Intravital imaging and its impact on cancer research in the past, present and future

Stephanie Kappes
Student number 3565882

Utrecht University
Master Molecular and Cellular Life Sciences

Examiner: Jacco van Rheenen
Daily supervisor: Saskia Ellenbroek

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Summary

The molecular processes underlying cancer have been subject to extensive research for many years. While substantial progress has been made, the overall understanding of the complex and dynamic disease remains incomplete, and key relationships remain unelucidated as traditional research methods exhaust their limits. Intravital microscopy has proven powerful in overcoming these boundaries. The technique which was first described in the mid-nineteenth century allows direct observation of dynamic processes in living organisms. Recent developments in fluorescent technology, genetic engineering of model organisms and microscopy have revolutionized intravital imaging and boosted its popularity. In the past few years, cancer-related intravital microscopy studies have mushroomed, exploiting the unique possibilities that the technique offers, such as real-time single cell tracking *in vivo*. The rapid developments taking place in the field of intravital microscopy predict a fruitful future for cancer research.

1 Introduction

Cancer is one of the main causes of death. According to the GLOBOCAN 2008 estimates, cancer killed 7.6 million people in 2008. This number is expected to rise to an approximate 13.1 million by 2030, and will continue rising if we fail to develop treatments that are more effective than those currently in clinical use. In order to develop more effective cancer treatments, we have to expand our so far incomplete understanding of the disease. However, the types and causes of cancer are diverse, and disease development comprises a multitude of dynamic processes, making it a complex, multi-faceted disease.

The vast majority of cancers are cancers of the epithelium, whose development is characterised by several conserved stages (Figure 1): Cancer is initiated when mutations occur in normal epithelial cells that result in activation of oncogenes or inactivation of tumour suppressor genes, which are responsible for the regulation of cell growth and differentiation. Mutations in these genes, or mutations that alter the expression of these genes, disrupt the balance of cell proliferation and cell death that is crucial for the preservation of healthy, intact tissue. This can result in strong, uncontrolled increase in cell proliferation, or neoplasia. Neoplasia mostly leads to the formation of adenomas, benign outgrowths. At this point, epigenetic changes and genetic alterations can cause progression of the adenoma into a carcinoma *in situ*, a non-invasive, precancerous lesion with a high risk of becoming invasive. If further alterations take place, the carcinoma *in situ* can develop into an invasive carcinoma. At this stage, the tumour may metastasise (Tysnes and Bjerkvig, 2007). In the course of metastasis, some cancer cells manage to escape the primary tumour, enter the blood vessels (intravasation), spread throughout the body via the blood stream, exit the blood vessels (extravasation), migrate through the tissue, and seed metastases at distant sites (Roussos et al., 2011b).

Our current knowledge on cancer and its underlying mechanisms stems mainly from *in vitro* and *ex vivo* methods. These methods provide important tools to gain first insights into unknown mechanisms, as they model *in vivo* processes in a simplified way. However, when it comes to explaining processes and their underlying molecular mechanisms in more detail, such a reduction of the complexity of the studied system can present a limitation: while *in vitro* methods can never fully model the complex

host environment in which cancer naturally forms and progresses, thus not accurately reproducing *in vivo* events, *ex vivo* methods can only provide a snapshot of events at one point in time, obscuring the dynamics of the observed processes. A complete and accurate understanding of biological processes can only be obtained by *in vivo* studies.

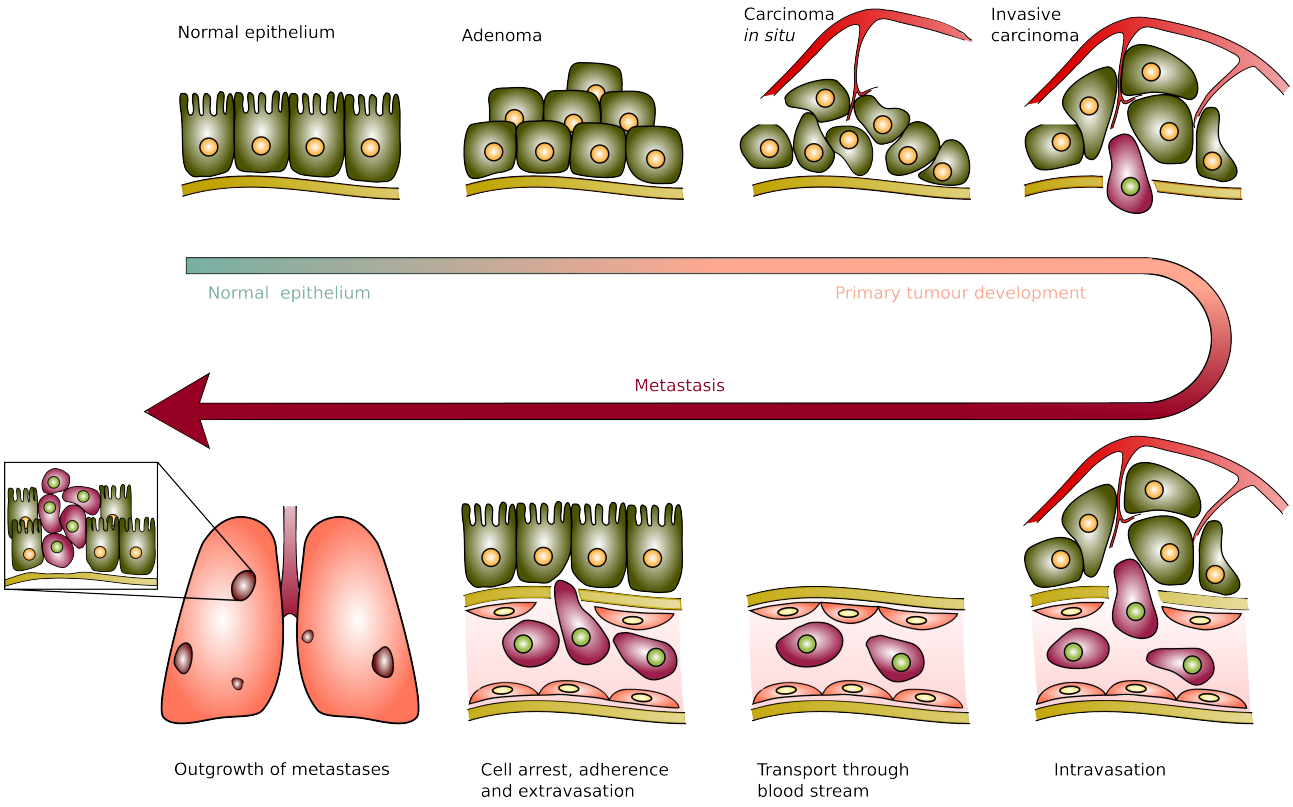

Figure 1. Several stages of tumour progression have been described that are conserved among the majority of epithelial tumours. In the first step, normal epithelium undergoes genetic transformation, leading to abnormal cell proliferation and the formation of an adenoma. By further mutations, the benign adenoma progresses into a carcinoma *in situ*, an early, pre-invasive form of cancer in which the basement membrane is still intact. By the time this stage is reached, tumour angiogenesis has started (Folkman, 1971). The carcinoma *in situ* becomes an invasive carcinoma when cells pass the basement membrane, marking the initiation of metastasis. During metastasis, carcinoma cells that have acquired invasive traits migrate towards blood vessels (invasion), possibly using a lymphatic route, and intravasate. They are then transported via the blood stream until the cells adhere to the vessel wall at a distant site and extravasate. The metastatic cells then migrate through the tissue and seed micro-, and finally macrometastases, which are the main cause of cancer-related death..

Following advancements in optical methods, genetic engineering and fluorescent technology, *in vivo* imaging by intravital microscopy (IVM) has gained crucial importance as a tool in cancer research in the past decades. This literature study aims

at summarising recent developments in the research performed using the latest intravital microscopy techniques to enhance the understanding of the mechanisms underlying different steps tumour development and progression, in particular metastasis. The results furnished by these studies may reveal mechanisms constituting potential targets for cancer therapies.

2 Intravital microscopy

2.1 Cancer research to date

One of the most widely used conventional techniques in cancer research is immunohistochemistry (IHC). Preparation steps preceding immunohistochemical analyses of animal tissues are the (inevitable) sacrifice of the test animal, collection of the tissues of interest, fixation, sectioning and eventually staining using antibodies and IHC reporters. Thereby, IHC captures a static image of the situation in the studied tissue at the time of animal sacrifice. IHC is a powerful method for, for example, analysis of protein expression, which can provide clues about the role of this protein in a certain cell type or process the cell is involved in. It has thereby helped to identify many proteins involved in different cancer processes, such as cathepsin D (cath-D). Overexpression and hyper-secretion of cath-D by a multitude of cancers, including breast cancer, ovarian cancer and melanoma, have been observed in immunohistochemical studies, and correlated with a poor prognosis in connection with a high incidence of clinical metastasis (Masson et al., 2010). Different hypotheses on the functions of the protein have been reported based on IHC (Berchem et al., 2002) and *in vitro* (Wang et al., 2011; Knopfová et al., 2012) studies, suggesting roles in apoptosis (Berchem et al., 2002) and cancer cell proliferation (Garcia et al., 1990; Ohri et al., 2007). However, a complete understanding of the function of cath-D has not been achieved, as evidenced for example by the failure to define the nature of its contribution to apoptosis (Masson et al., 2010). Elucidating the meaning of the findings obtained to date requires monitoring of cells and of expression and localisation patterns of proteins *in vivo* over time. The examples of IHC -based studies on cathepsin D present a showcase for problems that are encountered in cancer research, as it stresses the need for suitable techniques to study the dynamic processes that underlie the disease.

Intravital microscopy (IVM) has the potential to provide new insights into biological processes in their natural context, furnishing high (spatio-temporal) resolution data, down to the single cell level. Furthermore, modern tissue preparation techniques and novel imaging technologies causing minimal tissue damage allow for repeated imaging in the same, live animal, enabling time course IVM experiments for the study of

dynamic processes.

In this chapter, technical aspects of intravital microscopy will be discussed with a focus on its application in cancer research.

2.2 What is intravital microscopy?

Intravital microscopy denotes the imaging of exposed tissues in live, conscious or anaesthetised, animals. This experimental approach has first been described in the nineteenth century (Wagner, 1839; Waller, 1846). In its beginnings, IVM was suitable only for the study of microcirculation and vasculature in semi-transparent membranes that could be illuminated by the lamp of a light microscope, such as the prepuce or the frog tongue, or tissues that could be surgically exposed. Today, thanks to developments in microscopy and fluorescent technologies, as well as novel tissue preparation techniques, a larger range of samples can be made accessible for and visualised by IVM, hereby making it an attractive tool to study a wide range of physiological and pathological processes, including the different stages of cancer development.

The main added value of IVM to biology research is its ability to build a bridge from *in vitro*/*ex vivo* findings to their actual relevance in processes occurring *in vivo* by allowing their observation in live, intact organisms. The range of processes that can be studied by IVM, and the types of information that can be obtained about these processes, are practically limitless, and include functional studies such as the assessment of blood-flow rate and vascular permeability, anatomical measurements, including tumour or organ size, and cellular imaging for the tracking of different cell types, including cancer cells, immune cells and stem cells (Pittet and Weissleder, 2011).

2.3 Requirements for IVM

There are four requirements for performing any informative IVM study resulting in a realistic picture of biological events taking place in their natural environment: (1) molecular probes for detection, (2) a suitable model system, (3) a microscope and detection system, including computer software for the analysis of the acquired data, and (4) tissue preparation that allows visualisation (Jain et al., 2002; Bonapace et al.,

2012).

2.3.1 Molecular probes

IVM as a technique allows the study of processes *in vivo*, thereby providing a realistic picture of biological events in their natural environment. To fully exploit this opportunity, the availability of (fluorescent) reporters, with which sufficient resolution and contrast can be achieved *in vivo*, is of paramount importance. Without fluorescent technologies using tags such as *Aequorea victoria* green fluorescent protein (GFP) and its different colour derivatives [e.g., red fluorescent protein (RFP), yellow fluorescent protein (YFP), cyan fluorescent protein (YFP)] or a selection of exogenous fluorescent probes, IVM as we know it today would not be possible.

The use of exogenous probes for IVM has been described in a wide array of publications, some of which will be highlighted below (Pittet and Weissleder, 2011). Exogenous probes offer a vast range of possibilities for contrast generation. For instance, they allow for visualisation of the blood stream (e.g. high molecular weight fluorescent dextrans which are injected into the blood stream and, due to their size, cannot exit intact blood vessels) or cell-type specific tagging (fluorescent antibodies or synthetic fluorescent probes). Some fluorescent dyes are used to stain cells *ex vivo* before transfer into the animal (e.g. CMTMR, CFSE), while others are injected directly into the animal prior to imaging (e.g. fluorescent dextrans, quantum dots; (Pittet and Weissleder, 2011). Exogenous dyes provide a quick way to label cells or cellular components. However, their signals are usually unenduring (Zomer et al., 2011). Endogenous labelling on the other hand is more time-consuming, but provides a lasting signal. Labelling of specific cell types or tissues can be achieved by fusing fluorescent proteins (FPs) to cell type-specific proteins. By use of inducible promoters, expression of the FP-protein fusion can be further restricted. An elegant example for an endogenous reporter under control of an inducible promoter has been described by (Snippert et al., 2010) Snippert and colleagues generated the *R26R-Confetti* Cre-reporter allele, an invaluable tool for lineage tracing and single cell tracking (Figure 2). This was achieved by insertion of Brainbow2.1, which encodes for the four fluorescent proteins GFP, CFP, RFP and YFP, with an upstream CAGG promoter, which ensures widespread high levels of expression throughout cell types and development, flanking LoxP-sites allowing for Cre-mediated recombination, and a neomycin resistance/polyA

cassette acting as a transcriptional roadblock, into the *Rosa26* locus. Upon Cre activation and expression, recombination takes place by which the roadblock is removed, whereupon each cell carrying the *R26R-Confetti* allele will stochastically express either one of the following four fluorescent labels: nuclear GFP, membrane-associated CFP, cytoplasmic RFP or cytoplasmic YFP. Tissue specificity of confetti labelling can be achieved by submitting the expression of Cre recombinase to the control of a tissue-specific promoter. This system can be further optimised for example by expression of a fusion protein encoding for Cre recombinase coupled to a modified version of the oestrogen receptor ligand binding domain [Cre-ER(T2)] making Cre-mediated recombination (hence color induction) inducible by injection of tamoxifen.

Another achievement in fluorescent technology that has had a major impact on IVM is marked by photoswitchable FPs, such as Dendra2. Dendra2 has been generated by introduction of a single point mutation into the octocoral *Dendronephthya sp.*-derived protein Dendra in order to improve its maturation properties and the intensity of its fluorescence emission. Prior to photoconversion, Dendra2 emits green light. Its fluorescent state is modified irreversibly upon exposure to 405 nm light, resulting in a strong red shift of its excitation and emission spectra. Cells can be labelled by fusion of Dendra2 to endogenous proteins. The switch of fluorescence emission from green to red allows selective cell fate tracking of photoconverted cell populations (Gurskaya et al., 2006; Chudakov et al., 2007; Zhang et al., 2007; Gligorijevic et al., 2009).

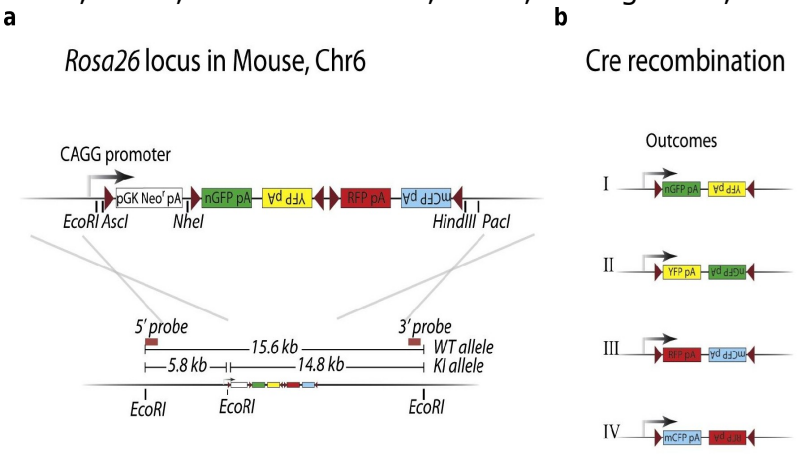

Figure 2. a The R26R-Confetti Cre-reporter allele at the *Rosa26* locus. b The four possible cases after Cre recombination (adapted from (Snippert et al., 2010)

Cell labelling can also be achieved by expression of luciferases, which naturally occur in some organisms, e.g. different sorts of bacteria and marine organisms. The most commonly used luciferases are firefly luciferase (Wet et al., 1987), renilla luciferase

(Lorenz et al., 1991) and several bacterial luciferases. Luciferases do not emit light themselves, but catalyse an oxidation reaction that releases energy in the form of light. The *lux* operon that carries the luciferase gene in bacteria also encodes the enzymes required for the biosynthesis of the bacterial luciferase substrate whose oxidation produces bioluminescent emission (Meighen, 1991). However, when using firefly or renilla luciferase, the substrates have to be injected prior to imaging.

2.3.2 Model systems

Most microscopy techniques used for intravital imaging rely on fluorescent labelling of cells in order to generate the contrast necessary to visualise and track single cells or specific cell populations, such as tumour and/or stromal cells in the case of cancer research. Different mouse models with fluorescently labelled tumours have been described for the use in intravital microscopy. A widely used technique for obtaining FP-expressing tumours in mice is the injection of fluorescent tumour cell lines into the test animal. This approach constitutes an easy and straight-forward way to obtain fluorescent tumours in living animals, because cell lines can be easily engineered to express fluorescent protein fusions (e.g., Bouvet et al., 2002) and, largely being derived from aggressive tumour cell subpopulations, result in rapid outgrowth. Some important and commonly used tumour cell lines are MTLn3 cells, 4T1 cells and C26 cells. MTLn3 cells are derived from the clonal selection of lung metastases from rats with oestrogen-independent mammary tumours. These tumours are highly metastatic and have been shown to metastasise to the lymph nodes and lungs. As shown by the spread of MTLn3 tumours to the lungs and lymph nodes, as well as the cytokeratin and vimentin expression pattern that these carcinomas exhibit, MTLn3 tumours behave similarly to a basal-like subtype of human breast cancer (Lichtner et al., 1989). The 4T1 cell line is derived from lung metastases that originated from a highly metastatic spontaneous mammary tumour in BALB/c mice. Both arterial and tail vein injection, as well as orthotopic implantation into the mammary fat pad, result in metastases in lung, liver, bones and brain within a period of a few weeks (Aslakson and Miller, 1992). C26 cells are a mesenchymal cell line originating from a chemically induced BALB/c mouse undifferentiated colon carcinoma. They are highly metastatic, and the metastases that they form are mesenchymal as well (Corbett et al., 1975). The genome of C26 cells bears a mutation that is common in cancer, namely the K-

Ras D12 mutation that results in constitutively active K-Ras (Morris et al., 2010).

Moreover, the use of cell line-based tumour models enables the study of human cancer *in vivo* in immune suppressed or deficient mice, for example by injection of the MDA-MB-231 cell line. The MDA-MB-231 cell line was isolated from pleural effusions of a human breast cancer patient in 1973. The cells exhibit epithelial-like morphology and an invasive phenotype *in vitro*. *In vivo*, MDA-MB-231 cells have been shown to form tumours in the mammary fat pad of nude mice. Furthermore, experimental metastases have been observed upon tail vein injection of MDA-MB-231 cells (Cailleau et al., 1974).

However, the use of tumours generated by use of cell lines has several drawbacks. Firstly, tumour cell lines are isolated from their primary source in late stage cancer and then grown in tissue culture where the conditions differ strongly from those in the natural tumour environment, possibly altering cell metabolism. And secondly, the strongly malignant tumours that develop from these cells generally grow much faster than natural tumours and are studied within a few weeks of implantation. Under natural conditions, the interactions between primary human or animal tumours with the host microenvironment can take months or years to be established. Tumours generated by injection of cell lines might thus not be able to develop these interactions, which can lead to artefacts (Rockwell, 1980).

Genetic mouse models present a more realistic system for the study of cancer. The generation of fluorescence-labelled genetic tumour mouse models, however, is time-consuming, expensive, and technically challenging (Zomer et al., 2011). Nonetheless, genetic mouse models that can be used for cancer research by intravital microscopy have been established. An example of a widely used mouse model of breast cancer is the MMTV-PyMT model, in which the polyoma middle T (PyMT) antigen, which functions as an oncogene, is expressed under the control of the mouse mammary tumour virus (MMTV) promoter. The MMTV promoter ensures exclusive expression of PyMT in the mammary glands, where it results in epithelial transformation and the formation of multifocal adenocarcinoma. Metastatic spread occurs to the lymph nodes, and with a high incidence to the lungs. The tumour progression in this model is very similar to the progression of human breast cancer (Fantozzi and Christofori, 2006).

2.3.3 Microscopy techniques

The first IVM studies in the mid-nineteenth century were carried out using simple wide-field light microscopes. By now, wide-field microscopy has largely been replaced by new imaging techniques that strongly outperform the former in terms of resolution and contrast, partly owing to the immense progress that fluorescent technologies have seen in the past decades.

Imaging of the fluorescent probes described in chapter 2.3.1 requires specialised microscopy techniques. Nowadays, the most widely used techniques for *in vivo* imaging of animal tissues are confocal and two- or multiphoton microscopy, which both detect fluorescent probes and allow the recording of 3D stacks by optical sectioning. Furthermore, they largely meet the requirements on spatial and temporal resolution imposed by the processes most commonly studied by IVM. A list of commonly used intravital imaging techniques with their respective properties can be found in 1.

Confocal microscopy

In confocal microscopy, a focused laser beam is used to scan the sample point by point, rather than illuminating the entire sample at once as in wide-field microscopy. The wavelength is chosen such that a single photon is sufficient to excite a fluorophore (Figure 3a). Therefore, the rate of fluorophore excitation is linearly dependent on the photon flux, i.e. the strongest fluorescence signal is emitted by fluorophores in the focal plane, however, fluorescence emission occurs throughout the full trajectory of the laser beam. High spatial resolution is achieved by eliminating out-of-focus light by means of a pinhole. Brightness can be enhanced by enlarging the pinhole, however, at the expense of resolution. A drawback of the confocal approach is that resolution decreases quickly with increasing imaging depth, due to scattering and absorption of the photons in the animal tissue beyond the focal point. Confocal imaging is therefore limited to a depth of about 100 µm (see also 1).

Multiphoton microscopy

Multiphoton microscopy (MPM) does not make use of an aperture to obtain a focussed image of a single plane, but instead relies on the use of low energy photons, so that two photons are necessary for the excitation of a single fluorophore (Figure 3b). Due to the need for two photons, the probability for fluorescence emission increases

quadratically with excitation intensity, rather than linearly as in one-photon excitation systems. Therefore, absorption and fluorescence emission are strictly confined to the focal plane, where the beam is extremely focussed and the photon density is high, thus eliminating the need for a pinhole. The focal volume in which the excitation intensity is high enough for the quasi simultaneous absorption of two photons by a single fluorophore is in the sub-femtolitre range. To obtain sufficient photon density, pulsed near-infrared (NIR) lasers are used rather than the continuous wave lasers used in confocal microscopy. The low-energy IR photons experience less scattering and absorption, thereby allowing imaging at larger depths than in confocal microscopy, up to 1 mm into the tissue (Theer et al., 2003). In addition, the use of longer wavelength photons and the reduction of the excitation volume to the focal point come with reduced phototoxicity and photobleaching, thereby limiting tissue damage and making MPM specifically suitable for long-term imaging.

Repeated imaging of a sample in multiple imaging sessions over a longer period of time also requires finding or creating a "landmark" in the tissue in order to have a point of reference for the study of dynamic processes such as cell movement. Due to the excitation wavelength used in multiphoton imaging being in the IR range, multiphoton platforms allow second harmonic generation (SHG) imaging of non-centrosymmetric structures, such as collagen, without the need for fluorescent labelling (Campagnola et al., 2002) and can thereby provide such a point of reference. During SHG, two photons passing through a structure lacking a centre of symmetry are effectively combined to form a single photon, the energies of the original photons adding up. This results in light emission at exactly half of the excitation wavelength. SHG emission from collagen structures, which are highly stable in terms of position and structure, forms a suitable frame of reference. Therefore, imaging of e.g. type I collagen surrounding tumour tissue and metastasis-prone organs provides a roadmap that can be used to identify the exact same positions over multiple imaging sessions.

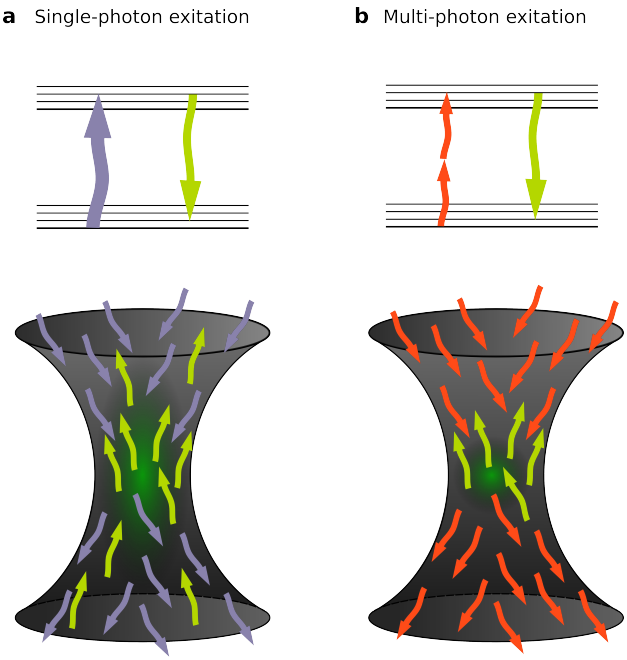

Figure 3. Single- and multi-photon excitation. a The probability of fluorophore excitation by single, high-energy photons is a linear function of the photon flux, resulting in a large excitation volume and a substantial out-of-focus signal. b When the excitation wavelength is such that two photons are required for fluorophore excitation, the excitation probability decreases quadratically with the photon flux, restricting fluorescence emission to a small focal volume, thereby limiting phototoxicity and background signals.

Multi-channel imaging

Standard confocal microscopes can be used for multi-channel imaging, by which cell types labelled with different fluorophores can be discriminated and their interactions studied. Meanwhile, multi-channel imaging in multiphoton microscopy has been complicated by the limited number of fluorescent probes that can be separated into different channels. Efforts to achieve multi-channel multiphoton imaging mainly focus on two approaches. The first approach consists of selecting fluorophores that can be detected by individual channels in standard multiphoton platforms, while being compatible with the excitation wavelength range of the latter (Kawano et al., 2008). The second approach relies on the construction of a "broadband" multiphoton microscope by extending the range of excitation wavelengths of the system using an optical parametric oscillator (OPO), which by now has led to a commercially available system (TriM Scope II, LaVision) (Entenberg et al., 2011).

Temporal resolution

The study of dynamic processes may require, depending on the speed at which they occur, a higher temporal resolution than can be obtained using regular laser scanning confocal microscopy or multiphoton microscopy. For example, the study of immune cell dynamics or events within the blood stream demand fast image acquisition, while maintaining high spatial resolution. Furthermore, fast scanning limits image distortion

caused by tissue motion, such as respiratory movements. The spinning disk confocal microscope combines high resolution imaging with a frame rate of, in theory, up to 1000 frames per second (Nakano, 2002). This is made possible by simultaneous illumination and acquisition through multiple pinholes arranged in a spiral pattern on a rapidly rotating disk. Due to the previously unattained acquisition rates achieved by the spinning disk confocal microscope, the latter has been used for the study of various physiological and pathological processes (Grayson et al., 2001; Egeblad et al., 2008; McDonald et al., 2010)

Technique	Resolution	Time	Depth	Multi-channel imaging	Invasiveness/ toxicity
PET imaging	1-2 mm	Min–hours	No limit	No	None
MR imaging	10-100μm	Min–hours	No limit	No	None
CT imaging	50μm	Min	No limit	No	Non-invasive/ substantial radiation toxicity
Confocal laser scanning microscopy (CLSM)	Subcellular (<0.5μm)	Sec	<100μm	Yes	Invasive/ substantial phototoxicity
Spinning disk confocal microscopy	Subcellular (<0.5μm)	Millisec-sec	<100μm	Yes	Invasive/ substantial phototoxicity
Multiphoton microscopy	Subcellular (<0.5μm)	Sec	<1mm	Yes	Invasive(/low phototoxicity
Bioluminescence	>1mm	Sec-min	>2cm	Yes	None

Table 1. Commonly used intravital imaging techniques (adapted from (Condeelis and Weissleder, 2010) (Beerling et al., 2011) and (Zhou et al., 2011).

2.3.4 Tissue preparation techniques

Most of the microscopy techniques used for IVM require tissue preparation prior to imaging of internal tissues, because, as mentioned above, they don't reach large imaging depths in animal tissue due to light scattering (see also 1). Several approaches have been developed to clear the optical path between the sample and the microscope objective.

Acute tissue preparations

Surgical exposure is the most straightforward preparation method for *in vivo* imaging. An example of surgical exposure is the skin flap method, in which a small incision is made and the skin is folded back in order to directly expose the studied tissue to the microscope objective (Figure 4a). Imaging of some organs may require exteriorisation for the duration of the imaging session. Acute exposure has one major drawback, which is its invasiveness. The latter imposes limitations on the duration of imaging sessions and on the possibility of repeated imaging. Surgically exposed or exteriorised tissue preparations typically allow imaging of internal tissues in anaesthetised animals for several hours, the exact duration depending on the tissue. Imaging sessions can be extended to up to 40 hours when vital signs are closely monitored (Egeblad et al., 2008). Repeated imaging requires closing and subsequent reopening of the preparation which bears a high risk of infection, and can induce an inflammatory response that can result in artefacts.

Imaging windows

This problem has been solved in part by the invention of transparent observation windows. The first use of such a window was described by (Sandison, 1924) who developed the rabbit ear chamber for IVM in the early twentieth century. Observation windows were subsequently adapted for implantation at different sites and in different organisms, resulting in, amongst others, the hamster cheek pouch chamber (Sewell, 1966), the mouse dorsal skinfold chamber (Algire, 1943; Algire and Legallais, 1949) and even chambers that allowed the observation of the human microcirculation, implanted into the upper arm of the subjects (Brånemark et al., 1964). By eliminating the need for acute exposure during imaging and thereby the high risk of infection, observation windows allow prolonged intravital imaging sessions and repeated imaging for, depending on the type of window and its location, several weeks to

several months, creating the opportunity to follow tumour development over time in a single animal.

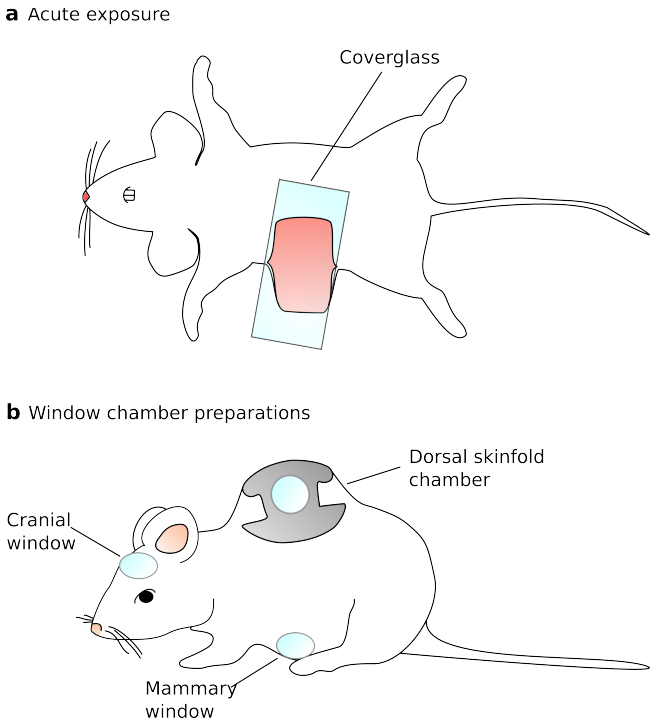

Figure 4. Schematic overview of animal preparation methods for IVM. a Acute surgical exposure using the skin flap method. b Three widely used imaging window preparations in cancer research.

Based on Algire's pioneer work, a new model of the dorsal skinfold chamber was developed by Lehr et al. (1993) in which two parallel frames are implanted, clamping the double layer of skin (Figure 4b). One layer of skin is completely removed in the circular area that will constitute the observation window, exposing the remaining layer which is protected by a glass cover-slip incorporated into one of the frames. Imaging using this chamber can be taken up 48 hours after its implantation, whereas its predecessors require a recovery period of 4 to 6 weeks after surgery. While results obtained by imaging *in vivo* in the dorsal skinfold chamber are likely to be much more significant than *in vitro* findings, this model still has two major flaws: firstly, the dorsal skinfold is not the natural environment for most tumours that are implanted at this site, and secondly, tumour growth is restricted to two dimensions. In order to achieve conditions even closer to those in which tumour growth takes place in nature, tumours have to be studied orthotopically. For this purpose, imaging windows have been

developed for the different sites, e.g. the cranial imaging window for the visualisation of orthotopic brain cancer models (Yuan et al., 1994), and the mammary imaging window for imaging of breast cancer (Shan et al., 2003; Kedrin et al., 2008). Recently, a new window preparation, the abdominal imaging window, has been developed in the van Rheenen group. This window allows for imaging of several internal organs, including liver, pancreas, spleen, and the intestine, and therefore holds great promise for the orthotopic study of metastasis to these organs, as well as a wide range of different primary cancers (Ritsma et al., manuscript in revision, personal communication).

Every IVM study has individual demands on factors such as resolution and duration of imaging sessions, influencing the choice of preparation and imaging method. For instance, measurement of tumour growth over time does not require high spatial or temporal resolution, however, it may be necessary to image the same animal for weeks or months, making non-invasive methods in the intact animal, e.g. MRI or bioluminescence (see 1), a good choice. By contrast, tracking of single cells in a mammary tumour for several days calls for a mammary window preparation, because this will allow prolonged and repeated imaging at (sub-)cellular resolution.

3 IVM in examples of present cancer research

Many genes and proteins involved in cancer have been identified by *in vitro* and *ex vivo* methods. However, these methods cannot shed light on the detailed mechanisms in which they act, because they can only draw a static picture of the events in cancer, e.g. by *ex vivo* histology, or a highly simplified (and possibly somewhat inaccurate) one, by *in vitro* experiments. Therefore, our understanding of the molecular mechanisms underlying cancer is sketchy, and stems mainly from inference rather than direct observation. The advent of intravital imaging enabled the study of dynamic events in the live, intact host, furnishing understanding of *in vivo* events, such as – in the very beginning – regeneration of damaged vasculature. Ever since, a gradual development of IVM techniques and suitable model systems has taken place and continues to improve intravital imaging as an increasingly versatile, powerful and reliable tool for *in vivo* investigations. As such, IVM has been an invaluable asset for cancer research and has resulted in many new insights. In the following, discoveries from different fields of cancer research that have been made possible by IVM and

recent advancements in IVM techniques will be presented and discussed.

3.1 The anti-tumour immune response

It is thought that there are lymphocytes which are engaged in a perpetual patrolling action, termed immunosurveillance, to find and eliminate constantly arising transformed and potentially malignant cells in which intrinsic cellular tumour suppressor mechanisms have failed (Burnet, 1957; Dunn et al., 2002; Schreiber et al., 2011). When transformed cells that have adopted certain hallmark criteria of malignancy (Hanahan and Weinberg, 2000) and manage to evade immunosurveillance, neoplastic growth can take place. However, the immune system remains active in later stages of carcinogenesis and tumour progression. The anti-tumourigenic activities of the native and adaptive immune system, taken together, are referred to as the anti-tumour immune response. The dynamic nature of the immune response, which includes signalling events and immune cell motility, has hindered a good understanding of the role of the immune system in cancer progression in the past. The possibility of intravital imaging at the sub-cellular level has greatly assisted the progress in the study of the dynamics and the molecular underpinnings of the anti-tumour immune response.

Several different cell types of the immune system are known to infiltrate tumours as part of the tumour-associated immune response. However, an ambivalent role of the immune system in cancer has been stated in the literature, as not all involved cell types counteract tumour growth and progression (Zal and Chodaczek, 2010). A selection of immune cell infiltrates in tumours, including macrophages, neutrophils and $CD4^+$ helper T cells, has been correlated with a poor prognosis, suggesting that these cells assist cancer progression. However, tumour infiltrates of several other immune cell populations, such as natural killer cells and other types of lymphocytes, have been associated with a favourable prognosis (Lohela and Werb, 2010). The opposing functions of different immune cell populations might be responsible for the inefficacy of the anti-tumour immune response (Shimizu et al., 1999; Yamazaki et al., 2011) and its controversial role stresses the importance of accurate investigation by IVM of the host immune system in cancer.

Natural killer (NK) cells and cytotoxic T lymphocytes (CTLs), can recognise and

eliminate stressed cells, such as infected cells or tumour cell (Vivier et al., 2012), and have consistently been correlated with a favourable disease prognosis. Target cell recognition by NK and cytotoxic T cells is mediated by receptors expressed by the effector cells. One receptor expressed by NK cells is the NKG2D receptor which binds a variety of ligands that are overexpressed in many cancer cells. Expression of the NKG2D ligand Rae-1β by neoplastic cells has been shown to result in increased NK cell infiltration and dissemination throughout the tumour, which appears to be mediated by augmented recruitment, or enhanced survival or proliferation of NK cells at the tumour, rather than changes in the NK cell niche. Once within the Rae-1β-expressing tumour, NK cells seem to be locally activated and display cytotoxic activity toward their target cells (Deguine et al., 2010). In order to investigate the dynamics of NK cells in Rae-1β-expressing neoplasms, Deguine and colleagues performed intravital MPM on surgically exposed tumours originating from Rae-1β EL4 lymphoma cells that had been injected subcutaneously into the leg of test mice. Their study revealed that the motility of NK cells is strongly enhanced in Rae-1β tumours compared to tumours which do not express NKG2D ligands. Comparison with $CD8^+$ T cells showed that these two populations display distinct patterns of motility. While $CD8^+$ T cell motility decreased strongly in areas of high tumour cell density, NK cell motility was hardly dependent on the latter. A possible explanation for this observation lies in the target cell killing dynamics of NK and $CD8^+$ T cells. Deguine et al. observed by IVM that the CTLs engage in a long-lasting contact with targeted tumour cells, which might be the reason that they are retained at the border of cancer cell-rich regions. By contrast, NK cells maintain contact with their target cells only shortly, which allows them to remain motile and disseminate.

Fisher et al. (2011) investigated the process of tumour infiltration by $CD8^+$ T cells. Paralleling with the inefficacy of the anti-tumour immune response that has been stated beforehand, by default, only few $CD8^+$ T cells can exit the blood vessels to infiltrate the tumour mass. However, simulation of acute inflammation by systemic thermal therapy (STT) of the host, i.e. elevation of the core temperature to 39.5°C for several hours, was shown to increase $CD8^+$ T cell infiltration in various tumour types in mice, while reducing infiltration by $CD4^+CD25^+FoxP3^+$ regulatory T cells, which are correlated with poor prognosis. Fisher and colleagues studied the mechanism by which $CD8^+$ T cells extravasate from tumour vessels to infiltrate the neoplastic lesion during

STT by use of intravital imaging in the dorsal skinfold chamber. They found strong increases in $CD8^+$ T effector cell rolling and cell arrest that took place uniquely in tumour vessels and led to increased $CD8^+$ T homing in the TME, mediated by E/P-selectin, which was required for the rolling interactions, and ICAM-1, a ligand for LFA-1 integrin that was required adhesion and extravasation. $CD8^+$ T cells homed preferably in peripheral regions of the tumour in spite of equal ICAM-1 concentrations in the vessels of tumour centre and periphery. This may coincide with the restriction of $CD8^+$ T cells to regions of lower cancer cell density that has been described by Deguine et al. (2010).

Fisher and colleagues found expression of the trafficking molecule ICAM-1 by the tumour vascular endothelium to be constitutively low, limiting $CD8^+$ T cell extravasation, but was increased by inflammation-induced (or STT-induced) interleukin 6 (IL-6) trans-signalling, thereby allowing for more CTLs to infiltrate the tumour. However, constitutive IL-6 trans-signalling, which takes place in tumours and assists their growth, did not promote T cell trafficking, even though taking place in the same microenvironment. The authors suggest a model where the anti-tumour activity of $CD8^+$ T cells is limited by their trans-endothelial trafficking rate, which can be augmented by activating local IL-6 trans-signalling through induction of acute inflammatory conditions.

The above studies illustrate how IVM can answer not only the "what" but also the "how" and the "why" of cancer processes.

3.2 The tumour microenvironment

The tumour microenvironment (TME) consists of extracellular matrix components such as collagens and fibronectins, different populations of stromal cells, including fibroblasts, lymphocytes, dendritic cells, macrophages and other myeloid cells, as well as growth factors and proteases. In addition, oxygen and metabolites make up part of the TME. Tumour biology is strongly dependent on the host microenvironment (Fukumura et al., 1997; Lohela and Werb, 2010). For example, the TME influences tumour characteristics such as the structure of the microvasculature and the metastatic potential of cancer cells, as well as therapeutic responsiveness of the

tumour. Therefore, obtaining a detailed overview of the TME and its interaction with tumour cells is crucial in order to understand various events in cancer. Even though *in vitro* models of the tumour microenvironment have been described (Krasny et al., 2010), studies performed outside the live host cannot take into account the full complexity of the host microenvironment and its interaction with the tumour. Furthermore, the stromal microenvironment evolves with tumour progression (Egeblad et al., 2008, 2010). By use of intravital imaging, the natural TME and its progression can be visualised, thus providing access to more realistic models of tumour-host interactions.

Nakasone et al. (2012) have studied the impact of extrinsic factors of the TME on chemoresponsiveness to doxorubicin by spinning disk confocal microscopy in MMTV-PyMT mice cross-bred with ACTB-ECFP and c-fms-EGFP reporter mice. In these mice, all cells are labelled with ECFP for contrast, and myeloid cells – the most abundant stromal cell type – are labelled with EGFP, as expression of the *c-fms* gene is restricted to cells of this lineage. Furthermore, different exogenous dyes are used in this study, e.g. tumour stages are visualised by leakage of intravenously injected dextrans, and cell death is made measurable by staining dead cells with intraperitoneally injected propidium iodide.

After observing a clear, but non-linear correlation between tumour stage and doxorubicin-induced cell death, Nakasone and colleagues found that doxorubicin-responsiveness of isolated cells from these tumours did not depend on the stage of the tumour they originated from *in vitro*. By marking leaky vasculature with *Ricinus communis* agglutinin I, the authors found a correlation between tumour stage and vasculature leakage that corresponded to the correlation between tumour stage and doxorubicin-dependent necrosis. It is therefore suggested that the increased sensitivity towards doxorubicin that occurs in intermediate tumour stages compared to early and late stages is connected to vascular leakage. Additionally, high CFMS-EGFP$^+$ myeloid cell infiltration into the tumour appeared to correlate with increased vascular leakiness. Myeloid cells can regulate vessel stability through matrix metalloproteinases (MMPs), such as MMP9, which are expressed at high levels in tumour infiltrating myeloid cells. Investigating this link, Nakasone and colleagues observed increased sensitivity towards doxorubicin in MMP9 null host environments, and increased myeloid cell infiltration in doxorubicin treated tumours. They found the elevated rate

of myeloid cell infiltration to be mediated by recruitment of these cells through a stromal CCL2/CCR2 chemokine/chemokine receptor axis. Doxorubicin-responsive tumours (thus, tumours in an intermediate stage) became even more sensitive to the drug in absence of CCR2 in the host environment that seemed to be due to the effect on tumour infiltration by myeloid cells, and not leaky vasculature.

By discovering the above described effects of molecular changes in the TME, Nakasone and colleagues have found potential ways to improve the efficacy of current chemotherapies against cancer by changing the host microenvironment of primary mammary tumours. This study illustrates how IVM techniques can reveal not only the dynamics of processes occurring at the cellular level, but also provide information on the signalling pathways involved in mediation of the latter.

3.3 Metastasis

The ability of tumours to metastasise is a hallmark of virtually all types of human cancer, and – metastases being the main cause of human cancer deaths – for a large part accounts for the deadliness of the disease. For metastasis to occur, tumour cells need to acquire certain traits, such as motility, survival and invasiveness (Beerling et

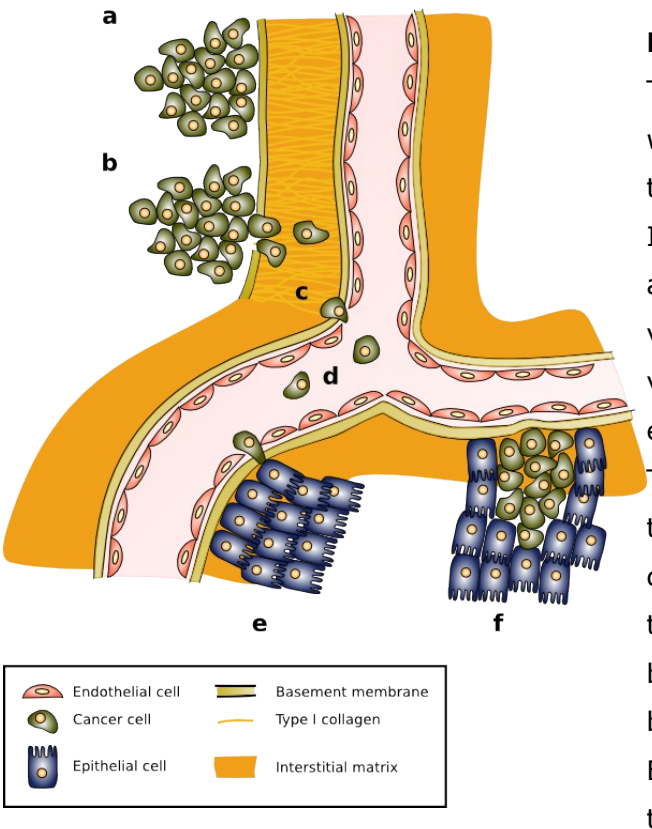

Figure 5. Steps of the metastatic process. a The pre-invasive lesion: A small portion of cells within the primary tumour acquire invasive traits that allow them to metastasise. b Invasion: Cells cross the basement membrane and interstital matrix to migrate towards blood vessels. c Intravasation: Cells enter the blood vessel through basement membrane and endothelial layer. d Hematogenous spread: Tumour cells are transported to distant sites by the blood stream. e Extravasation: After cancer cell arrest and adhesion to the vessel wall have taken place at a distant site, the cells exit the blood vessel through endothelial cell layer and basement membrane. f Outgrowth: Extravasated cancer cells seed a secondary tumour.

al., 2011). The origin of invasive traits so far remains elusive (Karnoub et al., 2007), and only few cells within the primary tumour acquire those traits to eventually kick off the metastatic cascade. The metastatic cascade consists of a series of dynamic processes/steps including invasion, intravasation, survival and transport through the blood stream and extravasation (Figure 5). IVM permits the direct observation these steps, making intravital imaging a tool ideally cut out for the study of metastasis. It is thus not surprising that the first groundbreaking IVM studies on metastasis were done as early as the late 1950s and early 1960s [e.g., (Wood, 1958) visualisation of metastasis in the rabbit ear chamber); (Zeidman, 1961) investigation of movement and arrest of cancer cells in capillaries of the rabbit intestine by means of surgical exteriorisation)]. As a result of the progress that has been made in the past decades in the fields of microscopy, sample preparation and cancer model systems, discussed in chapter 2.3.2, as well as in the field of cancer biology, today, IVM enables the study of metastasis and its individual steps in unprecedented detail.

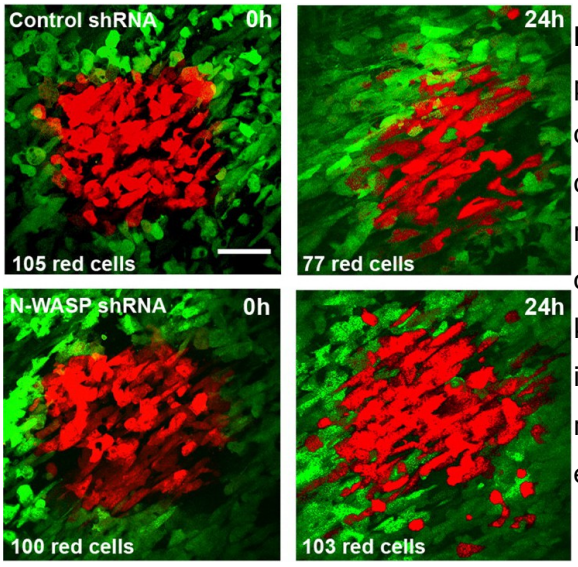

Figure 6. Intravital imaging of Dendra2-photoswitched tumour cell populations in proximity of a tumour vessel. Cells treated with control shRNA disappear into the vasculature as evidenced by a reduction in number and tumour cell count in the circulation (not shown here). Cells with N-WASP knockdown exhibit impaired invasion and intravasation, and do not show a reduction in number after 24 hours. (Adapted from (Gligorijevic et al., 2012)

A wide array of publications can be found that cover the initial steps of metastasis, invasion and intravasation. For instance, (Gligorijevic et al., 2012) have investigated the role of neural Wiskott–Aldrich syndrome protein (N-WASP) and N-WASP-mediated invadopodium formation during metastasis. Invadopodia are membrane protrusions that are characterised by their high proteolytic activity and have been observed *in vitro* when invasive cancer cells were cultured on substrates resembling the extracellular matrix (ECM) or basement membrane. Invadopodia have been suggested to be involved in degradation and remodeling of the ECM – a process controlled by proteases, in particular matrix metalloproteases (MMPs). Tumour cells need to

remodel the ECM in order to overcome barriers imposed by, and migrate through the latter. Gligorijevic and colleagues studied the N-WASP-dependent formation and function of invadopodia using stable MTLn3 rat mammary adenocarcinoma cell lines, *in vitro* and *in vivo* as tumour allografts in rats or xenografts in mice. They found that N-WASP is required for the formation of invadopodia *in vivo*, likely by actin reorganisation, as cells in which N-WASP is suppressed manifest reduced invadopodia and F-actin assembly. *In vivo*, tumour allografts in which N-WASP is suppressed manifest decreased capability to invade, a lower carcinoma cell count in the blood flow, and reduced metastasis in the lungs of test animals. Using IVM photoconversion, Gligorijevic et al. were able to show that N-WASP is required for migration of tumour cells through the tissue and intravasation. The photoconversion experiment was done using tumour allografts from cells labelled with Dendra2. After exposure to 405 nm light, Gligorijevic and colleagues observed that the number of cells in photoswitched control populations in the vicinity of tumour vessels decreased, suggesting that they intravasated into the tumour microvasculature. Meanwhile, the number of cells in populations in which N-WASP was suppressed remained largely the same (Figure 6). IVM further unveiled that GFP-labelled tumour cells in which N-WASP was suppressed were rounded, less polarised, formed fewer protrusions and exhibited reduced motility compared to control cells in which N-WASP was active. Using IHC, the protrusions in the control cells were identified as invadopodia by detection of typical invadopodia components, and the degraded ECM area was quantified using antibodies against collagen fragments created by different MMPs, showing that when N-WASP was suppressed, a tenth of the area degraded in control tumours was cleaved, showing that N-WASP-dependent invadopodia are required for ECM remodeling in invasion and for intravasation.

Another study that uses a combination of IVM and other tools for the investigation of the initial steps of metastasis was carried out by (Kedrin et al., 2009). It is well established that the expression of the epidermal growth factor (EGF) receptor family is upregulated in many malignancies, and is correlated with a poor prognosis. Members of the EGFR family, including the EGFR or ErbB1, ErbB2, ErbB3 and ErbB4, are known to be involved in cell processes such as proliferation, apoptosis, survival, invasion and differentiation, both under physiological and pathological conditions (Burgess, 2008).

Using intravital MPM to track single cancer cells, (Kedrin et al., 2009) have found that combined inhibition of EGFR and ErbB2 phosphorylation in three different tumour models in mice (MTLn3E xenograft tumour, the transgenic PyMT model, and a human tumour cell line xenograft, MDA-MB-231) strongly decreases the number of motile tumour cells within the primary tumour and its surrounding tissues. The observation of motile tumour cells within the tumour mass *in vivo* has only become possible thanks to IVM, because cell motility cannot be assessed by *ex vivo* methods such as IHC. In static histological samples, the only tumour cells that could be identified as motile were those which had already escaped the tumour mass.

Kedrin and colleagues further observed a strongly diminished number of cancer cells in the animal blood due to decreased intravasation of tumour cells. By additional experiments, in which they selectively inhibited EGFR or ErbB2, they were able to show distinct roles of the two receptors in the early steps of metastasis. As shown by IVM tracking of single cells, cell motility and invasion within the primary tumour were strongly reduced following selective EGFR inhibition. However, a stastitically significant reduction of intravasated cells could only be observed after prolonged inhibition of EGFR, suggesting that EGFR inhibition does not affect intravasation directly, but rather indirectly by inhibiting cell motility and invasion. Because combined EGFR/ErbB2 inhibition resulted in an immediate reduction of intravasation, ErbB2 was suspected to play a role in the intravasation step, which Kendra and colleagues confirmed using an *in vitro* assay.

The importance of EGF receptor function and its underlying mechanisms, have further been studied by (Smirnova et al., 2012). ErbB2 is known to form a heterodimer with another member of the EGF receptor family, ErbB3. The latter binds heregulin β-1 (HRGβ1), and further contains six binding sites for PI3 kinase. Overexpression of ErbB3 in mammary tumor cells has been shown to significantly enhance chemotaxis toward HRGβ1 and overall metastatic potential of these cells (Xue et al., 2006). (Smirnova et al., 2012) found that ErbB3-HRGβ1-dependent chemotaxis and invasion *in vitro* require PI3K signalling. Using immunohistochemical analysis, they also showed that upregulated ErbB3 enhances spontaneous metastatic potential, intravasation and lung seeding *in vivo* in orthotopic mammary tumour xenografts in immunosuppressed mice in a PI3K-dependent manner. After establishing the role of PI3K in ErbB3-HRGβ1-mediated invasiveness, Smirnova and colleagues used intravital

MPM of acutely exteriorised orthotopic GFP-labelled mammary tumour xenografts in order to study the importance of ErbB3/PI3K for invasion in the primary tumour environment, finding that ErbB3 overexpression significantly enhances tumour cell motility and polarisation.

The inhibitory effect of ErbB2 inhibition on intravasation described by Kendra et al. and the enhancement of intravasation that follows ErbB3 overexpression observed by Smirnova et al. suggest an important role for the ErbB2/ErbB3 heterodimer in this process. The two studies suggest that the activation of the PI3K pathway by ErbB3, and possibly the ErbB3/ErbB2 heterodimer, plays an important part in the initial steps of metastasis.

The protein Mena, which had previously been suggested to be involved in invasion and intravasation, was studied by (Roussos et al., 2011a) They found that the two isoforms of Mena, Mena invasive ($Mena^{INV}$) and Mena11a, are both active in several steps in early metastasis, hoewever with different functions. Roussos and colleagues found that, while $Mena^{INV}$ increases dissemination, Mena11a decreases dissemination. IVM showed that $Mena^{INV}$ promotes streaming cell movement, rather than random cell movement, which makes cells move faster and more efficiently. Consistently, enhanced EGF-dependent invasion was observed in $Mena^{INV}$-expressing carcinoma cells *in vivo*. IVM also helped elucidate that the streaming motion of carcinoma cells requires macrophages and paracrine signalling between tumour cells and macrophages, and that streaming occurs directionally towards blood vessels. Using Dendra2 photoconversion, Roussos and colleagues found that intravasation is elevated in $Mena^{INV}$-expressing cells compared to Mena11a-expressing cells. However, this is only the case when perivascular macrophages are present and assist intravasation by paracrine signalling.

Not only the early steps, but also later events in the metastatic cascade can be investigated by means of intravital imaging. In a recent study by Arpaia et al. (2012), they investigated the molecular mechanisms that underlie the previously proposed link between Cav1 and Rho-GTPases and cancer and metastatic progression. They describe a function for caveolin-1 (Cav1) and the Rho-GTPase RhoC in survival and extravasation of metastatic cells, elucidated by IVM.

Arpaia and colleagues establish a crucial role for Cas1 and RhoC, and more specifically

the interaction of these two proteins, in different processes required for cell motility and invasiveness *in vitro* in a B16 melanoma cell line and a cell line generated from explanted MMTV–PyMT mammary epithelial tumor cells, and *in vivo*. For instance, $p130^{Cas}$ phosphorylation is dependent on Cas1-RhoC interaction. $p130^{Cas}$ is an important signalling molecule that is required for Rac1 activation which, in turn, is involved in migration and cytoskeletal remodeling of many different cell types. According to the findings of Arpaia and colleagues, activation of Rac1 is necessary for F-actin fibre polymerization or stabilization *in vivo*, as depletion of Rac1 activation results in reduced stress fibres, while actin remains present in puncta dispersed throughout the cell. The consequence of this *in vivo* is a reduced capability of the cells to migrate and invade. Furthermore, Arpaia and colleagues observe that activation of the kinase phosphorylating $p130^{Cas}$, Src, is dependent on Cas1-RhoC interaction. This explains the impairment of migration and invasion seen in cells in which Cas1-RhoC interaction is disrupted, as FAK and Pyk2, which are involved in cell migration, invasion and adhesion, are two direct downstream targets of Src. Activation of Erk1/2, which promotes cancer cell migration and invasion, and is activated by Src via Ras, is equally reduced upon disruption of Cav1-RhoC interaction, which Arpaia and colleagues were able to explain by lowered levels of Ras-GTP in these cells. Furthermore, they found a drastic reduction of α5-integrin expression – whose function is required for cell adhesion, migration and growth – when Cav1-RhoC interaction was depleted.

These findings suggest that the interaction of Cav1 and RhoC is at the bottom of a whole signalling cascade mediating cell motility, invasion and adhesion, and that its disruption might impair cancer metastasis *in vivo*. Arpaia et al. have tested this hypothesis, first *in situ* by injection of cancer cells into the mouse tail vein, showing that cells lacking Cav1-RhoC interaction do not cause any detectable metastases, then *in vivo* by IVM, which provided an explanation for this phenomenon by unveiling a higher attrition rate of these cells during extravasation. IVM showed that cells in which Cav1-RhoC interaction was disrupted, morphological alterations occurred either during or after extravasation, coinciding with their decreased survival rate.

Kienast et al. (2010) have studied extravasation and outgrowth of metastases in the brain of immunodeficient nude mice by MPM through a cranial imaging window. The

use arterially injected RFP-labelled human melanoma (MDA-MB-435 and A2058) and lung cancer (PC14-PE6 and HTB177) cell lines enabled the observation of differential behaviour during the late steps of metastasis displayed by lung carcinoma and melanoma cells. For contrast generation, the host vasculature was made visible by endogenous GFP-labelling of the endothelium and additional injection of FICT-dextran. By analysing the behaviour of individual cells of the different cell lines over time and correlating cell fate to the preceding behaviour of the same individual cells, Kienast and colleagues were able to identify factors underlying successful metastasis and particularly inefficient processes that present rate limiting steps of metastasis formation. Their results show that for both melanoma and lung cancer cells initial prolonged cell arrest and early extravasation (i.e. within three days of entering the blood stream) are necessary conditions for the formation of metastases. Cell arrest appears to be caused by trapping in vessel branches rather than vascular wall adhesion. The extravasation process likely occurs through small holes in the vessel wall as suggested by video microscopy observation of extravasating cancer cells. Furthermore, only cells that perpetuated a perivascular position directly adjacent to the abluminal endothelial cell layer were observed to proliferate into macrometastases. Up to this point, lung carcinoma and melanoma cells display a similar behaviour, though differing in some minor points. Once extravasation has taken place, however, they adopt different growth patterns. Melanoma cells co-opt microvessels for nutrient supply. Their proliferation occurs along these vessels, and consequently, small changes are induced in the latter, mainly formation of loops. When the cell cluster reaches a critical diameter, the local microvasculature undergoes more extensive changes, and angiogenesis is induced. In contrast to the co-optive growth of metastasising melanoma cells that relies on pre-existing vasculature, lung carcinoma cells display an angiogenic growth pattern. Lung carcinoma metastases as observed by Kienast et al. are formed by fusion of separate proliferating cancer cell clusters, whereupon the critical diameter for induction of major changes in the local vasculature and angiogenesis is reached, resulting in fast outgrowth. Lung cancer cells, which – unlike melanoma cells – constitutively express vascular endothelial growth factor A (VEGF-A) adopt melanoma cell-like co-optive growth when angiogenesis is suppressed by bevacizumab-mediated inhibition of VEGF-A, and eventually become dormant, explaining the therapeutic effect of the drug.

By tracking the fate of individual cells during metastasis formation, this pure IVM study by Kienast et al. truly takes advantage of the benefits that intravital imaging offers.

4 Discussion

As demonstrated by the examples listed above, IVM has evolved from a method for visualisation of only vasculature in live organisms into a technique that has become instrumental for today's cancer research because it can unveil dynamics and signalling pathways involved in different cancer processes *in vivo*. Due to its unique ability to capture spatio-temporal information in living systems, intravital imaging – alone and in combination with "traditional" techniques – has been able to answer a large number of questions that were previously unresolvable. The progress that has taken place in the fields of microscopy, molecular probes, and genetic engineering of model systems has been requisite for IVM to become the powerful and versatile tool that it is today.

Current limitations of IVM

Unquestionably, however, many mysteries remain to be solved with regards to cancer and its underlying processes. Even though IVM has proven able to provide information on signalling events in cancer processes, as evidenced for example by the study carried out by Nakasone et al. (2012), described in chapter 3.2, this is only a recent development. Techniques for the study of signalling by microscopy *in vivo* require further elaboration in the future in order to obtain more detailed information and elucidate the full signalling cascades involved in cancer processes. In addition to improved spatial and temporal resolution, whose frontiers are being pushed constantly, this will ask for model systems that allow direct conclusions on the interrelationships between signalling events and cancer cell behaviour, for example by introduction of inducible oncogenes or signalling proteins into the cancer cells (Beerling et al., 2011; Ritsma et al., 2012).

Next to developments aiming at "zooming in" even closer on the studied processes and thereby obtaining more detailed information at ever smaller levels, there is the need for both model systems and technologies that allow imaging in conditions even closer to nature than possible at this point. By making use of IVM, we are already lengths ahead of *in vitro* and *ex vivo* methods, nonetheless, there are several points which leave room for improvement, or that might bear artefacts that we are simply not able to assess. This starts with the molecular probes that are used to label cells or cellular components for detection by IVM. One of the most important labelling

techniques is the use of protein-FP fusions. However, protein-FP fusions might behave differently from the endogenous protein due to their larger size, possible occlusion of interaction sites, wrong sorting or even misfolding of the protein. While there are assays that enable assessment of protein sorting and binding with known interaction partners, it cannot be excluded that binding to so far unknown interaction partners might be disrupted, possibly interfering with signalling and cell behaviour. Molecular probes are, however, indispensable for direct observation of any process by IVM, making them a necessary evil. Artefacts caused by FP-labelling may be reduced by design of lower weight FPs. Another limitation imposed by the need for fluorescent labelling is the limited number of fluorophores available that can be distinguished by current imaging platforms. This problem has been approached both by improving microscopy platforms for detection of larger numbers of probes and by ameliorating existing or developing new fluorescent probes. A relatively new development in this area are quantum dots whose emission spectrum can be tuned to the individual needs of a certain study, thus, they can be used in combination with other fluorescent proves while avoiding interference of different signals (Stroh et al., 2005).

The model systems frequently used in cancer research by IVM are not quite ideal yet, either. Often, cancer research by IVM involves tumours grown from cell line allografts or xenografts, mainly because they are easy to use and do not require time consuming breeding, as opposed to genetic model systems. As already discussed in chapter 2.3.2, tumours generated by cell line injection do not perfectly model natural tumours, because their metabolism and behaviour are dependent on the stage in which they were isolated and further influenced by culturing in cell culture medium. Moreover, cell line xenografts can only be done in immunodeficient animals, as otherwise immune clearance of cancer cells takes place before tumour growth can occur. Suppression or absence of a functioning immune system is very likely to cause artefacts because the immune system plays an important, though elusive, role in cancer progression.

Genetic mouse models present more realistic model systems and, even though their preparation is time-consuming and technically demanding, the generation of new genetic model systems is reported on a regular basis. With IVM becoming an increasingly important and wide-spread tool for cancer research, the amount of genetic mouse models specifically generated for this purpose will increase more and

more quickly, which will eventually speed up research and thus generation of knowledge on cancer.

In addition to artefacts that are intrinsic of the model, artefacts can be produced by preparation and during imaging of the animal. Especially acute preparations tend to produce inflammatory reactions that might distort observations. Inflammation of the tumour tissue was not observed in the mammary imaging window (Kedrin et al., 2008). By contrast, avoiding inflammation in the cranial imaging window is virtually impossible, thus, avoiding inflammatory reactions that possibly interfere with observations through this window is virtually impossible (Shih et al., 2012). During imaging, conditions might be altered by anaesthesia of the studied animal. However, aside from aspects of animal protection, anaesthesia is required to limit movements that can cause image distortion. Techniques that can acquire images at higher rates may be able to evade distortion by motion to a certain degree (e.g. spinning disk confocal microscopy, see 1). So far, however, the resolution that can be achieved at frame rates high enough to compensate for strong movements such as would be expected in conscious animals, is not sufficient for many processes of interest in cancer research.

Future perspectives

Several big questions in cancer so far remain unanswered, including the existence and origin of a cancer stem cell (CSC) (Sampieri and Fodde, 2012) and the meaning of epithelial to mesenchymal transition (EMT) for metastasis in human cancers *in vivo* (Bastid, 2012). Such questions are predestined for study by IVM because single cell tracking and lineage tracing *in vivo* are required. The recent advancements that have been made in microscopy techniques and genetic model systems for IVM allow for direct observation of such events and will likely provide answers in the coming years. Recent publications have been able to establish (causal) relationships between molecular events within the tumour and stroma, cancer progression *in vivo* and drug efficacy, thereby interconnecting different fields in tumour biology research. In addition to answering discrete questions, IVM will thereby lead to a more comprehensive understanding of cancer and metastasis.

While IVM has proven invaluable as a tool for fundamental research on cancer, it can also be particularly helpful for the development or improvement of new anti-cancer

drugs because it offers the possibility of assessing the effects of therapeutics over a course of time. Furthermore, IVM has been used to decipher the mechanisms underlying current cancer therapies. A good understanding of the mechanism of action of cancer therapies can be helpful for their improvement or for development of new therapies. For instance, Abedinpour and co-workers (2011) were able to show that tumour regression upon androgen withdrawal treatment, which has been a standard treatment for metastatic prostate cancer for several decades, relies on disruption of the tumour vasculature. With this knowledge, improvements as well as complementary treatments can be found in a goal-oriented manner. Work has also been done on improvement of drug delivery efficacy. Using a combination of mathematical modelling and intravital imaging, van de Ven and colleagues (2012) made efforts to optimise delivery of nanoparticle-based cancer drugs, taking into account physiological barriers imposed by cancer.

With the increasing use of intravital imaging in cancer research, developments in the areas of microscopy methods and model systems will take place increasingly fast and will thereby fuel the research on cancer mechanisms *in vivo*, as well as the development of cancer treatments.

5 References

Abedinpour, P., Baron, V.T., Welsh, J., and Borgström, P. (2011). Regression of prostate tumors upon combination of hormone ablation therapy and celecoxib in vivo. Prostate *71*, 813–823.

Algire, G., and Legallais, F. (1949). Recent Developments in the Transparent-Chamber Technique as Adapted to the Mouse. J. Natl. Cancer Inst. *10*, 225–&.

Algire, G.H. (1943). An adaptation of the transparent chamber technique to the mouse. J. Natl. Cancer Inst. *4*, 1–11.

Arpaia, E., Blaser, H., Quintela-Fandino, M., Duncan, G., Leong, H.S., Ablack, A., Nambiar, S.C., Lind, E.F., Silvester, J., Fleming, C.K., et al. (2012). The interaction between caveolin-1 and Rho-GTPases promotes metastasis by controlling the expression of alpha5-integrin and the activation of Src, Ras and Erk. Oncogene *31*, 884–896.

Aslakson, C.J., and Miller, F.R. (1992). Selective events in the metastatic process defined by analysis of the sequential dissemination of subpopulations of a mouse mammary tumor. Cancer Res. *52*, 1399–1405.

Bastid, J. (2012). EMT in carcinoma progression and dissemination: facts, unanswered questions, and clinical considerations. Cancer Metastasis Rev. *31*, 277–283.

Beerling, E., Ritsma, L., Vrisekoop, N., Derksen, P.W.B., and van Rheenen, J. (2011). Intravital microscopy: new insights into metastasis of tumors. J. Cell. Sci. *124*, 299–310.

Berchem, G., Glondu, M., Gleizes, M., Brouillet, J.-P., Vignon, F., Garcia, M., and Liaudet-Coopman, E. (2002). Cathepsin-D affects multiple tumor progression steps in vivo: proliferation, angiogenesis and apoptosis. Oncogene *21*, 5951–5955.

Bonapace, L., Wyckoff, J., Oertner, T., Van Rheenen, J., Junt, T., and Bentires-Alj, M. (2012). If You Don't Look, You Won't See: Intravital Multiphoton Imaging of Primary and Metastatic Breast Cancer. Journal of Mammary Gland Biology and Neoplasia.

Bouvet, M., Wang, J.W., Nardin, S.R., Nassirpour, R., Yang, M., Baranov, E., Jiang, P., Moossa, A.R., and Hoffman, R.M. (2002). Real-time optical imaging of primary tumor growth and multiple metastatic events in a pancreatic cancer orthotopic model. Cancer Res. *62*, 1534–1540.

Brånemark, P.-I., Aspegren, K., and Breine, U. (1964). Microcirculatory Studies in Man By High Resolution Vital Microscopy. ANGIOLOGY *15*, 329–332.

Burgess, A.W. (2008). EGFR family: Structure physiology signalling and therapeutic targets. Growth Factors *26*, 263–274.

Burnet, M. (1957). Cancer—A Biological Approach. Br Med J *1*, 841–847.

Cailleau, R., Young, R., Olivé, M., and Reeves, W.J., Jr (1974). Breast tumor cell lines from pleural effusions. J. Natl. Cancer Inst. *53*, 661–674.

Campagnola, P.J., Millard, A.C., Terasaki, M., Hoppe, P.E., Malone, C.J., and Mohler, W.A. (2002). Three-Dimensional High-Resolution Second-Harmonic Generation Imaging of Endogenous Structural Proteins in Biological Tissues. Biophysical Journal *82*, 493–508.

Chudakov, D.M., Lukyanov, S., and Lukyanov, K.A. (2007). Tracking intracellular protein movements using photoswitchable fluorescent proteins PS-CFP2 and Dendra2. Nature Protocols *2*, 2024–2032.

Condeelis, J., and Weissleder, R. (2010). In Vivo Imaging in Cancer. Cold Spring Harb Perspect Biol *2*,.

Corbett, T.H., Griswold, D.P., Jr, Roberts, B.J., Peckham, J.C., and Schabel, F.M., Jr (1975). Tumor induction relationships in development of transplantable cancers of the colon in mice for chemotherapy assays, with a note on carcinogen structure. Cancer Res. *35*, 2434–2439.

Deguine, J., Breart, B., Lemaître, F., Di Santo, J.P., and Bousso, P. (2010). Intravital imaging reveals distinct dynamics for natural killer and CD8(+) T cells during tumor regression. Immunity *33*, 632–644.

Dunn, G.P., Bruce, A.T., Ikeda, H., Old, L.J., and Schreiber, R.D. (2002). Cancer immunoediting: from immunosurveillance to tumor escape. Nature Immunology *3*, 991–998.

Egeblad, M., Ewald, A.J., Askautrud, H.A., Truitt, M.L., Welm, B.E., Bainbridge, E., Peeters, G., Krummel, M.F., and Werb, Z. (2008). Visualizing stromal cell dynamics in different tumor microenvironments by spinning disk confocal microscopy. Dis. Model. Mech. *1*, 155–167.

Egeblad, M., Nakasone, E.S., and Werb, Z. (2010). Tumors as organs: complex tissues that interface with the entire organism. Dev. Cell *18*, 884–901.

Entenberg, D., Wyckoff, J., Gligorijevic, B., Roussos, E.T., Verkhusha, V.V., Pollard, J.W., and Condeelis, J. (2011). Setup and use of a two-laser multiphoton microscope for multichannel intravital fluorescence imaging. Nature Protocols *6*, 1500–1520.

Fantozzi, A., and Christofori, G. (2006). Mouse models of breast cancer metastasis. Breast Cancer Research *8*, 212.

Fisher, D.T., Chen, Q., Skitzki, J.J., Muhitch, J.B., Zhou, L., Appenheimer, M.M., Vardam, T.D., Weis, E.L., Passanese, J., Wang, W.-C., et al. (2011). IL-6 trans-signaling licenses mouse and human tumor microvascular gateways for trafficking of cytotoxic T cells. J Clin Invest *121*, 3846–3859.

Folkman, J. (1971). Tumor angiogenesis: therapeutic implications. N. Engl. J. Med. *285*, 1182–1186.

Fukumura, D., Yuan, F., Monsky, W.L., Chen, Y., and Jain, R.K. (1997). Effect of host microenvironment on the microcirculation of human colon adenocarcinoma. Am J Pathol *151*, 679–688.

Garcia, M., Derocq, D., Pujol, P., and Rochefort, H. (1990). Overexpression of transfected cathepsin D in transformed cells increases their malignant phenotype and metastatic potency. Oncogene *5*, 1809–1814.

Gligorijevic, B., Kedrin, D., Segall, J.E., Condeelis, J., and van Rheenen, J. (2009). Dendra2 Photoswitching through the Mammary Imaging Window. J Vis Exp.

Gligorijevic, B., Wyckoff, J., Yamaguchi, H., Wang, Y., Roussos, E.T., and Condeelis, J. (2012). N-WASP-mediated invadopodium formation is involved in intravasation and lung metastasis of mammary tumors. J. Cell. Sci. *125*, 724–734.

Grayson, M.H., Chaplin, D.D., Karl, I.E., and Hotchkiss, R.S. (2001). Confocal fluorescent intravital microscopy of the murine spleen. Journal of Immunological Methods *256*, 55–63.

Gurskaya, N.G., Verkhusha, V.V., Shcheglov, A.S., Staroverov, D.B., Chepurnykh, T.V., Fradkov, A.F., Lukyanov, S., and Lukyanov, K.A. (2006). Engineering of a monomeric green-to-red photoactivatable fluorescent protein induced by blue light. Nature Biotechnology *24*, 461–465.

Hanahan, D., and Weinberg, R.A. (2000). The Hallmarks of Cancer. Cell *100*, 57–70.

Jain, R.K., Munn, L.L., and Fukumura, D. (2002). Dissecting tumour pathophysiology using intravital microscopy. Nat. Rev. Cancer *2*, 266–276.

Karnoub, A.E., Dash, A.B., Vo, A.P., Sullivan, A., Brooks, M.W., Bell, G.W., Richardson, A.L., Polyak, K., Tubo, R., and Weinberg, R.A. (2007). Mesenchymal stem cells within tumour stroma promote breast cancer metastasis. Nature *449*, 557–563.

Kawano, H., Kogure, T., Abe, Y., Mizuno, H., and Miyawaki, A. (2008). Two-photon dual-color imaging using fluorescent proteins. Nature Methods *5*, 373–374.

Kedrin, D., Gligorijevic, B., Wyckoff, J., Verkhusha, V.V., Condeelis, J., Segall, J.E., and van Rheenen, J. (2008). Intravital imaging of metastatic behavior through a mammary imaging window. Nat. Methods *5*, 1019–1021.

Kedrin, D., Wyckoff, J., Boimel, P.J., Coniglio, S.J., Hynes, N.E., Arteaga, C.L., and Segall, J.E. (2009). ERBB1 and ERBB2 have distinct functions in tumor cell invasion and intravasation. Clin. Cancer Res. *15*, 3733–3739.

Kienast, Y., von Baumgarten, L., Fuhrmann, M., Klinkert, W.E.F., Goldbrunner, R., Herms, J., and Winkler, F. (2010). Real-time imaging reveals the single steps of brain metastasis formation. Nat. Med. *16*, 116–122.

Knopfová, L., Beneš, P., Pekarčíková, L., Hermanová, M., Masařík, M., Pernicová, Z., Souček, K., and Smarda, J. (2012). c-Myb regulates matrix metalloproteinases 1/9, and cathepsin D: implications for matrix-dependent breast cancer cell invasion and

metastasis. Mol. Cancer *11*, 15.

Krasny, L., Shimony, N., Tzukert, K., Gorodetsky, R., Lecht, S., Nettelbeck, D.M., and Haviv, Y.S. (2010). An in-vitro tumour microenvironment model using adhesion to type I collagen reveals Akt-dependent radiation resistance in renal cancer cells. Nephrol. Dial. Transplant. *25*, 373–380.

Lehr, H.A., Leunig, M., Menger, M.D., Nolte, D., and Messmer, K. (1993). Dorsal skinfold chamber technique for intravital microscopy in nude mice. Am J Pathol *143*, 1055–1062.

Lichtner, R.B., Julian, J.A., Glasser, S.R., and Nicolson, G.L. (1989). Characterization of cytokeratins expressed in metastatic rat mammary adenocarcinoma cells. Cancer Res. *49*, 104–111.

Lohela, M., and Werb, Z. (2010). Intravital imaging of stromal cell dynamics in tumors. Curr. Opin. Genet. Dev. *20*, 72–78.

Lorenz, W.W., McCann, R.O., Longiaru, M., and Cormier, M.J. (1991). Isolation and expression of a cDNA encoding Renilla reniformis luciferase. Proc Natl Acad Sci U S A *88*, 4438–4442.

Masson, O., Bach, A.-S., Derocq, D., Prébois, C., Laurent-Matha, V., Pattingre, S., and Liaudet-Coopman, E. (2010). Pathophysiological functions of cathepsin D: Targeting its catalytic activity versus its protein binding activity? Biochimie *92*, 1635–1643.

McDonald, B., Pittman, K., Menezes, G.B., Hirota, S.A., Slaba, I., Waterhouse, C.C.M., Beck, P.L., Muruve, D.A., and Kubes, P. (2010). Intravascular Danger Signals Guide Neutrophils to Sites of Sterile Inflammation. Science *330*, 362–366.

Meighen, E.A. (1991). Molecular biology of bacterial bioluminescence. Microbiol. Rev. *55*, 123–142.

Morris, J.P., Wang, S.C., and Hebrok, M. (2010). KRAS, Hedgehog, Wnt and the twisted developmental biology of pancreatic ductal adenocarcinoma. Nature Reviews Cancer *10*, 683–695.

Nakano, A. (2002). Spinning-disk Confocal Microscopy — A Cutting-Edge Tool for Imaging of Membrane Traffic. Cell Structure and Function *27*, 349–355.

Nakasone, E.S., Askautrud, H.A., Kees, T., Park, J.-H., Plaks, V., Ewald, A.J., Fein, M., Rasch, M.G., Tan, Y.-X., Qiu, J., et al. (2012). Imaging Tumor-Stroma Interactions during Chemotherapy Reveals Contributions of the Microenvironment to Resistance. Cancer Cell *21*, 488–503.

Ohri, S.S., Vashishta, A., Proctor, M., Fusek, M., and Vetvicka, V. (2007). Depletion of procathepsin D gene expression by RNA interference: a potential therapeutic target for breast cancer. Cancer Biol. Ther. *6*, 1081–1087.

Pittet, M.J., and Weissleder, R. (2011). Intravital imaging. Cell *147*, 983–991.

Ritsma, L., Ponsioen, B., and van Rheenen, J. (2012). Intravital imaging of cell signaling in mice. IntraVital *1*, 0–8.

Rockwell, S. (1980). In vivo-in vitro tumour cell lines: characteristics and limitations as models for human cancer. Br J Cancer Suppl *4*, 118–122.

Roussos, E.T., Balsamo, M., Alford, S.K., Wyckoff, J.B., Gligorijevic, B., Wang, Y., Pozzuto, M., Stobezki, R., Goswami, S., Segall, J.E., et al. (2011a). Mena invasive (Mena(INV)) promotes multicellular streaming motility and transendothelial migration in a mouse model of breast cancer. J. Cell Sci. *124*, 2120–2131.

Roussos, E.T., Condeelis, J.S., and Patsialou, A. (2011b). Chemotaxis in cancer. Nature Reviews Cancer *11*, 573–587.

Sampieri, K., and Fodde, R. (2012). Cancer stem cells and metastasis. Semin. Cancer Biol. *22*, 187–193.

Sandison, J.C. (1924). A new method for the microscopic study of living growing tissues by the introduction of a transparent chamber in the rabbit's ear. The Anatomical Record *28*, 281–287.

Schreiber, R.D., Old, L.J., and Smyth, M.J. (2011). Cancer Immunoediting: Integrating Immunity's Roles in Cancer Suppression and Promotion. Science *331*, 1565–1570.

Sewell, I.A. (1966). Studies of the microcirculation using transparent tissue observation chambers inserted in the hamster cheek pouch. J. Anat. *100*, 839–856.

Shan, S., Sorg, B., and Dewhirst, M.W. (2003). A novel rodent mammary window of orthotopic breast cancer for intravital microscopy. Microvasc. Res. *65*, 109–117.

Shih, A.Y., Driscoll, J.D., Drew, P.J., Nishimura, N., Schaffer, C.B., and Kleinfeld, D. (2012). Two-photon microscopy as a tool to study blood flow and neurovascular coupling in the rodent brain. J. Cereb. Blood Flow Metab. *32*, 1277–1309.

Shimizu, J., Yamazaki, S., and Sakaguchi, S. (1999). Induction of tumor immunity by removing CD25+CD4+ T cells: a common basis between tumor immunity and autoimmunity. J. Immunol. *163*, 5211–5218.

Smirnova, T., Zhou, Z.N., Flinn, R.J., Wyckoff, J., Boimel, P.J., Pozzuto, M., Coniglio, S.J., Backer, J.M., Bresnick, A.R., Condeelis, J.S., et al. (2012). Phosphoinositide 3-kinase signaling is critical for ErbB3-driven breast cancer cell motility and metastasis. Oncogene *31*, 706–715.

Snippert, H.J., van der Flier, L.G., Sato, T., van Es, J.H., van den Born, M., Kroon-Veenboer, C., Barker, N., Klein, A.M., van Rheenen, J., Simons, B.D., et al. (2010). Intestinal crypt homeostasis results from neutral competition between symmetrically dividing Lgr5 stem cells. Cell *143*, 134–144.

Stroh, M., Zimmer, J.P., Duda, D.G., Levchenko, T.S., Cohen, K.S., Brown, E.B., Scadden, D.T., Torchilin, V.P., Bawendi, M.G., Fukumura, D., et al. (2005). Quantum dots spectrally distinguish multiple species within the tumor milieu in vivo. Nature

Medicine *11*, 678–682.

Theer, P., Hasan, M.T., and Denk, W. (2003). Two-photon imaging to a depth of 1000 ? m in living brains by use of a Ti:Al2O3 regenerative amplifier. Opt. Lett. *28*, 1022–1024.

Tysnes, B.B., and Bjerkvig, R. (2007). Cancer initiation and progression: Involvement of stem cells and the microenvironment. Biochimica Et Biophysica Acta (BBA) - Reviews on Cancer *1775*, 283–297.

van de Ven, A.L., Wu, M., Lowengrub, J., McDougall, S.R., Chaplain, M.A.J., Cristini, V., Ferrari, M., and Frieboes, H.B. (2012). Integrated intravital microscopy and mathematical modeling to optimize nanotherapeutics delivery to tumors. AIP Adv. *2*,.

Vivier, E., Ugolini, S., Blaise, D., Chabannon, C., and Brossay, L. (2012). Targeting natural killer cells and natural killer T cells in cancer. Nat. Rev. Immunol. *12*, 239–252.

Wagner, R. (1839). Icones physiologicae; tabulae physiologiam et geneseos historiam illustrantes (Voss).

Waller, A. (1846). XLIV. Microscopic examination of some of the principal tissues of the animal frame, as observed in the tongue of the living Frog, Toad, &c. Philosophical Magazine Series 3 *29*, 271–287.

Wang, J., Chen, L., Li, Y., and Guan, X.-Y. (2011). Overexpression of cathepsin Z contributes to tumor metastasis by inducing epithelial-mesenchymal transition in hepatocellular carcinoma. PLoS ONE *6*, e24967.

Wet, J.R. de, Wood, K.V., DeLuca, M., Helinski, D.R., and Subramani, S. (1987). Firefly luciferase gene: structure and expression in mammalian cells. Mol. Cell. Biol. *7*, 725–737.

Wood, S. (1958). Pathogenesis of Metastasis Formation Observed Invivo in the Rabbit Ear Chamber. Archives of Pathology *66*, 550–568.

Xue, C., Liang, F., Mahmood, R., Vuolo, M., Wyckoff, J., Qian, H., Tsai, K.-L., Kim, M., Locker, J., Zhang, Z.-Y., et al. (2006). ErbB3-dependent motility and intravasation in breast cancer metastasis. Cancer Res. *66*, 1418–1426.

Yamazaki, S., Okada, K., Maruyama, A., Matsumoto, M., Yagita, H., and Seya, T. (2011). TLR2-dependent induction of IL-10 and Foxp3+ CD25+ CD4+ regulatory T cells prevents effective anti-tumor immunity induced by Pam2 lipopeptides in vivo. PLoS ONE *6*, e18833.

Yuan, F., Salehi, H.A., Boucher, Y., Vasthare, U.S., Tuma, R.F., and Jain, R.K. (1994). Vascular Permeability and Microcirculation of Gliomas and Mammary Carcinomas Transplanted in Rat and Mouse Cranial Windows. Cancer Res *54*, 4564–4568.

Zal, T., and Chodaczek, G. (2010). Intravital imaging of anti-tumor immune response and the tumor microenvironment. Semin Immunopathol *32*, 305–317.

Zeidman, I. (1961). The Fate of Circulating Tumor Cells I. Passage of Cells Through Capillaries. Cancer Res *21*, 38–39.

Zhang, L., Gurskaya, N.G., Merzlyak, E.M., Staroverov, D.B., Mudrik, N.N., Samarkina, O.N., Vinokurov, L.M., Lukyanov, S., and Lukyanov, K.A. (2007). Method for real-time monitoring of protein degradation at the single cell level. BioTechniques *42*, 446–450.

Zhou, Z.N., Boimel, P.J., and Segall, J.E. (2011). Tumor-stroma: In vivo assays and intravital imaging to study cell migration and metastasis. Drug Discov Today Dis Models *8*, 95–112.

Zomer, A., Beerling, E., Vlug, E.J., and van Rheenen, J. (2011). Real-time intravital imaging of cancer models. Clin Transl Oncol *13*, 848–854.