Models of cancer cell migration and cellular imaging and analysis

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Abstract

The present topic focuses on the different cellular imaging technologies available to monitor and to characterize the migratory behavior of cancer cells. To this aim, we consider two aspects that greatly influence the choice of an adapted imaging technology. The first one concerns the different levels of investigation which can be considered (and combined) to efficiently analyze cell migration, going from a global analysis of the behavior of a cell population to a focus on the motility apparatus of a single cell. The

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second aspect relates to the biological models available to provide information about the process of tumor cell dispersal through imaging. We then analyze the pros and cons, as well as the complementary aspects, of these different models by taking into account their ability to mimic the in vivo reality and the resulting imaging requirements. The following two sections are dedicated to descriptions of the different imaging technologies available for the monitoring of cell migration in the different environments proposed by the biological models, and the methods allowing for the quantitative image-based analyses of this cell process. Before concluding our review, we briefly describe a number of software packages available for cellular imaging and analysis.

1. Introduction: Why the study of cancer cell migration is of interest and which tools can be useful for this study

Cancer is a major health problem for humankind, and the approaches to its treatment have clear limitations (1). Oncology has one of the poorest records for investigational drugs in clinical development, with success rates that are more than three times lower than for cardiovascular diseases (2). Oncology, as a therapeutic area, is thus characterized by a desperate medical need for new drugs of novel types, i.e., not only drugs that kill cells and are consequently often toxic (3). The poor performance of most investigational drugs implies that the standard preclinical disease models are faulty or, at least, improperly used (3). As emphasized by Gibbs (1), the past 20 years have seen a tremendous increase in our knowledge of the molecular mechanisms and pathophysiology of human cancer. Many of these mechanisms have been exploited as new targets for drug development in the hope that they will display an enhanced level of antitumor activity and will be less toxic as far as the patients are concerned. Unfortunately, very few of these fundamental research-based hopes lead to clinical success. This failure could be (at least partly) due to the fact that a large majority of compounds that are entering clinical trials for cancer treatment have been selected in vitro on the basis of their cytotoxicity profile. However, cancer kills patients essentially because of the migratory nature of its cells. Indeed, it is now well established that cell migration plays pivotal roles in cancer cell scattering, tissue invasion and metastasis, i.e., processes which are essentially responsible for the dismal prognoses of a majority of cancer patients (4-7). Thus, as an alternative to treatments designed only to kill cancer cells, researchers must today focus on developing the means to reduce or even to prevent the migration of these cells.

As we reported recently (8), today there is no universally adopted pharmacological strategy dealing with the identification of new anti-cancer agents. On one hand, early pharmacological experiments must be carried out
in vitro following the approach developed by the US National Cancer Institute (NCI). In addition to the standard in vitro tests aiming to characterize the cytotoxicity of an investigational compound, the data reported above strongly motivate the use of tests characterizing its anti-migratory effects (9, 10). On the other hand, it can be dangerous to grant too much importance to in vitro data in terms of clinical predictability. Consequently, the in vivo approach is absolutely essential when the objective is to establish the pharmacological profile of a molecule with anti-cancer potential.

Understanding the biological mechanisms driving cancer cell migration and designing anti-migratory drugs to combat them thus constitute current needs in oncology (9). The design of anti-migratory compounds is a particularly promising approach because these compounds not only delay tissue invasion and the formation of metastases by cancer cells emerging from primary tumor sites (11-13), but also restore a certain level of sensitivity to apoptosis and/or autophagy in these slowly migrating cells (7, 14). As we showed in different studies, this restored sensitivity enables conventional treatments based on cytotoxic drugs to be more efficiently used (15-17). As mentioned above, the identification of anti-migratory compounds requires adapted in vitro and in vivo biological models, as well as efficient screening technologies. Concerning the latter, cellular imaging today clearly appears to be an efficient tool for a wide screening of cell behavior in general, and cell migration in particular. The recent advances and developments in microscopy, cell labeling and imaging technologies now allow cell monitoring in increasingly complex environments, which in turn allow the use of more realistic biological models for studying cancer cell migration. Combined with adapted methods of image analysis, this approach is able to provide direct, primary and quantitative information on the effects of various compounds on the migration of cancer cells, and also of other cell actors involved in cancer invasion (18).

2. Imaging cancer cell migration and associated cellular events: Combining different levels of investigation

In this section we briefly present different levels at which cell migration-related events can be observed, imaged and then analyzed. This description follows general to specific aspects, from an analysis of a global cell population to a focus on the motility apparatus inside a single cell, via intermediary stages centered on individual cell locomotion and related morphological characteristics.

2.1 Cancer cells as dynamic populations of similar objects colonizing their neighborhoods

A first level of investigation concerns the analysis of the migratory behavior of a population of cells taken as a whole. The global migration
property of a cell population usually refers to its ability to colonize its neighborhood. This ability is generally evaluated as the net increase in the total area covered by all the cells, or the distance covered by the migration front from the initial site after a set period of culture (Fig. 1A). This colonization ability is clearly affected by migration and growth. These two components should thus be taken into account when interpreting the results (19-21). Different assays using various biological models are available to carry out this global analysis, as detailed in section 3. At this first level of investigation, single cell locomotion is thus not considered, in contrast to the second level described below.

2.2 Cancer cells as individual moving (and dividing) objects

A second level of analysis focuses on the tracking of individual cells, aiming to reconstruct their trajectories from a set of successive positions. This task encounters a series of difficulties due to phenomena such as cell division (Fig. 1B), pathcrossing and clustering, in addition to the fact that a number of cells may enter and/or exit the observed microscope field(s). As detailed in section 5, recent methods in cell image analysis propose solutions to overcome these different difficulties.

While being more complex, the analysis of individual cell trajectories has a number of advantages (9). Firstly, it enables cell migration to be distinguished from cell growth. The effects of various factors (such as drugs or cell environment components) on cancer cell migration can consequently be analyzed in a more specific way than when performing global cell population analysis, in which effects on cell growth may mask or interfere with those affecting cell migration. In addition, by analyzing individual cell migration behavior, it is possible to identify subpopulations of cells presenting different migratory characteristics, as well as those presenting different response levels to a given treatment. Finally, establishing cell trajectories simplifies the detection of preferential directions followed by moving cells, e.g., in response to a diffused chemical agent having chemo-attractive or repulsive properties.

2.3 Characterizing phenotypic and morphologic features of cancer cells

During migration, cancer cells show a great variety of morphologic changes. Particularly in three-dimensional environments, these morphologic changes are characteristic of the various migration modes that the cells could adopt (with possible transitions between them) (9, 22). In the case of a single-cell migration mode, amoeboid-like migrating cells use a fast ‘crawling’ type of movement, requiring rapid cycles of morphologic expansion and contraction on the part of the cell body. In contrast, the mesenchymal mode of single-cell migration presents a succession of multiple stages involving cell polarization, protrusion extension, cell elongation and contraction processes to allow for cell
translocation. In the case of collective migration, cells maintain their cell–cell junctions and move as connected multicellular sheets, aggregates or clusters, in which a promigratory subset of cells at the leading edge can be identified (23, 24). Consequently, comparative analysis between the cellular ability to migrate and morphologic appearance (which can also greatly vary in standard 2D cell cultures, as illustrated in Fig. 1C) may provide interesting information on the cell migration process itself, as well as on the influence of the cell environment on this process, in addition to the possible anti-migratory effects of a given compound (24-27).
2.4 Focus on the motility apparatus of cancer cells

As mentioned above, the motility potential of cancer cells is directly related to cell deformability properties. An essential actor driving these properties is the actin cytoskeletal network (see chapter 3). Indeed, migrating cells use dynamic rearrangements of the actin cytoskeleton for the formation of protrusive structures and for generation of intracellular forces that lead to net cell translocation. Not only is the turnover of actin filaments important, but also their spatial organization (28, 29). It is thus particularly interesting to characterize the properties of these fibrillar actin (F-actin) filaments in a reproducible way. Fluorescence imaging enables one to easily visualize these filament networks (Fig. 1D). Such images can be analyzed to extract quantitative features characterizing the structural organization of these F-actin filaments, e.g., in terms of size, thickness, density and direction. In fact, relatively few studies are reported in the literature on these quantitative structural aspects (30-33). However, this kind of information could help to improve the comprehension of the cell motility process by establishing reliable links between actin network features and the motile potential of cells (34).

2.5 Combination of the investigation levels

Of course, the combination of different investigation levels is usually recommended to better characterize cancer cell migration processes and their response to potentially anti-migratory drugs. For example, some authors encourage the use of multi-assay strategies combining data obtained at either the cell population or the individual cell level (35), and also mixing two- and three-dimensional environments for cell migration observations (9). Others have paralleled observations carried out at the actin cytoskeleton level with those on cell migration (10, 34, 36-38).

3. Models of cancer cell migration: Improving the biological realism of the cell environment

Given the fundamental importance of in vivo cell locomotion, a number of in vitro methodologies have been developed to characterize this phenomenon more easily and to allow the study of the effects of endogenous or exogenous molecules on cell migration. In vitro tests are generally used to provide a range of initial information because in vivo tests are both more difficult and time- and money-consuming to perform, factors that limit the number of tests that can be run at any one time. In addition to this, quantification in in vivo tests is also generally more difficult. This is the reason why in vivo tests are generally used as the ultimate stage to confirm information provided by in vitro assays. In between, ex vivo tests use models based on organotypic tissue cultures able to better mimic the in vivo environment than the in vitro models.
3.1 Two- and three-dimensional in vitro models

Two-dimensional (2D) in vitro models are used to analyze the motility of a cell population in a 2D-environment, i.e., cells cultured on the surface of culture plates (or wells) either coated with a thick layer of matrix or left uncoated. Even though increasing evidence suggests that migration across planar substrates is very different from in vivo cell behavior (39, 40), 2D cell migration models continue to be in frequent usage for convenience’s sake. Furthermore, recent technological developments make possible the use of in vitro tests for the high-throughput screening of compounds for their effects on cell migration (41-43). However, observations of 3D cell cultures have previously shown that, when compared to cells cultured on a rigid 2D support, certain cell types cultured in a 3D gel exhibit completely different types of behavior in terms of gene expression, proliferation, shape, locomotion and multicellular organization (44-46). These data have motivated researchers to move towards a new stage in the design of cell migration assays, and this in its turn has led to more sophisticated methods which enable measurements to be made characterizing cell locomotion in a 3D gel. This section describes and discusses different 2D and 3D in vitro models that can be used for studying cell migration.

One of the most popular 2D models is the monolayer wound model, which consists of wounding a confluent monolayer of cells with an object, such as a pipette tip, a syringe needle, or even electrical currents (47). This makes the use of matrix-coated support difficult. The monolayer recovers and heals the wound in a process that can be observed over a period of between several hours and several days (depending on the type of cell, the conditions of the medium and, of course, the wound extent). This process can be monitored either by manually imaging samples fixed at particular moments (Fig. 1A), or by time-lapse microscopy.

Another interesting model is radial migration, one for which 2D and 3D versions exist. This kind of model studies radial cell migration by generating concentrations of cells (centered in rings, aggregated by centrifugation or cultured on microcarriers) that are either plated in a culture plate, which may be coated (2D models) or embedded in a very thin layer (3D models) of cell-free gel, often constituted of collagen or fibrin (48, 49). If rings are used in 2D models, they are then removed to allow cells to migrate from their points of origin. Radial migration can also be observed either by time-lapse microscopy or by manually imaging samples at particular moments. As detailed in the following sections, the 3rd dimension is often not taken into account in the quantification of 3D radial migration.

The 2D and 3D single-cell migration models are particularly well adapted in the case of biological mechanisms involving the migration of single cells, such as in the case of tumor spreading (50-52). This consists of the monitoring of the migratory behavior of single cells over time by means of time-lapse
video systems (Fig. 1B), with the aim to establish their individual trajectories (see section 5). This requires that the cell cultures observed be relatively far from confluence. While in 2D models cells migrate across rigid and planar substrates (possibly coated), in 3D models cells are embedded in matrix gels (Fig. 2A) and can be labeled by a fluorochrome to make their monitoring easier. Taking into account the 3rd dimension in the cell trajectories requires more sophisticated imaging techniques than are usually used in the case of 2D models, as described in the following section.

The **3D invasion models** usually consist of initially seeding cells onto the surface of a matrix gel and analyzing the migration of these cells into the gel. The **transwell cell invasion model** is one of the most commonly used models (also for chemoattraction, see below). It is based on porous membrane inserts coated with a thick layer of extracellular matrix and placed into wells. The pores of the inserts are small enough (5-12 µm size) to restrict active passage to cells that have been added to the upper compartment and which have to first degrade the matrix layer before migrating through the porous membrane. The processes of metastasis have also been studied with inserts overlaid with a monolayer of endothelial cells (or other cell types) to test the ability of cancer cells to penetrate into the previously established cell monolayer (53). This technique could also be applied to *ex vivo* models (see below). A second *in vitro* invasion model does not use inserts and consists of a collagen gel into which the depth of the cell migration is monitored (54). In contrast to the transwell model, this collagen invasion model, as well as the 3D radial migration model described above, does not require tumor cells to adhere to the filter membranes, to migrate through them or to attach themselves to their lower sides. As discussed recently (9), these requirements may cause certain artifacts in the evaluation of cell migration abilities.

This section would be incomplete without mentioning **2D and 3D chemotaxis models**. Chemotaxis is the directed movement of cells in response to chemoattractants (or repellents), and involves several complex and interrelated processes including motility, polarity and directional sensing. In fact, normal and pathological cells are able to detect the shallow gradients of extracellular molecules and to change their cell morphology and motility accordingly. In cancer development, chemotaxis is involved in different cell migration processes, which concerns the tumor cells themselves (55, 56) and also endothelial cells in the angiogenesis process. Indeed, this process of blood vessel formation from a pre-existing vasculature is initially driven by endothelial cell migration induced by chemotactic tumor-dependent stimuli (57, 58). In spite of the disadvantages mentioned above, transwell culture chambers are often used to study chemotaxis by placing a potential attractant in the lower compartment of the chamber. An interesting alternative is the Dunn chamber, which allows easy monitoring of single-cell chemotaxis. This
direct-viewing chamber enables a stable and linear chemical gradient to be maintained for relatively long periods (59-61).

### 3.2 Ex vivo organotypic culture models

Organotypic tissue cultures (of animal or human origin) provide more realistic environments with which to confront isolated or aggregated tumor cells (62-64). By plating organotypic cultures of tissue slices onto the porous filter of a transwell culture dish (Fig. 2B), this approach provides interesting 3D invasion models which better mimic the clinical reality than the standard transwell models (65). As detailed in section 4, advances in cell labeling and fluorescence microscopy enable the monitoring of the invasive behavior of labeled tumor cells by taking into account the interaction between tumor cells and host tissue in an organ- or locus-specific way (66). Furthermore, slices could be fixed immediately after imaging and subjected to standard immunohistochemical analysis to characterize the migrating cells as well as the surrounding structures (see section 4).

### 3.3 In vivo animal models

It must be kept in mind that a solid tumor is an organ composed of cancer and host cells embedded in an extracellular matrix (ECM) and nourished by blood vessels. A prerequisite to understanding tumor pathophysiology is the ability to distinguish and monitor each component in dynamic studies (67). As emphasized by Wang et al. (68), invasion of neighboring ECM tissue, the lymphatic system and blood vessels are key elements of tumor cell metastasis in many epithelial tumors. An understanding of the cell motility pathways that contribute to invasion in vivo can provide new approaches and targets for anticancer therapy. In vivo models provide tumor microenvironments that mimic the clinical situation and are thus needed to confirm results emerging from in vitro-based systems, in particular in the case of target validation processes or the analysis of the anti-cancer potentials of new molecules. The most used models include transplantable murine tumors grown in syngeneic hosts and xenografts of human tumors grown in immunocompromized rodents (69). It should be kept in mind that xenograft models require some degree of immunosuppression on the part of the host because mice with native immune systems will reject transplanted human tumors, preventing their growth. Xenograft models thus subvert a key function which consists of escaping the immune system.

**The syngeneic models** using conventional rodents are easier to carry out than human grafts in nude mice. These models thus occupy an important place in the field of anti-cancer pharmacology in order to obtain a first characterization of the potential in vivo antitumor activity of a given compound (8). For example, they can be useful for testing various concentrations and
various schedules and/or modes of administration of a given compound. We also showed that syngeneic models of rodent gliomas could be useful for the characterization of the benefit brought about by combining surgery and chemotherapy in the case of orthotopic glioma-bearing rats (70). In addition, these models, which preserve an intact tumor-host environment, are particularly valuable for the evaluation of therapies which require an immune response or target-specific components of blood vessels or the extracellular matrix (69, 71).

Figure 2. A) 3D in vitro migration models. In the gel invasion model, cells are placed on the surface of a collagen gel and their depth of migration into the gel is monitored by means of a phase-contrast microscope. In the radial migration model, cell-coated microcarriers are embedded in a (cell-free) gel where the cells are allowed to migrate for a set period of time. B) Ex vivo invasion model: Diagram illustrating brain slice preparation from a rodent, used as substrate in a transwell culture dish for studying cancer cell invasion in a realistic 3D environment. C-D) In vivo model: NMR imaging of a human Hs683 glioblastoma (brain tumor) developing into the brain of an immunocompromized mouse (see the hatched circle). One million Hs683 cells have been stereotactically implanted into the brain 4 weeks before NMR imaging. Figure 2D illustrates its postmortem histological analysis by means of haematoxylin-eosin staining of brain slices, which shows the tumor invading the rodent brain.
More sophisticated models of orthotopic xenografts of human cancers into immunocompromised rodents can be used to characterize the anti-tumor effects of various types of drugs on invading tumors (Fig. 2C-D) or metastasizing cancers. For example, the Hoffman group develops human cancer cell lines with a stable high-expression of green fluorescent protein (GFP), making it possible to visualize the development of metastases when GFP-expressing cell lines are grafted orthotopically into nude mice with severe combined immunodeficiency (see section 4). These in vivo models therefore permit the direct monitoring of the effect of a drug on the orthotopic development of human metastases in the mice (72-75). Hoffman even argues that rodent tumor models, including transgenic tumor models, and subcutaneously-growing human tumors in immunodeficient mice are not sufficient to represent clinical cancer, especially with regard to metastasis and drug sensitivity (76). He therefore proposes using surgical orthotopic implantation (SOI) to transplant histologically intact fragments of human cancers, including tumors taken directly from patients, into the corresponding organs of immunodeficient rodents (76). Hoffman reports that this SOI approach has been used for innovative drug discovery and mechanism studies and serves as a bridge linking preclinical and clinical research with drug development (76). We have also developed such SOI models with human cell lines and human tumor biopsies originating from human colon cancers (77), non-small-cell lung cancers (78) and gliomas (16, 70).

3.4 Complementary aspects of the different models

Cancer cell migration is a complex and adaptive process which is, in fact, highly influenced by the cell environment (22, 79). As we discussed recently (9), it seems to be indispensable to combine different in vitro models to obtain the maximum amount of information in the study of this cell process, as well as in the identification of potential anti-migratory drugs. In this latter context, recent technological developments have enabled multiple conditions to be analyzed simultaneously by means of different in vitro assays (41-43), thus making possible the high-throughput screening of compounds for their effects on cell migration.

Concerning the cell environment, the natural substrate for most cells in living organisms is the ECM, which is three-dimensional, complex and dynamic in its molecular composition. In the case of in vitro cell migration models, one can either coat supports in 2D models or prepare polymerized gels required for 3D ones (Fig. 2A). Natural ECM components, such as fibrin, collagen, laminin, etc., or hybrid matrices such Matrigel (a reconstituted basement membrane) are often used for these purposes. Synthetic biomimetic ECM analogs are now also available. Compared to their natural counterparts, these analogs advantageously enable different parameters to be controlled,
such as their exact compositions, the microstructure produced by polymerization and the availability and the identity of adhesive and proteolytically degradable sequences (80, 81). The control of these parameters is required for the standardization of in vitro cell migration models, an aspect that remains lacking today and which may consequently lead to high variability and questionable results (for more details see reference 9).

The choice and the design of an adapted environment to study in vitro cell migration are thus not trivial tasks. Compared to in vitro models, the advantages brought by ex vivo organotypic culture models are obvious in terms of biological and clinical relevance. Indeed, this approach offers the ECM composition and also the histological and anatomical structures specific of an organ (Fig. 2B), all of which constitute the natural and complex environment in which cancer cells (of the same origin, or of another in the case of metastases) spread.

However, reliable in vivo models are always needed to validate results obtained by means of in vitro and ex vivo models, particularly in the case of testing potential anti-cancerous compounds. As described above, complementary information can be obtained by combining different in vivo models in order to cover all the needs of pre-clinical stage research (69, 71, 82).

As the complexity of the different models for (cancer) cell migration increase, from 2D in vitro to in vivo animal models, so do both cell labeling and imaging requirements. These requirements, detailed in the following section, essentially depend of the level of transparency/opacity and the thickness of the different material supports used in the models.

4. Microscopy and image acquisition technologies for unlabeled and labeled specimen visualization and monitoring

4.1 Unlabeled cells

One of the main challenges in biology is the ability to observe essentially transparent (cell or tissue) materials. Solving this practical issue requires adapted methods which vary depending on whether the analyzed (transparent) materials are fixed or not.

In the case of fixed materials, standard staining techniques can be easily used to enhance the optical density of the region of interest. In the context of cell migration analysis, this requires the stopping of the experiments after a given period of time (end-point analysis), the fixation and then the staining of the cells. Depending on the purpose and the target of the analysis, different staining techniques are available. Standard cell staining methods (e.g., with cristal violet or toluidin blue) can be used if the aim is simply to identify the cell locations (or the cell number) on 2D transparent supports (in vitro models). Digitized images of the stained cultures can be easily acquired using standard
light microscopy and then submitted to image analysis for quantification (see section 5). This approach is usually employed in the case of the 2D radial migration and 3D transwell invasion models described in the previous section. In this latter case, the filters are removed from the wells and the cells, which remain on their upper sides, are removed first to stain the migrating cells attached to their lower sides (Fig. 3A). In the case of ex vivo models using human tumor cells cultured with animal tissue samples, tumor cell invasion is usually evidenced on histological slices by means of haematoxylin-eosin stainings or immunohistochemistry to reveal animal and tumor cell antigens (62, 63). Similarly, the postmortem analysis of stained tissues from animal models (Fig. 2D and 3B) enables end-point in vivo analyses of cell migration and metastasis processes (78, 83). In contrast, the end-point analyses of ex-vivo models confronting human tumor cells and human tissue samples usually require the prior labeling of living tumor cells, as described below (84).

Figure 3. A) Cell staining for migrating cell counting. Cristal violet staining of cancer cells having migrated through the filter of a transwell culture dish. B) Immunohistological staining showing galectin-1 expression in islets of human invasive glioblastoma cells (IGC) infiltrating the normal brain (NB) of an immunocompromized mouse after an intracranial orthotopic xenograft of human glioblastoma cells (see Fig. 2C). C) Time-lapse monitoring of unlabeled living cells: contrast-phase microscopes equipped with controlled incubators (to maintain the temperature at 37.0°C) and video acquisition systems allowing the monitoring of cultured cells over a number of days. D) Z-sectioning for time-lapse monitoring of cells into 3D gels: serial sections of the same XY field of vision obtained at each step in time (t) by means of a motorized Z stage.
Finally, immunofluorescent staining (e.g., targeting cell membrane proteins) or specific fluorescent markers (such as fluorescent phallacidin to label the fibrillar actin, see Fig. 1D) are particularly useful in the end-point analyses of the morphological features of migrating cells and their motility apparatus (7, 32, 34). In this case, standard (widefield) fluorescence microscopy is required for imaging (fixed and) stained cells.

In contrast, the monitoring of unlabeled living specimens requires other techniques. This is why microscopists have developed several optical tricks to exploit refraction differences that may exist between living material and its surrounding environment. Techniques such as phase-contrast microscopy and differential Normarsky (DIC) enable contrasted images to be obtained from transparent specimens. These techniques make possible the time-lapse monitoring of marker-free living cells. This approach usually consists of automatically recording frame sequences of living cell cultures through relatively inexpensive microscopes equipped with video acquisition systems (Fig. 3C). All the in vitro migration models based on cells cultured in transparent 2D environments can be easily monitored with this approach (85). In the case of transparent 3D environments (e.g., matrix gel), two kinds of imaging techniques are used, depending on the thickness of the 3D gel. In the case of cells embedded in a very thin gel layer, a simple time-lapse approach can be used (similar to the one used with 2D spatial environments) by focusing on a section in which a large number of cells are clearly visible. Quantitative measurements will thus be established on cell location projections on a horizontal plane (52). To take into account the 3rd spatial dimension in ticker gels, a useful alternative is based on image acquisition through a microscope equipped with a (software-controlled) motorized Z stage. At each time step, this results in a stack of serial frames focused at different depths (Z sections, as illustrated in Fig. 3D) on the same field of view (25, 86, 87).

4.2 Labeled living cells

Fluorescence-labeling techniques have been adapted to living cells (85, 88). More particularly, genetically encoded fluorophores, such as the green fluorescent protein (GFP) and its color-shifted genetic derivatives, can be used to tag biomolecules, making their tracking in living systems easier. This enables monitoring of cellular processes by means of live-cell imaging experiments based on fluorescence microscopy (66, 85).

If the aim is simply to mark cells in order to facilitate their tracking through less transparent or opaque substrates (such as tissue), simpler approaches can be used which do not require fluorescent protein fusion products. Of these, fluorescent vital dyes (e.g., the 1,1’-dioctadecyl-3,3,3’,3’-tetramethylindocarboxyamine perchlorate, also labeled Dil) are able to bind to cellular membranes of living cells and thus clearly delineate the entire cell
Cancer cell migration analysis

A very recent and efficient cell labeling technology consists of delivering fluorescent nanocrystals into the cytoplasm of live cells using a custom targeting peptide. Once inside the cells, these labels provide intense, stable fluorescence that can be traced through several generations. As the nanocrystals are not transferred to adjacent cells in a population, they thus provide an excellent tool for long-term (in vitro and in vivo) studies of live cells and tissues, including migration and invasion assays (89, 90). Furthermore, nanocrystals can be customized to concurrently image and differentiate different cell types in vivo (67). Finally, viral nanoparticles have been recently designed for the multivalent display of fluorescent dyes to image tissues deep inside living organisms (91). The bioavailable cowpea mosaic virus (CPMV) can be fluorescently labeled to high densities with no measurable quenching, resulting in exceptionally bright particles with in vivo dispersion properties that allow high-resolution intravital imaging (see below).

By enhancing only the object/target of interest, fluorescence microscopy has numerous advantages, such as allowing trivial image processing techniques (like classical image threshold, see section 5). However, more sophisticated approaches may be required to circumvent noise that might appear. It should also be noted that the transfer in cells of luminescent markers and the multiple cell irradiation consequently required for image acquisition might influence and modify cell behavior. Furthermore, the use of luminescent probes may limit the period of observation because of fading, whereas the new technology of fluorescent nanocrystals seems to be able to overcome some of these difficulties. Time-lapse fluorescent microscopy enables one to monitor labeled cell behavior in various (2D and 3D) environments (61, 79, 92), including ex vivo models, for which Z-sectioning can be used to analyze cancer cell invasion in tissue slices (66, 93). For reviews on fluorescence imaging techniques, see (94-96).

4.3 Advanced techniques and intravital imaging

As detailed above, cell migration imaging can be carried out by means of standard phase-contrast or fluorescence microscopy. More sophisticated techniques have now been developed in order to improve the ability to monitor living cells. Of course, these new, or even experimental, approaches are often costly and often require specific technical abilities to be efficiently used. Consequently, their use may be difficult in certain contexts, such as the high-throughput screening of anti-cancer compounds.

In imaging unlabeled living cells migrating in a 3D gel matrix, a promising approach is provided by digital holographic microscopy (DHM), which is able to globally record the structural information of 3D samples without any mechanical scanning. Recent developments using partially spatial coherent illumination avoid the noise artifacts of laser illumination currently
encountered in standard DHM (97). This makes possible the direct recording of information on the 3D structure of samples consisting of multiple objects embedded in scattering media, such as cell cultures in matrix gel (98). Only a small number (typically four) of successive frames have to be recorded. This advantageously avoids the storage of a large number of microscope images acquired over time by means of optical sectioning.

Concerning the monitoring of labeled living cells, a large number of non-invasive techniques involving 3D optical microscopy have been developed. These techniques generally focus on the study of dynamic cellular and molecular events inside single living cells or embryonic tissue (85), and include confocal microscopy and the related two-photon and multiphoton techniques (99), optical coherence or projection tomography (100, 101) and selective plane illumination microscopy (SPIM) (102). Confocal microscopy is also very useful in visualizing and characterizing the physical network properties of the 3D gels in which cells are made to migrate (44) and which can critically affect the cell migration process (9).

Intravital (in vivo) imaging is the subject of new experimental developments aiming to offer new information on cell migration and response to therapy. Several imaging technologies have been adapted to small animal research, including X-ray, magnetic resonance and radioisotope imaging (103). However, despite this plethora of visualization techniques, fluorescence imaging is emerging as an important alternative because of its operational simplicity, safety and relative cost-effectiveness (67, 103). In their review, Graves et al. (103) illuminate fluorescence imaging technologies that hold promise for small animal imaging. In particular, these authors focus on planar illumination techniques, also known as Fluorescence Reflectance Imaging (FRI), and discuss its performance and current use. They then discuss fluorescence molecular tomography (FMT), an evolving technique for quantitative three-dimensional imaging of fluorescence in vivo. This technique offers the promise of non-invasive quantification and visualization of specific molecular activity in living subjects in three dimensions (103). Finally, multiphoton microscopy combines laser-scanning microscopy with multiphoton fluorescence excitation to capture high-resolution 3D images of living tissue tagged with highly specific fluorophores (104, 105). In combination with animal models of cancer that use the quantum dot or GFP technology, this enables cell behavior in primary tumors in live animals to be observed directly (67, 104, 105).

4.4 Some key points to consider with imaging techniques

Before going on to quantitative image analysis methods in the following section, we want to conclude the present section by highlighting a number of key points. In addition to the different investigation levels described in section
Il at which cell events can be imaged and analyzed, the different imaging techniques can be characterized according to the following key points:

**The contrast method:** As mentioned above, cells are mainly transparent and thus require systems to generate contrast. Two kinds of approaches can thus be distinguished: on one hand, optically enhanced microscopy methods and, on the other hand, tagged cell imaging (by using fluorescent or other cell markers).

**Time monitoring:** Another consideration in imaging is the way time is taken into account. End-point cell analysis consists of analyzing samples after a period of time. This allows the samples to be fixed (in their current states) and a contrasting compound to be used to reveal/stain the targets of interest. Measures with time can also be achieved by stopping a number of cell culture replicates (carried out under the exact same conditions) after different time periods. In contrast, in a time-lapse analysis, the living cells are observed uninterruptedly over time, enabling continuous processes to be monitored.

**The acquisition depth:** Depending on the biological model used (see section 3), single images (2D) can be acquired, such as in the case of end-point applications, or image sequences for time-lapse (3D), or even sequences of image stacks (4D). These two latter cases are used in monitoring cell processes occurring over time in 2D and 3D environments, respectively (see Fig. 3D).

5. From cell images/movies to databases: Quantitative image analysis

Digital image processing and analysis is able to summarize a large amount of images into a few, hopefully meaningful and essentially numerical descriptors. As detailed below, cell image analysis is usually a chained process beginning with **low-level preprocessing**, followed by **segmentation** (i.e., extraction of the candidate objects from the background), the **postprocessing** of the candidate objects, and finally **feature extraction** that supplies a latter stage of **data analysis**. Some applications only focus on qualitative and visualization aspects, for which the process can be stopped after the pre-processing stage.

5.1 Low-level image (Pre)processing

Specific preprocessing steps are generally needed depending on the type of the acquired images. For instance, optical phase-contrast images are often subject to illumination problems (such as vignetting and illumination changes) and poor image contrast. As we showed previously (106, 107), a succession of image preprocessing steps are able to remediate these problems. These steps essentially include image background detection, background masking and local gray level histogram equalization. As illustrated in Figure 4A-D, the combination of these image-preprocessing steps ensures a good contrast.
between the inner part of the cells (darker areas) and the surrounding region (brighter areas).

Fluorescence microscopy generates different sources of noise that have to be suppressed. For example, the culture medium or substrate (such as tissue) may have autofluorescence abilities. The resulting noise can be subtracted by estimating the mean background contribution. Noise resulting from a short exposure time and/or a low excitation intensity (108) can be suppressed by means of image filtering tools (such as median or anisotropic filtering (109)). Finally, a part of the acquired fluorescence comes from out-of-focus planes and should be removed from the image to increase its sharpness. Deconvolution tools have been developed to solve this problem (88). Very briefly, deconvolution aims to numerically eliminate the optical contribution of out-of-focus light by applying the inverse of the optical transfer function of the system. This transfer function models the way (fluorescent) light coming out of the specimen is projected onto the sensor (i.e., the camera). This function may be difficult to establish a priori (e.g., because of unknown parameters and the complexity of the optical path) and usually has to be estimated (110). This approach may be advantageously combined with Z-sectioning acquisition techniques in standard (wide-field) fluorescence microscopy to constitute a good and cheaper alternative to confocal pinhole technology (111).

5.2 Image segmentation, object detection

Image segmentation consists of the partitioning of the image space into connected components belonging to either the objects of interest (e.g. cells) or the background. Two families of methods exist to carry out segmentation. Whereas the first is based on the grouping of pixels sharing certain similarities (according to a criterion defined with respect to the application), the second exploits the borders existing between the objects and the background. The simplest way of exploiting pixel similarities is to use a gray level threshold to select the pixels belonging to the objects. This requires the images to present a good level of contrast between the objects and the background, a condition which is not guaranteed in unlabeled cell imaging (see Fig. 4E-F). The threshold determination can be based on different techniques relating to various, but often statistical, concepts (e.g. area, mean gray level, maximum entropy, clustering, etc.) (88, 112). To efficiently segment cells from the background, authors have combined border detection with watershed transformation (24, 106, 113). The watershed transform is a powerful method of image partitioning but is highly sensitive to the presence of small variations in the images (114). This often results in image over-segmentation, i.e., the generation of a lot of small regions (Fig. 4G-H). Different methods were thus developed to circumvent this problem (114).
Figure 4. A-D) Illustration of low-level image pre-processing steps applied to a phase-contrast cell image. B) Contrast enhancement and illumination correction of image A achieved using a local equalization. C) Background detection (by thresholding the gradient image). D) Final image obtained by applying the background mask detected in image C on image B. E-H) Illustration of object segmentation in a phase-contrast cell image. F) Bad result obtained by thresholding image E. G) Detection of local minima in image E to initiate its watershed transformation. H) Watershed result showing the detection of the cell border with over-segmentation of the object which can be resolved by means of additional methods (see text).
5.3 Postprocessing of the candidate objects

Postprocessing stages are often needed after image segmentation in order to better identify the objects of interest. These stages aim to separate neighboring objects which remain grouped after segmentation, to merge two parts of the same (over-segmented) object, to fill small holes, to remove small objects, etc. These tasks are usually achieved by means of the so-called “morphological” operators which are usually applied to binary (i.e. segmented) images (24, 41, 115). While these stages are particularly useful for evaluating certain measurements, such as the object count, they are not necessary for others, such as the measurement of surfaces (see below). Morphologically specialized filters can also be used to enhance the characteristics of biological objects, such as neurites (115) or actin fibers (32). These filters thin the objects until their morphological “skeletons”, constituted of one-pixel-thick lines, can be identified. Helmke et al (109) used this approach to characterize intermediate filament networks in living cells (see below).

5.4 Feature extraction

We review here a number of quantitative features that can be extracted from cell images that are able to provide information on cell migration processes.

5.4.1 Cell colonization measurements

As mentioned in section 2, the global migration property of a cell population usually refers to its ability to colonize its neighborhood, such as analyzed in the scratch-wound and the radial migration assays described in section 3. This ability can be easily monitored by analyzing phase-contrast time-lapse images or by end-point analyses of fixed and stained materials (see section 4). The net increase in the total area covered by the cells (and possibly its evolution with time) is evaluated by segmenting the surface occupied by cells from the background (Fig. 1A). In addition, this segmentation process enables the borders of the colonized areas (e.g. the wound edge or the migration front) to be identified in these different assays, enabling the measurement of the rate of advance and/or the linear distance covered by cells in the migration front (47, 116, 117).

5.4.2 Object counting

To evaluate cell invasion using transwell chamber-based assays, it is common to stain and then count the cells which have migrated through the filter (Fig. 3A). A correct identification of these objects usually requires a combination of the segmentation and post-processing stages described above. This is also useful to count the number of migrating cells in a scratch-wound assay, i.e., the number of cells observed across the wound borders.
5.4.3 Cell trajectory

A particularly essential feature in the study of cell locomotion is the reconstruction of the trajectory covered by each cell from a frame to the following one in a sequence of images (Fig 1B). This is the aim of the analysis of 2D and 3D single cell migration models (see section 2). While interactive computer-assisted tracking has been used by many authors, automatic systems for (biological) object tracking are becoming increasingly popular, especially for analysis of cell migration in 2D environments (42).

A first approach to automate cell tracking consists of frame-by-frame segmented object tracking (86, 106). After a segmentation phase aiming to automatically detect object candidates, an interframe object-pairing is carried out in order to follow each object's displacement from one frame to the next. As the segmentation in each frame is usually independent from the other frames, it is possible to handle objects with a changing morphology or to take into account new objects entering into the frame. However, handling contiguous and overlapping objects may become difficult with this approach.

A second methodology deals with object model adjustment. This essentially consists of optimizing a parameterized model shape in order to fit the model to the targeted objects (i.e., each cell in a frame in this case). In contrast to the segmentation-based methods, this second group of methods does not require an interframe object-pairing stage in the processing of temporal sequences (for details, see 107). An example of this type of methods is “active contours” (or “snakes”), which also provides object segmentation as a secondary result (118-120). “Level sets” is a more general approach which is able to tackle object topology changes (121). Recently, we proposed a simpler and efficient method for single cell tracking by combining a mean-shift algorithm and model adjustment (107). Certain model-based approaches are able to maximize the efficiency of the trajectory reconstruction process in long-term tracking by taking into account phenomena such as cell proliferation (see Fig. 1B) and pathcrossing (107, 119, 120).

5.4.4 Trajectory features

As illustrated in Figure 5A-B, each cell trajectory can be characterized by various features. These essentially consist of distances, areas and speeds. Characteristic distances related to a cell trajectory include the linear distance between the beginning and the end of the trajectory (TOTAL), the greatest linear distance (GLD) between the original position of the cell and the farthest position reached by the cell in its trajectory, and its generalization, which is the maximum distance (MAXDIST) between any two points of the cell trajectory. The HULL feature is the area of the convex hull (i.e., the smallest convex set that includes all the points of the cell trajectory). It should be noted that the observation times of different cells are not necessarily the same, e.g., due to
Figure 5. **A) Cell trajectory characterization.** Illustration of different quantitative features able to characterize a cell trajectory: A) The GLD refers to the greatest linear distance between the cell’s origin and any of its subsequent positions. HULL is the area of the convex hull polygon (i.e., the smallest polygon that includes all the points of the cell trajectory), and thus a measurement of the area covered by the cell trajectory. The MAXDIST is the maximum of all the possible distances between pairs of points of the cell trajectory. The average speed (see AS expression) is the average of the migration speeds of the cell computed in each time frame of the sequence (i.e., the mean trajectory length covered per time unit). B) **Persistence features:** For each trajectory interval (i) of a specific step size (labelled “step” and illustrated for step = 4), the direction persistence is evaluated as the ratio between \( T_{\text{step}}(i) \), which is the linear distance between the start and end positions of the interval, and \( S_{\text{step}}(i) \), which is the corresponding trajectory length. The logarithmic transforms of these ratios are used to compute the average over all possible intervals of the same size (i.e., the step value). This average expresses the tendency of a trajectory to be close to a straight line with respect to the chosen number of steps and decreases in negative values when the trajectory displays little persistence in direction. C) **Cell chemotaxis:** A relative representation of cell trajectories where all the original cell positions are set to the axis origin and illustrating cell attraction in the top direction. D) **Morphological features:** illustration of cell segmentation and feature extraction (see Table), quantifying the cell areas and perimeters, the lengths of the major and minor axes and the circularity indices (see text).
some of them entering or exiting the view field during the experiment. In order to compare cell trajectories with different observation times, each trajectory feature needs to be normalized by the respective valid observation time (107). In addition, the average speed (AS) is the mean distance covered by a cell per time unit (i.e., the trajectory length normalized by the observation time). In contrast to this latter feature, the speeds computed by normalizing GLD or MAXDIST are able to distinguish between cell trajectories constituted of many small movements around the initial cell location and those presenting larger displacements.

In order to describe the ability of a cell to maintain a certain direction over time, persistence was defined as the ratio between the linear distance between the two trajectory extremities (i.e., TOTAL in Fig. 5A) and the total length of the trajectory (122). This ratio equals 1 if the cell trajectory strictly follows a straight line and is smaller otherwise. Similarly to the diffusibility introduced by Mehes et al. (123), the feature labeled $R_{\text{step}}^{\text{avg}}$ in Fig. 5B expresses the partial tendency of a trajectory to be close to a straight line during a certain number of time steps. A trajectory for which this feature remains close to zero for increasing step sizes indicates that most of the trajectory is straight. On the contrary, fast descending (negative) values for increasing step sizes indicate that the trajectory displays little persistence in direction.

In chemotaxis experiments (see section 3), the directionality of the different cell trajectories is a key feature to analyze. A good visualization consists of plotting cell trajectories from an arbitrary and common point of origin (see Fig. 5C). This can be achieved by analyzing the distribution of the angles of the step vectors constituting the different cell trajectories or of other representative vectors, such as GLD, illustrated in Fig. 5A (61, 124).

5.4.5 Measurements related to special cellular events

While periods of time up to 1 day are generally sufficient to analyze cell trajectories in cell migration or chemotaxis studies, cell cultures must be observed for longer periods of time (3-4 days). It thus becomes possible to detect less frequent cell events, such as cell division or cell death. We recently described a semi-automatic approach for studying cell division events (125). This method is based on backward cell tracking (i.e., from the last to the first frame of the image sequence), in which a cell division corresponds to the fusion of two cell trajectories. Other authors advocate the use of the level set method and a complex track arbitrator scheme to rebuild cell lineage after cell division (126). The rarity of cell divisions makes this event difficult to detect on the basis of morphological features (compared to the numerous cell shape deformations which can be observed) and manual user interactions may be useful to obtain good performance. The literature also reports related studies in the fields of embryogenesis and clonal development (115, 127).
5.4.6 Shape and cell morphology

Once the objects of interest are segmented, a set of shape features can be extracted as illustrated in Fig. 5D (110). Cell shape descriptors in 2D environments include area and perimeter (24). The complexity of the cell shape can be expressed by means of a circularity index (equal to $4\pi \frac{\text{Area}}{\text{Perimeter}^2}$), which takes a value of 1 for a circle and decreases when the cell shape is more complex (Fig. 5D). This kind of index was also used to characterize a cell population (128). The “cell aspect ratio” is an index of shape elongation defined by the ratio of the length of the major axis by the minor axis of the ellipse that best fits the object (Fig. 5D). Zaman et al. (79) used this to characterize the 2D projections of the shapes of cells cultivated in a 3D gel. This index enabled amoeboid cells, which presented a spherical morphology and an aspect ratio of 1, to be distinguished from mesenchymal cells, which showed an elongated morphology and a greater ratio.

5.4.7 Cell cytoskeleton-related features

As a complement to the analysis of cell shapes, a deeper analysis of the cell motility apparatus, in particular the cell cytoskeleton organization, provides very useful information on the cell migration process (33). This analysis can be carried out on fixed and appropriately stained materials (Fig. 1D) or on living cells transfected with a cytoskeletal GFP-protein (see section 4). Visual inspection is not always enough to characterize structural differences due to experimental conditions affecting cell cytoskeletons (30). However, relatively few papers tackle the quantitative image analysis of cytoskeleton components (such as actin fibers) and their organization inside cells, whereas different tools for static and dynamic characterization are described in the literature but may be relatively complex. Briefly, Lichtenstein et al. (32) propose a “FiberScore” which is proportional to the number of labeled pixels assembled into fibers having particular orientations. Buno et al. (30) analyzed the distribution of different cytoskeleton fibers within labeled cells by means of densitometric (i.e. gray intensity) profiles submitted to interactive thresholding for image binarization. Structural features were then extracted and submitted to orientation analysis. Cytoskeletal organization can be also described by looking at cell texture and orientation (31). Fourier transform is one of the classical methods for texture description in image processing (112) and can be used to characterize actin distribution (33, 128). Finally, a number of authors propose tools to tackle the deformation or the dynamics of cytoskeleton filaments. Helmke et al. used a covariance-based measure applied to 3D stacks of deconvoluted fluorescent images (129). In a more recent study, they model actin fiber networks by their skeleton nodes (see the post-processing tools described above) and then quantify their deformations by stretch ratios. Delhaas et al. (130) characterized spontaneous
movements of cell cytoskeletons by matching consecutive cell images and building displacement maps.

5.5 Data storage and analysis

Following the evolution of acquisition devices, the size of the available images has increased incredibly over the last few years. From 500x700 pixels obtained by means of video frame grabbers, digital cameras nowadays provide several thousand pixels-wide images. Moreover, time-lapse applications, including Z-scanning acquisition (Fig. 3D) and multi-spectral imaging (e.g., combining phase-contrast and fluorescence imaging), multiply the needs in computing power and storage size. Fortunately, the power of computer and storage devices has increased following the well-known Moore's law (131). However, in some experiments, image compression is usually required to be able to store all the images recorded. As detailed below, different techniques of compression are available and their choices have to take into account the type of application and the subsequent image processing requirements.

Lossless compression uses image redundancy to algebraically diminish the memory required to code for the image without any loss. In this case, the compression ratio achievable is typically 2:1, and the subsequently uncompressed image is identical to the original one. In contrast, lossy compression is more efficient but requires irreversible simplifications of the image, and the subsequently uncompressed image is thus different from the original one. The simplified image is constructed by maximizing a resemblance criterion that generally aims to minimize the subjective (human) visual impact of the compression. Of course this process may degrade a number of features useful for automated image processing. Different levels of lossy compression can be achieved with tunable compression algorithms (e.g., JPEG (112)). It should be noted that phase-contrast microscopy images allow for a good compression ratio (typically 1:15) thanks to their nonuniform gray level distributions. For example, lossy compression can be used in applications where detailed cell shapes are not required, such as in the case of cell centroid tracking. Fluorescence images are also relatively easy to compress. However, in the case of low signal-to-noise ratios or for quantification purposes from gray level distributions, image compression (which introduces image artifacts) has to be avoided. Image compression is also very useful for visualization purposes. Time series sequences can be compressed by modern image compression algorithms like the MPEG4 method, which provides convincing visual results and high compression rates (typically more than 1:100) by exploiting temporal redundancy.

In this context, digital holographic technology (see section 4) strongly reduces the amount of data to be stored and the time required to record the full information of a 3D specimen as compared to more standard Z-sectioning
approaches. Indeed, this holographic technique requires only a small number (from 2 to 4) of successive video frames, from which a sample hologram is digitally reconstructed. This hologram is then able to provide a stack of slice images at varying focal planes (98).

Concerning data analysis, classical statistics can be used to characterize significance differences that may exist between different conditions. In this case, parametric tests (e.g., ANOVA or Student t-test) are often used. Non-parametric alternatives (e.g., Kruskall-Wallis and Mann-Whitney tests) can be more appropriate in the case of data distributions which do not always fit the parametric conditions (normality and variance equality), as we observed for the cell motility features illustrated in Fig. 5A which were extracted from our cell migration experiments (10, 107). When directionality is involved, specific statistical analyses for directional data are useful (132). For instance, chemoattractive properties of molecules or compounds were efficiently analyzed by means of Rayleigh vector distributions and related statistics (61, 124, 133). von Mises distributions were also used to describe fiber directions and thus to characterize cytoskeletal organization (31, 109). Finally, stochastic modeling methods are useful to more globally characterize cell migration processes. For this, Random Walk (50, 134, 135) and Hidden Markov models (87, 113) have already been used.

6. Available software packages for cellular imaging and analysis

There is a wide range of commercial software packages for cell imaging. However, a small number of them include tools for cell migration analysis. Furthermore, in terms of motility analysis, the available software packages are commonly restricted to the automatic tracking of labeled cells, as shown in Table 1. This restriction contrasts with the other interesting techniques developed for other purposes in cell image analysis research, as overviewed in the two previous sections. A reason for this could be that these other techniques were often developed under a restrictive set of conditions (e.g., a level of image quality or a set of predefined parameters) and hence require very similar conditions to function correctly. These requirements imply a lack of the robustness to varying image conditions that is otherwise expected of a commercial software package. Another reason for this focus on marked cell tracking is that the tracking of a blob (to which a labeled cell is usually reduced) is relatively robust after applying some standard contrast enhancement methods (see section 5), which are also widely available and automatic. Other more subtle techniques would require more knowledgeable parameter tuning, such as the tuning of dimension-dependent parameters in a model-based phase-contrast tracking method (107). This thus requires more
advanced image analysis knowledge than the contrast enhancement methods mentioned above. It is also worth noting that none of the software websites mentions supporting cell division.

The packages with marked cells tracking abilities export the resulting trajectories mostly as comma-separated coordinates, easily imported into any statistical software. Others only provide an interface that helps the user to track cells manually (e.g., the MtrackJ plugin for ImageJ). This is useful for working with unmarked or very noisy cell images. Packages like IQ or AxioVision Motility calculate features such as trajectory, speed and direction.

The analysis of wound healing assays is also supported by certain software packages. For example, CellProfiler and OpenLab (see Table 1) can analyze the cell-covered area vs. background area ratio. Labeled cell counting is also an interesting measurement in cell invasion studies (such as in the transwell chamber-based assay) and available in several packages. It should be noted that neither of these two features is advertised as a main feature of the package, probably because they are relatively simple segmentation problems.

As a commercial software package is often expensive, it can be useful to search first among the freely available packages. Table 1 includes different open source and free of charge systems, i.e., enabling both uses and modifications freely. All the features mentioned above are available in the free software packages mentioned in Table 1: cell counting (WCIF plugin bundle for ImageJ), tracking (CellTracker and ParticleTracker plugin for ImageJ) and wound healing (CellProfiler). Generally, the packages have a scripting language which allows the user to connect the software's image analysis primitives into a custom workflow. For example, a script would allow the user to bind measuring the contrast, enhancing it if necessary and running the tracking algorithm into an automatic workflow.

Usually, motorized or fluorescence microscopes require the purchase of a relatively expensive driving software package. This type of software often includes a number of generic image analysis capabilities that are often less efficient than task-specific systems and less customizable than general image analysis software package. It is worth noting that the open source MicroManager project (http://www.micro-manager.org) is a potential alternative for commercial microscopy software. It is able to control a limited set of supported devices, such as microscopes, stages and cameras.

Finally, some packages, such as Scion image and more recently ImageJ, are able to play the role of image analysis toolboxes for more advanced users. Both are free, offer common low-level image processing tools (especially ImageJ) and allow the development of new image analysis tools. ImageJ is the most widely used open source image processing toolbox in Java and has a large number of free (and mostly open source) plugins available (http://rsb.info.nih.gov/ij/plugins/index.html).
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This list is not exhaustive. Most of the software packages were not tested first hand and the information was gathered as advertised on the mentioned websites. Tracking is automatic if not mentioned otherwise.
7. Conclusions

Cancer cell observations have been extensively used for many years in a wide range of applications, including cell migration analysis and drug testing. Nowadays, computer assisted-microscopy allows the handling of considerably large amounts of image data of various origins and acquired during experiments lasting over several hours or several days. The combination of time-lapse video-microscopy with adapted image analysis methods constitutes an efficient tool for the screening of cell behavior in general, and cell motility and invasion in particular. From *in vitro* to *in vivo* investigations, new cell labeling and imaging techniques enable the monitoring of cell behavior in more complex models with increased biological and clinical relevance.

In the context of anti-cancer drug screening, it should be noted that cell imaging and analysis allow the observation and a first qualification of the effects of compounds on cell behavior (including cell locomotion processes). This information provides potential clues on the mechanism of drug action, which can guide the selection of further time-consuming and expensive biomolecular evaluations required to elucidate the true drug action mechanism. A similar approach is also useful in cell biology to investigate the roles played by molecular actors, such as the regulators of the cell cytoskeleton, in the spread of tumor cells and endothelial cell migration (which leads to tumor vascularization) so as to identify attractive targets for new chemotherapies.

We conclude by highlighting the fact that, in contrast to other cell processes, such as cell growth, there is no standardized or consensual way to study cell migration from a biological or pharmacological point of view. The present review describes various models, together with their possible side effects, on cell behavior. This requires one to set up a number of controls (e.g., on the matrix structure of gels used to study 3D cell migration) in order to provide robust and interpretable results. The quantitative analysis of cell migration also needs standardized tools which have to be as accessible as possible to biologists or pharmacologists, whereas multidisciplinary teams are very favorable in such a context, blending biology, optics, engineering and computer science.

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9. References