay was used to study T-cell infiltration in pancreatic carcinoma [120]; to demonstrate that tumor cell spheroids can influence monocyte differentiation to tumor associated macrophages [121]; or to prove that T-cadherin increased the invasion of endothelial cells into melanoma spheroids [122]. To the best of our knowledge this assay is not offered commercially.

4.7. Spheroid confrontation assay

The interaction and invasion behavior of two different 3D cell aggregates can be addressed with the spheroid confrontation assay [123]. In this approach, two preformed spheroids derived from different cell types (one being invasive the other one non-invasive) are cultivated side by side and eventually start to fuse (Fig. 3G). Thereafter, cells either infiltrate the opposing spheroid as single cells, or collectively, or display a non-invasive phenotype. In the non-invasion case a well-defined border is formed at the interface between the two cell types. One important advantage of this method is that the invasive properties of cells grown in 3D with well-established cell–cell interactions into another tissue-like structure composed of a different cell type can be studied. This is closely reflecting the in vivo situation in case of a carcinoma is invading a certain tissue or organ. The quantification of invading cells requires either confocal imaging or preparation for immunohistochemistry including paraffin embedding and tissue sectioning. If the cells express distinct markers, one can differentiate them immunohistochemically. Alternatively, the invading cell type can be fluorescently labeled prior to the confrontation and analyzed thereafter either by live imaging or after fixation and further processing. Therefore the main drawback of the assay is that it requires extensive post-experimental processing and special equipment. The prerequisite to form multicellular spheroids could represent another disadvantage. There are primary cells and cell lines, which do not form spheroids and therefore are not suitable for this kind of invasion assay. The spheroid confrontation method was first described to study the invasive properties of cells derived from human brain tumor explants into dermal cell spheroids representing the non-invasive, normal counterpart [124]. Another study analyzed the invasive capacity of colon carcinoma cells in contact with normal skin fibroblasts [125]. Recently, the spheroid confrontation assay was employed to demonstrate that NM23-H1 regulates contact inhibition of locomotion in invading glioblastoma cells [126]; that CXCL16 induces invasion of glial progenitors [127]; and that Endoglin enhances breast cancer cell invasion [128]. This assay format is not commercially available.

4.8. Spheroid gel invasion assays

When multicellular spheroids are embedded into 3D ECM such as collagen I or BME gels (Fig. 3H), non-invasive cancer cell lines stay as compact spheroids with a distinct border to the surrounding ECM and do not show any signs of invasion even after 2 weeks of cultivation [129]. Invasive cell lines (such as the HT-1080 sarcoma cell line, the ovarian carcinoma cell line SK-OV-3 or the breast cancer line MDA-MB-231) or endothelial cells start to invade into the surrounding matrix and display astral outgrowth from the spheroid [130] (Figs. 3H and 4B and C). Invasion can be followed by live imaging and quantified by measuring the invasive area over time in photomicrographs. The gels with the invading structures can be fixed and processed for immunofluorescence staining and confocal microscopy [118]. Alternatively, the gels can be enzymatically degraded and the cells isolated for flow cytometry analysis. A third possibility is to make protein lysates and perform Western blot analysis (Kramer, Walz unpublished). The main advantage of this assay is that cell movement through a 3D matrix closely mimics invasion in vivo. Importantly, invasion occurs from cell clusters with well-established cell–cell interactions rather than from single cells, as it is the situation in human cancers. A wide range of different ECM gels (e.g. collagen I, BME, fibrin) can be commercially obtained in their liquid forms and quickly solidified chemically or physically. Therefore different substrates are readily available depending on the research question. The outer border of spheroids placed in the gel can be easily detected in a standard inverted light microscope. In live imaging experiments kinetic measurements of cell invasion can be made. Finally, it is compatible with molecular biology analysis methods. Care must be taken to distinguish real invasion from cell movement on the surface of the gel. Some spheroids are occasionally embedded at the gel-medium interface or at the bottom in contact with the tissue culture plate and migrating cells always take the route of least resistance. If cells are situated at the gel-medium interface they migrate along the surface instead of taking the route through the gel. This falsely gives the impression of rapid invasion of that particular spheroid. These events have to be excluded from the analysis. Some experience is needed to distinguish the surface migration, which is characterized by movement of cells in a single plane and 2D cell morphology, from 3D invasion. Additionally the experimental effort is rather high compared to other assays and some pre-experience with 3D gel systems is required. The spheroid invasion system has already been used to define essential molecular pathways during 3D invasive growth [118,131] or to measure TGFbeta induced invasion [132]. In a very similar assay cell-coated microcarrier beads (see above in the migration section) can be embedded into ECM gels and invading cells can be detected. This assay format was used to investigate the destructive 3D motility in trophoblast-like cells [133]. It is mostly used to measure the formation of endothelial cell (EC) networks in 3D gels e.g. to demonstrate the secretion of pro-angiogenic factors by mesenchymal stem cells [134] or to show that ECM density influences EC capillary formation [135]. We employed this model to analyze EMT in hepatocellular carcinoma [136] and to clarify the function of lvP53A7 in ovarian carcinoma [137]. Using an advancement of the method we determined invasive properties and the molecular pathways activated by the interaction of colon carcinoma cells with stromal fibroblasts [129]. For that we dispersed fibroblasts in the collagen gel in addition to colon cancer spheroids. This setup allows the interaction of tumor cells with stromal fibroblasts in 3D, which induced cancer cell invasion in otherwise non-invasive lines. Currently we are using the spheroid invasion model to address the function of the mTOR signaling pathway [138–140] during invasion. So far there is no spheroid invasion assay commercially available.

4.9. Conclusions and perspectives

Since the acquisition of a migratory phenotype is the prerequisite for metastatic spreading, the determination of the
migratory and invasive potential of tumor cells and the molecular mechanisms behind this process is fundamental for proposing novel clinical strategies in cancer diagnosis, prognosis and drug development.

A wide variety of assays assess the migratory or invasive potential and activity of cells in vitro. These assays range from very simple and inexpensive ones to technical demanding or expensive solutions. However, the suitability of a specific method may be limited when considering a certain cell type(s) or specific research question. In this review, we offer researchers a concise and informative description of all available methods to measure invasion and migration potentials of cancer cells to employ in their investigations. We have in particular provided pragmatic description of all the available techniques and tools to choose from, including a depiction of the assay principle, advantages, drawbacks and limitations that will aid relative “newcomers” to the field in choosing the most appropriate methods for their work. To evaluate cell migration, with reference to our own experience, we recommend using the exclusion zone assay for several reasons. (1) There is no need for advanced cell biology technical knowledge. Cells are mostly seeded in 96 well plates as monolayers; the cell exclusion zone is defined by a physical barrier, avoiding damage to the cells. (2) The cell free zone is reproducibly of the same average size. (3) No special technical equipment is required; an inverted cell–migration microscopy equipped with a digital camera is sufficient. During migration of cells into the cell exclusion zone thereby decreasing it, the wells can be photographed using conventional phase contrast microscopy; the cell free area can be determined by image analysis tools (e.g. ImageJ, which can downloaded free of charge from http://www.rsweb.nih.gov/ij/).

(4) Optionally cells can be fluorescently labeled before seeding or at the end of the experiment; a mask can be applied and a fluorescence signal, which is proportional to the amount of migrated cells, can be detected in a microplate reader; this allows high throughput screening. (5) The assay allows kinetic assessment of migration and time lapse videomicroscopy is possible if desired. Therefore, actual migration velocity can be determined and the phenotype of the moving cells is recorded during the experiment. From our point of view these facts make the cell exclusion zone assay superior to the also widely used transwell migration assay, which reliably detects differences in cell migration, however offers less readout options for roughly the same cost input and work load. For a simple technique to measure invasion the same features pointed out above we support the use of the Platypus invasion assay instead of the widespread transwell invasion assay, despite being more laborious. If a close mimicry of the in vivo situation in tumors is desired, the following considerations should be taken into account. First, tissues and tumors are structures with established cell–cell contacts in the three dimensional space. Before being able to move out of these structures cells have to first bear down the close physical contact to their neighbors or move collectively and attach to a different substrate. This is best mimicked by the multicellular spheroid migration assay, which is from our experience a good compromise between a fairly close reflection of the in vivo situation and manageable experimental effort. Second, it is now obvious that the cellular components present in the interstitial space, which together comprise the tumor stroma (i.e. fibroblasts, myofibroblasts, pericytes, endothelial cells, immune cells) influence the invasive potential of cancer cells [113,141,142]. These interactions should be considered in in vitro assays to recapitulate the in vivo situation as close as possible. Some of the assays described here – i.e. the spheroid migration assay, the vertical gel invasion assay, spheroid gel invasion assay – can be used with heterotypic cell interaction and have already been demonstrated to work. Undoubtedly, these assays are labor intense and therefore error-prone and cannot be used in cellular high throughput screening. However we are convinced that they are the most sophisticated models to address cell migration and invasion in a highly relevant physiological setting and are therefore worth the additional effort.

Conflict of interest

There are no conflicts of interest.

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