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Advanced intravital subcellular imaging reveals vital three-dimensional signalling events driving cancer cell behaviour and drug responses in live tissue

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The integration of signal transduction pathways plays a fundamental role in governing disease initiation, progression and outcome. It is therefore necessary to understand disease at the signalling level to enable effective treatment and to intervene in its progression. The recent extension of *in vitro* subcellular image-based analysis to live *in vivo* modelling of disease is providing a more complete picture of real-time, dynamic signalling processes or drug responses in live tissue. Intravital imaging offers alternative strategies for studying disease and embraces the biological complexities that govern disease progression. In the present review, we highlight how three-dimensional or live intravital imaging has uncovered novel insights into biological mechanisms or modes of drug action. Furthermore, we offer a prospective view of how imaging applications may be integrated further with the aim of understanding disease in a more physiological and functional manner within the framework of the drug discovery process.

Introduction

Advances in imaging technology to monitor events at the subcellular or molecular level *in vitro* have provided unprecedented insight into the spatiotemporal regulation of biological processes such as cell migration, proliferation, survival and development. Our understanding of the subcellular events that underpin

Abbreviations
2D, two-dimensional; 3D, three-dimensional; CAF, cancer-associated fibroblast; CFP, cyan fluorescent protein; ECM, extracellular matrix; FLIM, fluorescence lifetime imaging microscopy; FRAP, fluorescence recovery after photobleaching; FRET, fluorescence resonance energy transfer; GFP, green fluorescent protein; IFP, infra-red fluorescent protein; MMP, metalloproteinase; PA, photo-activation; PB, photobleaching; PS, photoswitching; RFP, red fluorescent protein; SHG, second harmonic generation; TAM, tumour-associated macrophage; TGF, transforming growth factor; YFP, yellow fluorescent protein.

these processes has been accelerated by the use of fluorescent proteins [e.g. green fluorescent protein (GFP) and its variants] to selectively tag and localize virtually any protein. This application has allowed us to track biological events in real-time as they occur within the cell [1–3].

Initially, either cytosolic or whole cell expression of fluorescent proteins was used passively to track cell movement [4–7]. This rapidly progressed to the use of fluorescent proteins fused to a protein of interest either directly or via linker sequences, which, when combined with long-term imaging, allowed for a more active role for this technology [8,9]. Concurrently, specialized techniques using fluorescence to study cellular dynamics at the molecular level have emerged to provide yet another level of subcellular detail. These include quantitative techniques such as fluorescent recovery after photobleaching (PB) (FRAP), photo-activation (PA)/photoswitching (PS) and the study of protein–protein or intramolecular interactions using fluorescence resonance energy transfer (FRET) [3,10]. Recently, these specialized and quantitative techniques have been applied in a live *in vivo* disease context and, as such, are bridging the gap between our understanding of subcellular processes *in vitro* and real-time disease settings *in vivo*.

The transition from *in vitro* to *in vivo* imaging of disease progression has primarily emerged as a result of an increasing appreciation of the role of the surrounding host tissue, which provides a micro-environmental niche vital for normal organ homeostasis or often for disease progression itself. Imaging disease processes in a more native, functional and physiological setting is therefore critical if we are to accurately understand disease aetiology and develop effective treatment strategies [11,12].

In the present review, we highlight insights gained from intravital imaging, ranging from whole body imaging to single cell and subcellular events. We summarize how complementary approaches that exploit the topography or autofluorescent properties of the surrounding tissue can be utilized to our advantage when imaging *in vivo*. We also highlight recent advances in three-dimensional (3D) intermediate imaging platforms and optical window technology for longitudinal studies, and draw on the potential to utilize versatile intravital imaging models as more accurate pre-clinical tools for drug discovery prior to clinical investigation.

Insights from whole cell imaging *in vivo*

Early intravital imaging studies used cells expressing fluorescent proteins such as GFP to tag the entire cell and monitor cell morphology changes, track cell movement and velocity, or gauge vector-based persistence over time (Fig. 1A). Interactions or movement within the extracellular matrix (ECM) using confocal reflectance microscopy, in which a signal is derived from laser light reflected by features of the sample, was used to visualize the ECM architecture and provide context for the cellular dynamics under observation [13–15]. Fluorescently-labelled circulating tumour cells also allowed factors that limit the assessment of colonization, extravasation or tumour dormancy. These models of 'late stage metastasis' predominantly involved the tail-vein, intracardiac or intrasplenic injection of fluorescently-labelled cancer cells to monitor the arrest of cells in different target tissue or organs [4,6,7,13,16].

More recently, overexpression of GFP or fluorescent proteins in cells and their subsequent injection has given way to the rapid expansion of genetically-engineered mice that recapitulate human diseases in terms of histopathology and disease profile, and with these mice being crossed with tissue-specific GFP mice. In this setting, the ability to image recombined GFP-expressing cells within the host tissue of animals that possess an intact immune system has lead to more realistic imaging of cell behaviour and fate *in vivo*. Initial work of this type involved tissue-specific expression of GFP in an MMTV-PyMT based model of breast cancer where multiple stages of local invasion could be imaged simultaneously [17]. More recently, the use of Pdx1-specific expression of GFP in pancreatic progenitor cells allowed for cellular outgrowth within the developing primary tumours to be imaged, demonstrating a direct role for the tumour suppressor p53 in overcoming Kras-induced growth arrest/senescence in pancreatic cancer [18]. Because this model replicated the disease progression from precursor lesions to adenocarcinoma, a whole body fluorescence imaging system was used to repeatedly and non-invasively report upon the initiation of primary tumour occurrence and metastatic progression within the same animal for up to 20 weeks (Fig. 2) [18,19]. Tissue-specific expression of GFP *in situ* has also facilitated the study of bladder and renal cancer, as well as rhabdomyosarcoma in a similar manner [20–22].

Similarly, this type of tissue-specific targeting has been applied to many other fundamental biological processes. For example, Thy1-driven GFP within the central nervous system has been used to investigate axonal regeneration in spinal cord injury [23], whereas time-lapse imaging of the spinal cord of shiverer (Mbp mutant) mice to assess glial–axonal interactions was achieved using GFP-labelled murine neurospheres

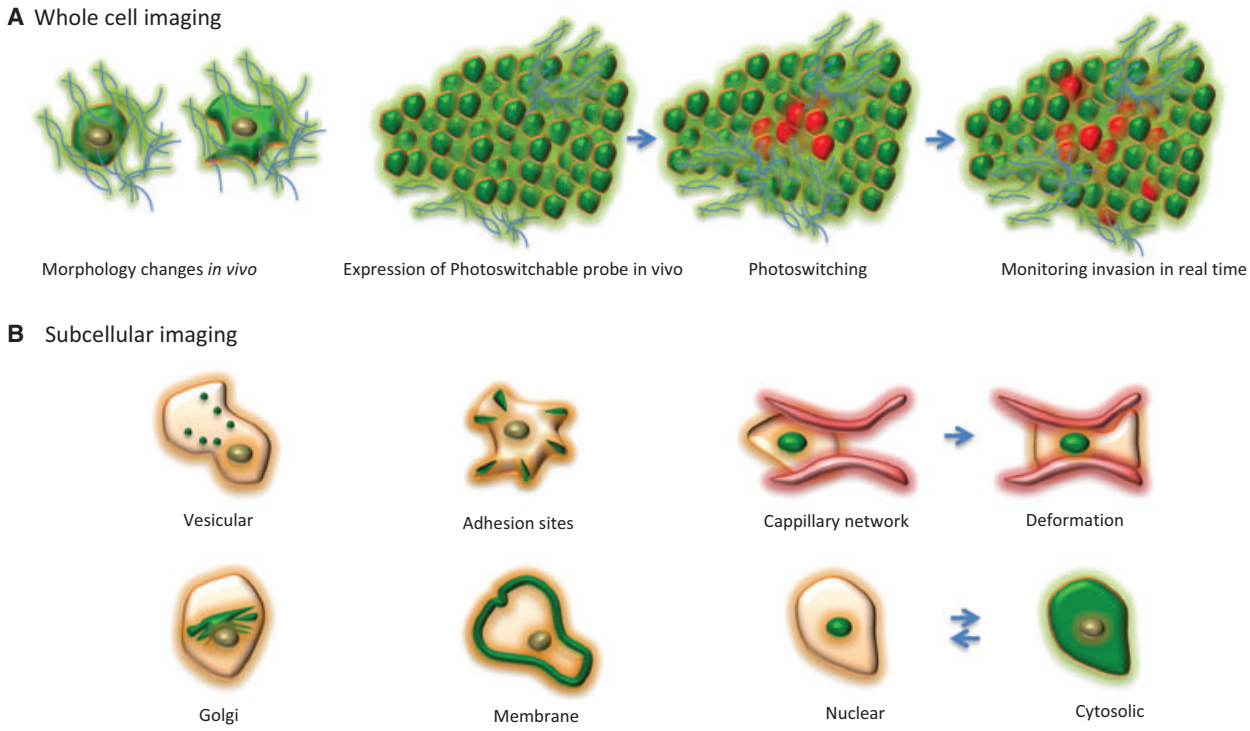

Fig. 1. (A) Schematic example of whole cell imaging to monitor cell morphology, speed or vector-based movement *in vivo* using PS fluorescent probes to track movement in real-time. (B) Schematic examples of subcellular imaging assessing parameters including (left to right), vesicular transport, adhesion dynamics, protrusion through microvasculature, Golgi localization during migration, plasma membrane localization or shuttling between cytosol and nuclei.

[24,25]. Live intravital whole cell imaging within lymphatic tissue *in situ* has been instrumental for assessing the cross-talk between components of the immune system upon activation of the immune cascade [26–28]. Tracking interactions between immune cells and tumour tissue *in vivo* has also highlighted the role that inflammation plays in the initiation, priming and progression of tumourigenesis, both in mammalian tissue and *Drosophila* [29–32]. GFP has also been used to target and identify Lgr5-positive stem cells in the intestine, colon, stomach and hair follicles [33–36], and has recently been used to reveal the role that reactive oxygen species production plays in Rac1-induced intestinal stem cell proliferation and colorectal cancer initiation [37]. Mammary gland development in the context of the Ets transcription factor ELF5 has also been assessed in longitudinal studies using this form of whole cell imaging [38]. Finally, responses to therapeutic or molecular intervention in both prostate and pancreatic cancer have been assessed using this approach, highlighting the potential early application of fluorescent intravital imaging models for the translational and pre-clinical drug discovery process [18,20,39,40].

Dual whole cell imaging *in vivo*

In cancer studies, whole cell imaging is not restricted to monitoring tumour cells alone, and tagging other cell types with another fluorophore has revealed a vital interdependence between cancer cells and the surrounding stromal environment [32,41,42]. For example, a symbiotic relation between squamous cell carcinoma cells and cancer-associated fibroblasts (CAFs) has been revealed, whereby CAFs are required to lead the way during collective invasion [15]. Here, 3D imaging revealed a protumourigenic role for fibroblast remodelling of the ECM to make tracks in the surrounding tissue, thereby creating a more permissive microenvironment for cell invasion [14,15,43].

Requirement for tumour-associated macrophages (TAMs) to be in close proximity to cancer cells for efficient metastasis to occur has also been demonstrated using whole cell imaging [44–47]. Macrophages constitute an important component of the tumour microenvironment and facilitate processes such as angiogenesis, tumour cell migration and ECM breakdown [47]. In murine mammary tumours, macrophages have been shown to be predominantly present at the

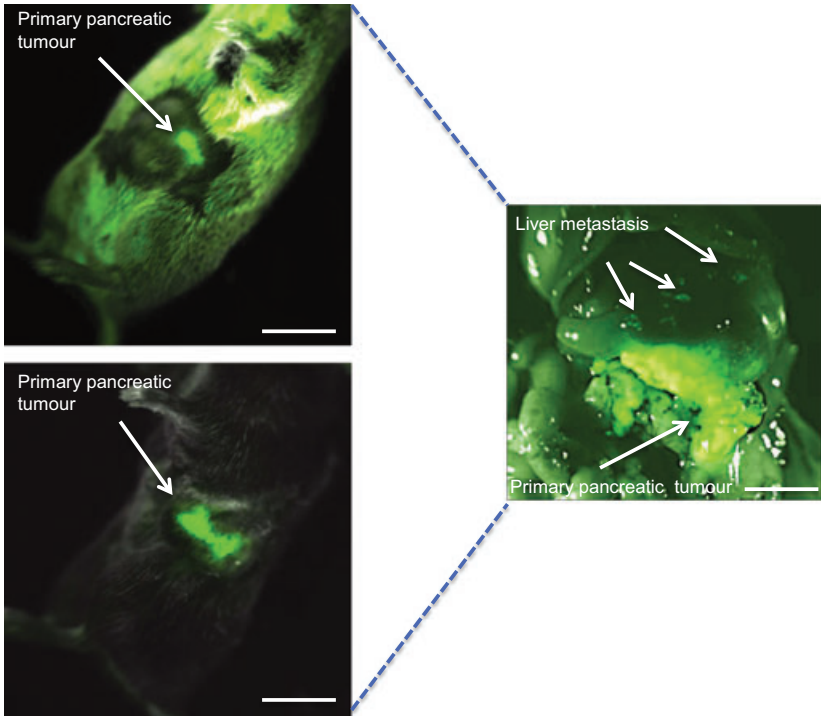

Fig. 2. Example of whole cell imaging at the whole body animal imaging level using genetically engineered GFP mice. Expression of GFP is driven by Pdx1-Cre in the pancreas to monitor disease progression from primary pancreatic tumour to micrometastasis in the liver. Scale bar = 10 mm. Green, GFP.

tumour margins and, to a lesser extent, deeper inside the tumours [46]. Moreover, in the PyMT model of breast cancer, they confer an invasive phenotype to carcinoma cells, allowing for increased motility, polarization and invasion towards local vasculature [29,45,46,48,49]. This, along with TAMs mediating an angiogenic switch in tumours, highlights the interdependent role of tumour cells and the surrounding tissue types at late and early stages of the metastatic cascade [45,49]. Dual imaging of cancer cells and associated pancreatic stromal stellate cells using whole cell imaging has also recently been shown to significantly improve the efficiency of pancreatic cancer cell metastasis when accompanied by stellate fibroblasts [50]. Finally, co-culturing cells and identifying cells that have undergone molecular manipulation by tagging them with different coloured fluorophores has revealed the existing hierarchy during complex processes such as tumour cell invasion [51,52]. In this way, cells that overexpressed Lim kinase or neural Wiskott–Aldrich syndrome protein have been shown to lead the way during 3D invasion when mixed with wild-type, control cells of a different colour [51,52].

In a recent elegant study, Cre-mediated recombination in the intestine was employed using brainbow technology inserted into the Rosa26 locus [25] to generate cells expressing either GFP, yellow fluorescent protein (YFP), red fluorescent protein (RFP) or cyan fluorescent protein (CFP) to allow discrimination between clonal progeny within the intestine [53]. Using this technique, lineage tracing and the symmetry of stem cell division could be examined for the first time *in vivo* [53]. The use of complementary approaches to track cell lineage was also employed using 3D duodenal crypt culture explants or organoids supplemented with Wnt-ligand and essential growth factors [53–55]. This allowed the 3D manipulations or examination of stem cell tracking in a semi-*in vivo* environment prior to fullscale *in vivo* exploration of this biological process [54]. Simultaneous labelling of cells with different colours and tracking their fate has recently been adopted for the first time to monitor this process in real-time both in the intestinal stem cell niche, using new abdominal intravital windows [56,57], as well as to study cancer stem cell plasticity in mammary tumours, using mammary optical windows [58]. It is therefore clear that whole cell intravital imaging is continuing to provide significant insight into complex biological processes in live tissue, especially when used in conjunction with real-time imaging approaches such as optical window technology.

Insights from subcellular imaging *in vivo*

Early subcellular intravital work involved dual-labelling of different compartments within the same cell (e.g. nucleus versus cytoplasm) and revealed new layers

of detail regarding factors that determine cancer cell behaviour *in vivo* (Fig. 1B). For example, dual-labelling of the nucleus and cytoplasm with different fluorophores demonstrated that the diameter of the nucleus governs whether a cell can squeeze or negotiate its way through microvessels present in small capillary networks in secondary tissue sites. Cells were shown to deform their cytoplasm during this process; however, remodelling the shape or size of the nucleus was the limiting factor controlling cancer cell trapping and dissemination in various organs [4,59–63].

Other studies have lead to similar limitations being observed during tumour invasion through dense extracellular matrices, and have highlighted the inherent plasticity of tumour cells during 3D invasion. When presented with structural and physical factors that do not permit passive cell movement within tissue, tumour cells adopt different modes of invasion to overcome these obstacles [64–67]. As a result, tumour invasion has now been classified into distinct modes of movement, including amoeboid, mesenchymal or collective invasion. Amoeboid movement is characterized as single cell spheroid locomotion, whereas mesenchymal motility involves spindle-like, elongated and metalloproteinase (MMP)-mediated remodelling of the surrounding ECM to facilitate further movement. Collective invasion involves a multicellular sheet-like movement of a number of cells and involves both proteolytic-dependent and independent mechanisms, as described above [68]. Importantly, the aptitude of tumour cells to switch between distinct modes of invasion enables tumour cells to rapidly conform to their surrounding environment. This malleable capacity of cells to change shape during invasion would not have been observed if studied using two-dimensional (2D) cell culture as a surrogate model for cell movement within live tissue [3].

Adjustment to different modes of invasion upon molecular or therapeutic intervention may provide an alternate mechanism for invading tumour cell resistance to various drug treatment strategies, such as MMP-targeted therapies [69]. In line with this, intravital subcellular monitoring using GFP-tagged proteins during breast cancer motility has been performed using a Smad2-GFP fusion protein that shuttles from the cytoplasm to the nucleus upon tranforming growth factor (TGF)β signalling. Real-time imaging revealed TGFβ signalling activity and Smad2-GFP translocation to the nucleus during single cell movement, whereas Smad2-GFP remained in the cytoplasm in non-motile or collectively moving cells [70]. This implies that transient spatial TGFβ signalling is an important determinant of tumour motility and may regulate the mode and efficiency of cell invasion *in vivo*. As described for MMP targeting above, this work has implications regarding therapeutic targeting and resistance to TGFβ in invasive cancer [69,70].

Assessment of other subcellular events during cell invasion *in vivo* such as myosin light chain dynamics or actin cytoskeletal remodelling using 'Lifeact' have provided further insight into *in situ* biological processes [65,71–73]. In a recent study, intravital imaging was employed to visualize secretory granule exocytosis and fusion events in live salivary gland [74]. Adrenergic signalling was shown to be sufficient for eliciting a maximal exocytosis response independent of muscarinic signalling, as previously reported. In addition, it was found that the contractile actomyosin coat is recruited to the granules post-fusion and is critical in controlling the delivery phase of granule exocytosis in live tissue [74]. Similarly, using multiphoton-based intravital microscopy, endocytosis of fluorescently-labelled dextrans was used to monitor the dynamics of endosomal-lysosomal trafficking events *in vivo* [75]. High-resolution, *in vivo* imaging allowed the overall uptake kinetics, cargo progression and fusion events between distinct compartments to be monitored in live tissue [75]. Such subcellular transient events would be difficult to establish from 'snap shot' or whole cell imaging, which can often generate contradictory hypotheses in the absence of this spatiotemporal information. This type of analysis suggests that we are at the beginning of a new phase of *in vivo* imaging, in which subcellular spatial resolution can be realised to help decipher biology at the molecular level in terms of 'when and where' events are taking place. This detail then allows us to correlate the phenotypic/biological consequences associated with these events in an environment that more faithfully recapitulates the disease in question, thereby facilitating drug targeting studies.

In a similar manner to tumour cell remodelling, the dynamic rearrangement of the ECM has also been explored *in vivo* using second harmonic generation (SHG) imaging of fibrillar structures such as collagen fibres. Using SHG imaging, distinct tumour-associated collagen signatures have been identified in intact tumours tissue [76]. Furthermore, the orientation or integrity of ECM fibres was shown to impact upon the ability of cancer cells to migrate in this setting, thereby revealing a clear plasticity and cross-talk between tumour cells and the surrounding environment [77]. In line with this, recent work by Weigelin and Friedl [78] demonstrated that SHG and third harmonic generation imaging of the tumour landscape allowed other components of the tumour microenvironment to be

visible, such as erythrocyte flow inside vessels or the presence of adipocyte boundaries [78]. This label-free subcellular imaging also allowed the detection of tissue space and tracks to be observed *in vivo* that cancer cells could potentially use for rapid invasion [78]. This form of topological imaging takes advantage of the subcellular fluid phase properties of different cell types *in vivo*, and could also be used in the future to visualize cancer cell migration in a more physiologically relevant context in mammalian tissue using label-free methods of detection, as described previously for zebrafish [79].

Insights from advanced molecular dynamic techniques *in vivo*

A number of techniques have been developed that assist the analysis of dynamic molecular processes *in vitro* and have recently been adapted for live applications. These involve techniques such as FRAP to monitor cell–cell or single cell adhesion dynamics, PA/PS to track the movement of tumour subpopulations *in vivo* and FRET to monitor intra/intermolecular interactions in live tissue [80]. Advanced imaging modes such as fluorescent lifetime imaging microscopy (FLIM) or anisotropy are being used more readily to image events at fast acquisition rates with the aim of applying this detailed quantitative analysis to the drug discovery field. Here, we draw upon some of these recent applications *in vivo* and highlight their advantage and potential use in translational research.

FRAP involves live cell imaging of a fluorescently-tagged protein within a cell in which a defined region of interest is subjected to PB and the recovery of fluorescence in the bleached area is subsequently examined. The level and rate of recovery, referred to as the mobile fraction and recovery rate, respectively, allow the relative levels of mobile to immobile fraction of the tagged protein to be calculated, at the same time as providing real-time information on the speed at which the process occurs (Fig. 3A, B) [10,80]. The use of FRAP to assess the dynamics of

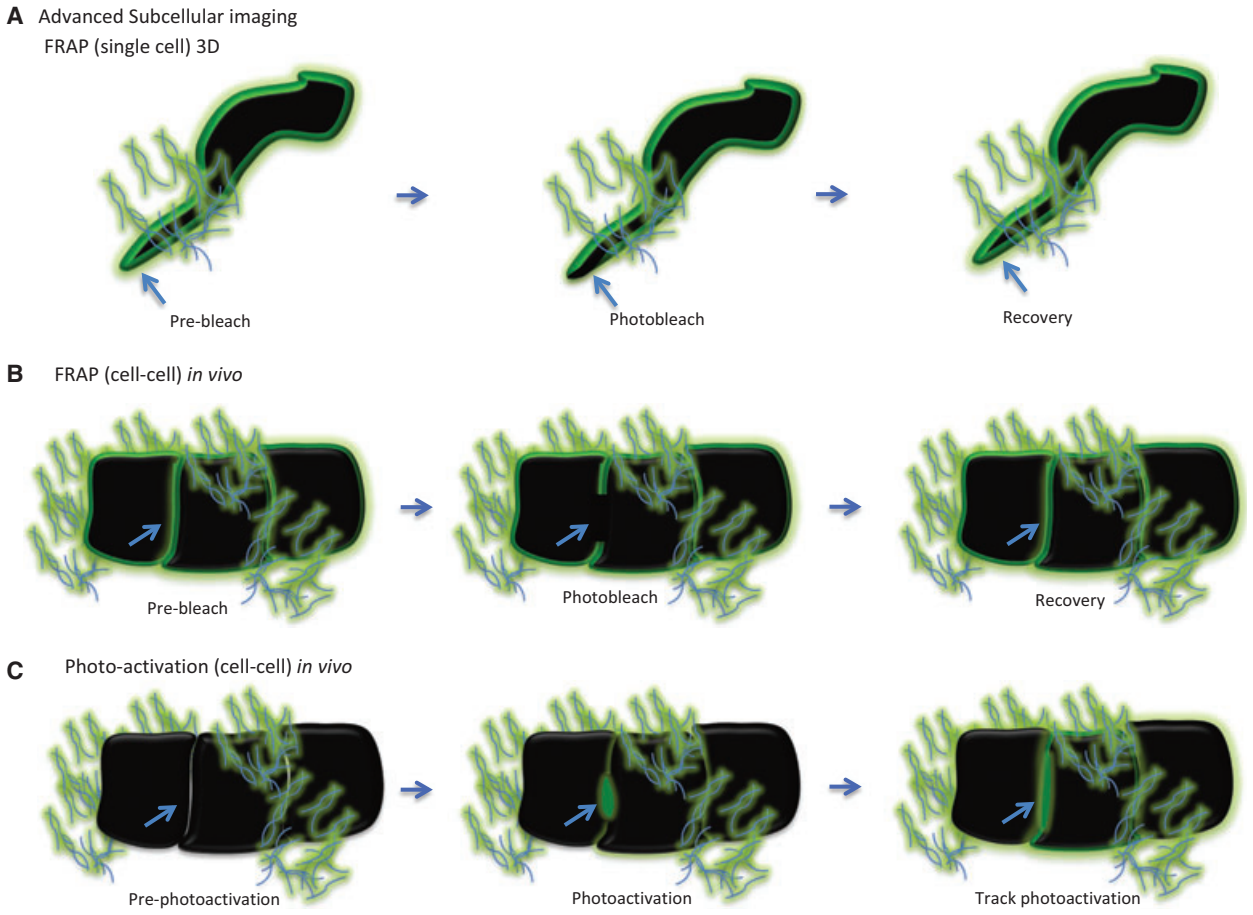

Fig. 3. Schematic examples of advanced subcellular imaging techniques including single and dual cell FRAP or subcellular PA.

fluorescently-tagged proteins at a subcellular level *in vitro* has been extensive; however, quantitative investigations in three-dimensions or *in vivo* are only beginning to emerge [10,81]. For example, recent work using 3D FRAP has revealed the importance of subcellular trapping of metalloproteinases at leading protrusions during invasion to efficiently guide and direct matrix remodelling during tumour cell invasion [52]. FRAP of fluorescently-tagged MT1-MMP within invasive breast cancer pseudopods showed that neural Wiskott–Aldrich syndrome protein is required to deliver F-actin to pseudopods and to capture or stabilize MT1-MMP spatially at the protrusive tips. Moreover, this coupling of MT1-MMP at protrusive edges correlated with the disruption and breakdown of dense ECM components and enhanced 3D invasion. This work highlights the insights that can be gained from performing imaging such as FRAP in a 3D background [52].

In line with single cell-based FRAP analysis, FRAP of molecules involved in a multicellular context such as cell–cell interactions governed by cadherins has also been achieved in *Drosophila* and, more recently, in mammalian tumours (Fig. 3B) [82,83]. E-cadherin junctions play a prominent role in governing early remodelling stages of epithelial to mesenchymal transition and their deregulation can lead to the destruction of the epithelial architecture preceding intravasation from primary tumours [84]. In *Drosophila*, FRAP of E-cadherin-GFP has been utilized to address the cross-talk between E-cadherin and the actin cytoskeleton, where independent subpopulations of actin pools were shown to drive lateral cell movement or clustering of E-cadherin to maintain tissue architecture [82]. Similarly, we recently demonstrated that the complex dynamics of E-cadherin can also be assessed in an *in vivo* mouse model of cancer using FRAP of E-cadherin-GFP [81,83]. Initially, we showed that the dynamics of E-cadherin are significantly different when cells are plated on intermediate 3D substrates that allow integrin engagement, such as cell-derived matrix compared to *in vitro* culture. Moreover, we revealed that E-cadherin dynamics are also significantly faster *in vivo* than *in vitro*, and that the recovery of E-cadherin is associated with the migration rate of cells at the edge of the tumour subpopulation [83]. Importantly, the efficacy of the clinically approved Src inhibitor, dasatinib, to impair the turnover of cell–cell adhesions and tumour breakdown was also shown to be significantly greater *in vivo* than *in vitro* [83,85].

Quantitative imaging of molecular dynamics *in vivo* therefore highlights important limitations of 2D modelling as a substitute for living tissue and suggests that caution should be taken when extrapolating data from *in vitro* experiments to the *in vivo* setting, especially in a clinical context [86]. The use of intermediate systems such as cell-derived matrix or organotypic assays allows the engagement of cells in a 3D setting and offers flexibility and imaging control not always available *in vivo*. Moreover, they can span the common ground between the biological effects *in vitro* that may be relevant in live tissue (a detailed description of advantages and disadvantages of intermediate imaging platform, is provided elsewhere [3]).

In a similar way to FRAP, photo-activatable GFP-tagged proteins have considerably improved the quantification of molecular dynamics *in vitro* and are gradually being applied in 3D and *in vivo* studies. PA is essentially the inverse of FRAP, whereby PA-GFP is activated by a concentrated beam of 405 nm excitation light. This allows whole cells or certain compartments/subcellular pools of PA-GFP-tagged protein to be spatially and temporally activated and traced over time, provided that a counterstain of a different colour is used to classify the region or compartment of interest prior to activation (Fig. 3C). In this way, integrin internalization and recycling to the plasma membrane [87–89], the dynamics of focal adhesion/ERM proteins [90] and the dynamics of the plasma membrane itself have been studied on various 3D substrates [91]. Accordingly, the first application of PA at the subcellular level *in vivo* has been performed to track membrane dynamics using photo-activatable GFP linked to the plasma membrane targeting sequence of H-Ras [83]. Importantly, the dynamics of the plasma membrane, which plays a vital role in signal transduction events and key biological processes such as vesicle trafficking, are significantly less mobile in live animals compared to parallel *in vitro* studies [83]. This work shows that a lack of continuity can exist between 2D and 3D systems, which represents a key obstacle that must be overcome if we are to effectively translate molecular findings from bench-to-bedside in the future [86].

Along similar lines to PA, PS variants of GFP such as Dendra have been derived to overcome the need for a counterstain during photo-activation studies. In the case of Dendra-2, 405 nm excitation of the fluorescent moiety induces an irreversible shift of fluorescence towards the red spectrum. This allows the protein of interest to be visible in the green channel prior to conversion, and thus its specific subcellular localization can be tracked before and after switching in a time dependent manner *in vitro* [10,92].

As shown in Fig. 1(A), PS and the subsequent monitoring of pre-selected tumour subpopulations have

been applied to track cell motility and invasion *in vivo* over long time periods [93]. This work demonstrated for the first time the potential application of PS for longitudinal drug discovery studies and, as such, has recently been applied to monitor the outcome of pharmacological targeting of Src or focal adhesion kinase in live tumour tissue [94]. Using PS *in vivo*, perturbation of integrin signalling by dasatinib or the focal adhesion kinase inhibitor (PF-562, 271), an indirect cross-talk between integrin signalling and cell–cell junction stability was observed, in which integrin signalling controlled E-cadherin endocytosis and internalization [94]. This was subsequently shown to govern cell–cell adhesion strength *in vivo* and regulated collective cell movement in an invasive model of cancer. Intravital imaging in a more functional and physiological context using advanced techniques are therefore demonstrating subtle yet important outcomes of drug targeting on modes of invasion or cell behaviour *in vivo* that would otherwise be missed using *in vitro* platforms [3].

We are now entering a new phase of optical imaging, whereby the properties of photoreactive proteins can be exploited to directly manipulate protein activity itself. Recently, Wu *et al.* [95] fused a constitutively active mutant of Rac1 to the photoreactive domain of phototropin, causing sterical hindrance and impairing its interactions with downstream effectors [95]. Unwinding of the photoreactive domain of phototropin and uncaging of the fused protein, leading to Rac1 activation, could then be controlled is a spatiotemporal manner using 458 nm light. In this way, the subcellular activity of Rac1 was precisely manipulated to control fibroblast protrusion and migration *in vitro* [95,96]. As with other advanced techniques, light-mediated guidance has been extended to an *in vivo* setting and has recently been used in *Drosophila* to demonstrate light-dependent regulation and control of Rac1 during border cell migration [97]. This work has revealed a cross-talk between collective groups of cells during 3D coordinated movement of epithelia, with important implications on the migration of cells during metastasis, development and morphogenesis [97]. Similarly, photoreactive Rac1 was also used to assess the consequence of posterior/anterior signalling event during cell polarization in live zebrafish [98]. Light-assisted inactivation has also been utilized to assess the effect of spatial or temporal inactivation of adhesion proteins during cytoskeletal remodelling and migration [99]. Light-assisted manipulation of protein activity is therefore an ideal companion technology for leveraging additional value from genome-wide small-intefering RNA screens or large-scale quantitative proteomic or next generation sequencing studies [100,101]. The application of spatiotemporal light-mediated protein activation/inactivation technology in live mammalian tissue will therefore be an area of great interest in the future with regard to subcellular drug targeting studies.

Finally, the application of FRET for *in vitro* studies has significantly improved our understanding of 'when and where' molecular interactions or events take place in cells and is emerging as a useful technique for *in vivo* assessment of disease progression and the drug discovery process [102]. FRET is a nonradiative process involving the transfer of energy from a donor fluorophore to an acceptor fluorophore when in close proximity to each other. Ratiometric FRET analysis involves measuring the associated gain or loss of fluorescence emission of the acceptor upon specific excitation of the donor fluorophore. Recently, however, for fast or more complex FRET experiments, a change in the donor fluorescent decay rate is used to measure FRET and is referred to as FLIM. FLIM has become the method of choice in this setting because it is insensitive to artefacts caused by variations in fluorophore expression level and intensity, and is less sensitive to autofluorescence, both of which can interfere with ratiometric measurements in live tissue. Furthermore, individual fluorophores have unique decay characteristics and can be distinguished when multiple fluorophores are used within the same cell during multiplex analysis.

FRET or FLIM analysis has initially been used *in vitro* to monitor a number of distinct molecular processes, such as the formation of protein complexes, protein cleavage events, or to measure intracellular signalling events [102]. The design of intramolecular biosensors has also allowed kinase, metalloproteinase or RhoGTPase/adhesion activities to be monitored in real-time in response to external mechanical stimuli [103–106]. This technology is now being applied in a format applicable to the drug discovery process using time correlated single photon counting or frequency domain based-FLIM to generate high-content, rapid readouts of signalling events to drug screening approaches [107,108]. Moreover, Bakker *et al.* [109] have recently applied high-speed FLIM technology to 3D organotypic applications, thereby potentially improving the contextual output of FLIM-based screens [110]. New techniques that measure and take advantage of the rotation or change in acceptor-anisotropy of fluorescent probes during FRET are also emerging as alternative methods for rapid, high-resolution, high-content assessment of FRET [111]. Fluorescence anisotropy can be used to measure the rotation

of a given molecule by analyzing the polarization angle of the fluorophores emission. In the case of FRET, if an acceptor fluorophore is in close proximity to its donor (i.e. during FRET), it 'tumbles' less than if no FRET was occurring. In this recent study, Matthews *et al.* [111] take advantage of this and describe a prototypic screen that uses fluorescence anisotropy to read out FRET in response to drug intervention. It is shown that fluorescence anisotropy can be used to measure protein–protein interaction during hetero-FRET and that this can provide faster and higher-resolution readouts of FRET activity ideally suited to high content drug screening [111].

With regard to *in vivo* imaging, the decay rate of flurophores or autofluorescence of the surrounding environmental structures can change during physiological events, thereby providing a label-free readout of the tumour landscape when an assessment of the protein interaction of interest is taking place. For example, the intrinsic lifetime of ECM components or the metabolic activity within a spatially distinct microenvironmental niche in tumours can be assessed in this manner. Lifetime imaging has been used in this way to assess NADH status or redox potential in tumour tissue [37,112,113]. Moreover, it has revealed distinct autofluorescence differences between normal and malignant tumour tissue in both breast and colon cancers, applicable to human tissue microarray studies [114,115]. Similarly, local information on tumour activity at invasive borders versus the tumour core or necrotic regions has been revealed with this technique [116].

We and others have used FLIM/FRET to monitor protein interactions or activity during disease progression in live tissue and used this information to gauge efficacy of drug targeting in the context of disease. Initial work of this type monitored the interaction of protein kinase C α and the pro-inflammatory chemokine receptor CXCR4 in xenograft tumour tissue [117]. Here, differences in receptor binding were revealed when assessing tumour subpopulations from the surface to the interior of live tissue [117]. Similarly, single cell-based assessment of caspase-3 using FLIM-FRET within tumour subpopulations has been examined in response to chemotherapy, allowing drug resistant populations to be assessed *in vivo* [118].

More recently, we used a Rho-FRET biosensor in a live tumour model of pancreatic cancer to monitor subcellular signalling events during pancreatic tumour cell invasion [119]. We demonstrated that subcellular coordinated RhoA activity at the poles of cells drives invasion in a 3D context. We also revealed that this polarized activity of RhoA exists *in vivo* and that inhibition of RhoA at the tips of invading cells is sufficient to impair invasion, independent of its activity within the cell body (Fig. 4) [119]. Moreover, feedback cues from the surrounding tumour environment were shown to indirectly affect subcellular signalling event *in vivo* because RhoA activity was significantly enhanced within cells in close proximity to the tumour blood supply compared to cells distal to vasculature [120].

In parallel investigations using the Rho FRET-biosensor, other studies have revealed that coordinated RhoA signalling at the leading edge is required for efficient migration and invasion in other complex microenvironments [121,122], suggesting that application of FLIM/FRET imaging in three dimensions can reveal common mechanisms across a spectrum of disease states. In line with this, FRET analysis of the RhoGTPases Rac1 and RhoA in other organisms such as zebrafish has shown a similar polarized coordination during germ cell migration and embryonic development, emphasizing the broader applications of real-time *in vivo* FRET across species [123]. Lastly, Matsuda and colleagues [104] originally developed the Raichu probes for imaging Rho-family GTPases and subsequently used *in vivo* FLIM-FRET to report on differential activation of Rac1 and Cdc42 in a mouse model of glioma [124] and have recently incorporated this technology to create genetically encoded FRET reporter mice [125]. In combination with implantable imaging windows, or crossed with genetic models of human disease, this approach will enable more sophisticated investigation of cell behaviour in the organ or native tumour microenvironment of interest to be performed.

Advances in probe design and applications *in vivo*

Adaptations to current fluorescent proteins and the development or use of injectable quenched or unquenched probes are rapidly emerging to facilitate *in vivo* imaging. The penetration depth of fluorescence microscopy is limited by the scattering of excitation and emission light by tissue [126]. Scattering primarily occurs as a result of changes in the refractive index associated with compartmental boundaries within cells and tissue, including cell nuclei, membrane compartments, blood vasculature and ECM components. Scattering is wavelength-dependent, and occurs to a greater extent at shorter wavelengths, especially in the blue spectrum. Red fluorescent proteins are therefore superior for *in vivo* imaging because they allow signals to be imaged from deeper within tissue [3]. Red probes

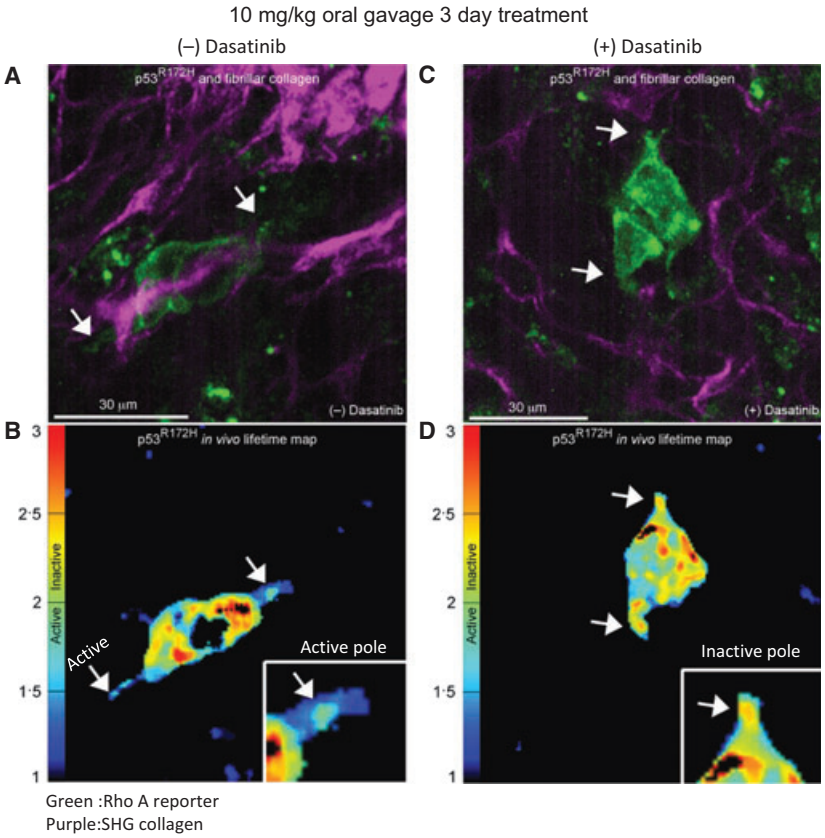

Fig. 4. Example of advanced subcellular imaging using FLIM-FRET technology *in vivo*. The subcellular signalling dynamics of RhoA were assessed in live pancreatic tumour tissue in the presence or absence of the anti-invasive drug, dasatinib, and revealed subtle changes in RhoA signalling that govern cell invasive efficiencies. Reproduced with permission [119]. Green, pancreatic tumour cells expression Rho Rachui probe; purple, SHG imaging of collagen/ECM components (top panels). With accompanying FLIM-based lifetime heat maps (lower panels), where yellow/red represents inactive regions and blue/green represents active regions.

also have the advantage of avoiding the high background associated with tissue autofluorescence, which is typically stronger in the blue and green spectrum.

Red probes such as tdTomato (an obligate dimer, well suited to cytoplasmic labelling of whole cells) and its derivative mCherry [127] along with improvements in monomeric RFPs, orange fluorescent proteins and YFPs derived from *Discosoma* sp, red fluorescent protein have been commonly used in conjunction with GFP for two-colour imaging *in vitro* [127]. Although these probes have also been used for *in vivo* imaging, their emission peaks (581 nm and 610 nm, respectively) are below the optimal spectral window of 700–900 nm, in which the absorbance and autofluorescence of tissue are minimized. Mutagenic strategies have therefore been used to stretch the emission range of fluorescent proteins derived from the sea anemone to above 600 nm, including mKate (emmission = 635 nm) [128], Neptune (emmission = 650 nm) [129] and eqFP670 (emmission = 670 nm) [130]. An interesting variant in this regard, is Long Stokes Shift mKate1, which has an excitation of 463 nm but emits at 624 nm. This means that Long Stokes Shift mKate 1 can be excited in the tuning range of a standard titanium-sapphire laser (< 1080 nm), and can be excited at the same wavelength as GFP for two-colour imaging [131].

Infra-red fluorescent protein (IFP) is a new type of fluorophore designed to overcome the emission limitations inherent to β-barrel-based fluorescent proteins [132]. IFP is based on phytochrome photoreceptors found in bacteria [133] and emits at 708 nm. Rather than an entire fluorophore, IFP only encodes a peptide backbone, which can bind the metabolite biliverdin that can either be scavenged from the cytoplasm or added exogenously. Recently, Filonov *et al.* [134] reported an IFP variant called iRFP, which is brighter than the original and overcomes the need for addition of biliverdin through higher affinity. Intense optimization of fluorescent proteins for FLIM have also recently resulted in an optimized variant of CFP called mTurquoise, which has an unusually long monoexponential lifetime (3.8 ns) and 93% quantum efficiency [135]. Adapting FRET biosensors to incorporate the use of red-far-red FRET pairs may also reduce light scattering, autofluorescence and absorption by tissue found at lower wavelengths and should provide a powerful tool for directly observing fundamental events at even greater depth in intact native tissue than can currently be

achieved using fluorescent lifetime of low wavelength biosensors such as CFP-YFP or GFP-RFP variants [120]. Similarly, a number of variants of PS probe are continuously being adapted for different spectral requirements [136,137].

Many useful dyes can also be injected directly into the bloodstream to label relevant structures in tissue. The simplest example of this is the use of passive fluorescent dyes, such as fluorescently-labelled dextran or quantum dots, as blood volume markers for imaging the vasculature (Fig. 5) [75,120]. Hoechst can also be injected intravenously, and will pass through the vascular endothelium to label nuclei in many tissue types, including the intestinal crypt [138]. Some injectable dyes provide functional information beyond simple localization. For example, probes have been made that unquench (i.e. become fluorescent) when they are cleaved by specific enzymes. Using this approach, probes have been designed to detect apoptosis [139], the activity of matrix metallopeptidases [140] or cathepsins [141] in live tissue.

Finally, important features of the tumour microenvironment, including pH, reactive oxygen species and partial oxygen pressure, can be measured *in vivo* and can be used to read out key biological processes that can drive disease. Many fluorophores are sensitive to pH, including fluorescein and several variants of GFP [142]. Linkage of the autophagosomal marker LC3 with GFP (which is pH sensitive) and mRFP (which is not) has been used to track fusion of autophagosomes with acidic lysosomes [143]. This approach could be modified to identify cells in acidic tumour environments, which has recently been shown to drive invasion *in vivo* [144]. Moreover, an LC3 GFP-RFP based transgenic mouse has been generated that could be crossed with various tumour models to provide a readout of autophagy in context of the local tumour niche [145]. The GFP variant HyPer has also been engineered to report on hydrogen peroxide using ratiometric imaging [146]. We have recently found that the fluorescence lifetime of this probe is sensitive to hydrogen peroxide, meaning that HyPer is also a potentially suitable probe for monitoring hypoxia using *in vivo* FLIM (E. McGhee, P. Timpson and K. Anderson, unpublished observations). Lastly, several approaches exist for imaging oxygen levels in complex *in vivo* environments, including porphoryn-based probes [147] and polymer nanoparticles [148], which have recently been shown to be sensitive enough to measure transient decreases in pO2 between individual red blood cells traversing capillaries [149]. The application of these probes to the study of cancer in areas of hypoxia, which is known to drive invasion, will help to clarify how changes in the local tumour niche can influence disease initiation, progression and the response to therapy.

Advances in longitudinal studies using intravital imaging window technology

The techniques described above, including whole, single or subcellular imaging, have provided a significant insight into our understanding of cell behaviour *in vivo*. The long time scale and sporadic nature of cell behaviour during disease progression or upon drug treatment, however, cannot always be captured using standard skin flap or terminally anaethetized *in vivo* disease models because they typically only allow imaging ranging from 4 to 24 h. New methods are therefore required to allow us to monitor biology as it takes place over longer time scales.

The use of observation windows facilitates such longitudinal imaging studies and is revealing biological consequences or responses to drug treatment that occur on a time scale ranging from days to months *in vivo*. The application of dorsal skin fold chambers

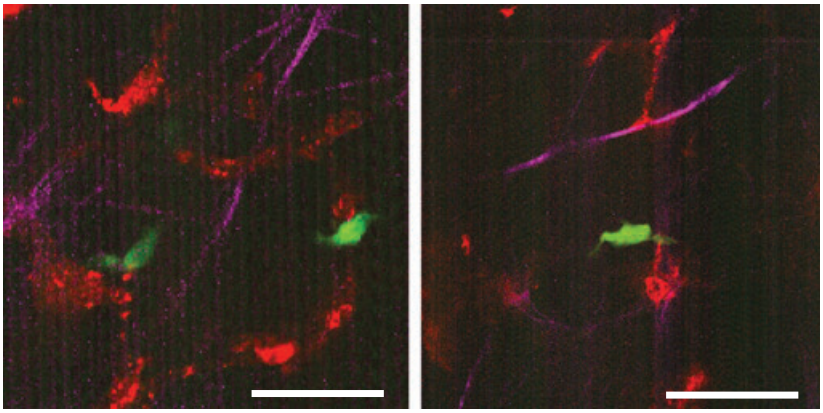

Fig. 5. Example of whole cell imaging in the context of host tissue vasculature using quantum dots as a contrast agent. Scale bar = 50 μm, Green, invasive pancreatic tumour cells; red, Qdot 655 (Molecular Probes, Carlsbad, CA, USA), marking blood supply; purple, SHG imaging of collagen/ECM components.

in which cells can be established between glass and the underlying tissue has initially helped in this respect [150]. By applying controlled anaesthesia and surgically implanting observation windows, repeated non-invasive real-time imaging allows cell behaviour to be monitored over longer time scales [150,151]. Moreover, observation windows have facilitated therapeutic intervention studies in many tumour types and SHG imaging has also been used to monitor the role that ECM integrity plays in limiting drug delivery to the tissue of interest [140].

Cranial or spinal imaging chambers have been employed to image biology for long time periods during brain metastasis or spinal injury [152,153], whereas cell dynamics in subcutaneous and breast cancer tissue have been examined using mammary intravital imaging windows [93]. Dendra-2 and PS technology was used to track subpopulations of orthotopically-injected tumour cells for up to 3 weeks in breast tissue in the context of the host tumour landscape and revealed that cancer cell motility was enhanced in vascular environments containing TAMs compared to avascular regions [93]. Recently, in a model of collectively invasive cancer, a similar optical windows approach was used in conjunction with PB, PA or PS to quantitatively read out changes in cell–cell or cell–ECM adhesion dynamics in response to distinct anti-metastatic treatments targeting integrin signalling [94]. Long-term chronic imaging revealed a subcellular regulation between integrin signalling and E-cadherin dynamics, which impacted upon tumour susceptibility to anti-invasive drugs [85,94].

A number of organs remain anatomically inaccessible to imaging with current optical windows, including the liver, intestine, spleen, lung, pancreas and kidney, and therefore require the mouse to be surgically opened to expose the organ of interest for short-term, high-resolution, imaging purposes (Fig. 2) [154]. Long-term, high-resolution imaging in these vital organs to monitor cancer initiation and progression has been a key challenge in the field. Moreover, these organs are common sites of secondary metastasis and therefore the long-term dynamic processes of early tumour colonization remain relatively unknown. In this regard, Ritsma *et al.* [56,57] have developed an abdominal intravital imaging window to overcome this limitation, which involves a titanium window being surgically secured within the abdominal wall, allowing imaging of single-cell invasion events within abdominal organs to be monitored for up to 5 weeks [56,57]. Here, titanium was used as a light, inert and corrosion-resistant alloy to minimize inflammation and any problems associated with the surgical implantation of foreign bodies in animal tissue. Biological processes could be repetitively monitored over long time courses in the liver, spleen, pancreas, intestine and kidney, revealing subtle changes in cell behaviour that would otherwise have been missed using short-term imaging. Similarly, repetitive long-term assessment of islet transplantation in the pancreas or intestinal stem cell plasticity, using simultaneous labelling of cells with different colours and tracking their fate, was achieved for the first time using such an approach [58].

Hepatic colonization of colon cancer cells was also studied using abdominal windows and, importantly, a 'pre-metastatic' migratory state was identified, which facilitates early 'homing' or colonization of cancer cells in the liver. This was subsequently followed by a 'micrometastatic' state in which migration levels or homing behaviour subsided after an appropriate niche was found in the secondary tissue. This early homing phenomena of recently extravasated cancer cells to find an appropriate environment for outgrowth was also shown to be a susceptible to early anti-migratory drug treatment, reducing the formation of subsequent micrometastasis [56]. Such work therefore pinpoints a potential achilles' heel in the early stages of tumour metastasis for future pharmacological intervention [56]. Improved titanium mammary windows were also recently used in conjunction with simultaneous cell tagging to monitor stem cell plasticity and fate over long time scales at the adenoma or carcinoma stage of breast cancer progression [58], which could otherwise not have been achieved using short-term *in vivo* imaging studies. Finally, combining imaging techniques with longitudinal studies using imaging windows reduces the number of animals needed for pre-clinical studies and may help in the drug discovery process by allowing us to image the efficacy of therapeutic interventions in a non-invasive repetitive manner within the same animal.

Advancing drug discovery through intravital imaging

High attrition rates of promising candidate drugs during clinical development as a result of lack of efficacy or unanticipated toxicities present major challenges to the translation of the drug discovery process [155,156]. This threatens the ability and motivation of pharmaceutical and biotechnology companies to invent truly novel medicines against an unmet clinical need in favour of lower-risk strategies that develop 'follow-on' drugs against pre-validated drug target classes. Conventional target-directed strategies poorly predict clinical outcomes and a reduction in pursuing alternative

drug discovery approaches has resulted in many academic and industrial drug discovery organizations pursuing the same (and limited) drug target space using standard pre-clinical models that poorly predict efficacy [157]. Thus, alternative drug discovery paradigms and technologies that support the development of novel therapeutic classes with increased predictivity of clinical success are urgently required.

The application of intravital imaging together with recent advances in optical reporter design and pre-clinical fluorescent model development are well placed to support and drive alternative and innovative drug discovery strategies. Emerging intravital imaging technologies (as outlined in the present review), along with longitudinal optical windows, may also support the development of novel therapeutic classes, with the core aim of reducing animal numbers, enhancing clinical efficacy and improving the pre-clinical predictions of clinical efficacy of new drugs. Here, we summarize some aspects of the drug discovery process in which integration of early *in vivo* imaging of a disease could improve the predictive benefit of pre-clinical studies (we have separated the drug discovery pipeline into five key sections and outline how intravital imaging can help in the respective settings; as summarized in Table 1).

Predictive pre-clinical models

The ability of any single murine model or 3D intermediate system to better predict clinical outcomes remains to be determined and it is more likely that the more complex transgenic, orthotopic or 3D modelling systems will recapitulate particular disease segments rather than the entire spectrum of human disease heterogeneity. For example, the application of intravital imaging of tumour angiogenesis using targeted endothelial probes or vascular pooling agents (e.g. quantum dots or fluorescent-conjugated BSA) enables direct assessment of intratumour vascular integrity and function to be performed [75,120]. Such models recapitulate particular disease segments not replicated *in vitro* and enable the evaluation of multiple drug classes that cover broad areas of new target space within the tumour and/or tumour-associated host cell populations (Table 1).

Target validation

A confounding factor to any target hypotheses and validation study is the examination of a disease in a context under which the biology normally occurs. We propose that early validation of targets under a more

Table 1. The potential of intravital imaging when integrated early into the pre-clinical drug discovery pipeline. Earlier integration of intravial imaging into the drug discovery process may improve the predictive benefit of pre-clinical studies. Techniques used *in vivo* include FRAP, FRET, PA, PS, FLIM, SHG, mammary intravial window (MIW), spinal intravital window, abdominal intravital window (AIW), cranial intravital window (CIW), anisotrophy (AN) and whole cell imaging (WCI). Representative examples of intravital applications to drug discovery are provided.

Aspects of drug discovery pipeline	Application of intravital imaging	Intravital imaging techniques
Predictive pre-clinical modelling	Evaluating drug response deep within live tumour models including more physiologically relevant orthotopic or transgenic models that recapitulate the pathophysiology and genetics of human disease. Applying quantitative intravital imaging of cellular phenotypic response to reverse engineer *in vitro* screening assays and replicate cellular physiology observed within *in vivo* environments.	FRAP (73,84), PA (73,84), PS (83,84), FLIM/FRET(94,95,104–107), AN (97), SHG (124) WCI (17–22, 36,122), MIW (83,84,51), AIW (50,51), CIW (124)
Target validation	Validate target hypothesis in the context of integrated pathway signalling networks that exists within the intact cellular- tissue- and patho-physiology of live *in vivo* environments.	FLIM-FRET (104–107)
Lead identification & optimization	Evaluation of lead compounds to provide *in vivo* proof-ofconcept data prior to subsequent medicinal investigations. *in vivo* testing to support lead optimization/chemical design activities based upon quantitative *in vivo* endpoints.	FRAP (73,84), PA (73,84), PS (83,84), FLIM-FRET (104,105), SHG (124), WCI (36, 49–51,83), MIW (83,84,51), AIW (50,51)
Predictive Pharmacodynamics & pharmacokinetics	High resolution imaging of pharmacodynamic and pharmacokinetic biomarkers at sub-cellular, cellular and tissue levels in live *in vivo* environments.	FRAP (73,84), PA (73,84), PS (83,84), FLIM-FRET (104,105), WCI (36, 49–51,83), MIW (83,84,51), AIW (50,51)
Drug combination studies	Evaluation and development of rational drug combination strategies including optimal dose ratio setting and scheduling.	FRAP (73,84), PA (83,84), PS (83,84), FLIM (94,95,104–107) WCI (36)

appropriate *in vivo* context utilizing highly quantitative intravital imaging techniques and specific functional biosensors of target activity (e.g. FRET probes) provides a more robust and indepth understanding of target biology in the context of integrated pathway networks that exists within intact *in vivo* tissue environments (Table 1) [119–122,124,125]. Such methods frontload the validation of targets in live tissue systems, thereby building the necessary confidence to merit the significant investments required to embark upon novel target-directed drug discovery strategies.

Lead identification and optimization

The application of techniques such as PA, PS and PB using intravital imaging methodology provides quantitative readouts of drug activity and mechanism-of-action *in vivo* (Table 1) [56,83,93,94]. Such technologies significantly speed up the evaluation of drug responses *in vivo,* whereas, in conjunction with recoverable anaesthesia protocols and optical windows for intravital imaging, they enable longitudinal studies and significantly reduce the number of animals required to evaluate lead compounds with the necessary statistical robustness. These advances in intravital imaging provide a realistic fit with lead optimization cycles to provide a new opportunity for optimizing drugs based upon *in vivo* efficacy and/or proof-of-mechanism data. Such an approach extends the phenotypic drug discovery paradigm from *in vitro* cell-based screening to more select and physiologically relevant *in vivo* screens.

Predictive pharmacodynamics and pharmacokinetics

High-resolution imaging of pharmacodynamic and pharmacokinetic biomarkers at subcellular, cellular and tissue levels in live environments by intravital imaging may provide a new opportunity for exploring drug uptake and retention within cells and tissue. In most pre-clinical studies, the intratumoural uptake of the drug is not considered, with plasma drug concentrations being used as a surrogate. A significant advance in understanding drug behaviour *in vivo* would be to directly correlate the uptake and retention of drug(s) with a biosensor that reads out the target of interest. Future application of FRET biosensors *in vivo* may help in this respect to predict drug retention, drug-target engagement and physiological responses in both diseased tissues and systemic host tissue (Table 1) [107,108,119,120]. The application of such predictive models may enable the optimization of more effective disease tissue targeting strategies, at the same time as allowing an assessment of systemic liabilities on host tissue physiology or potential adverse toxicities to be monitored. This approach may also facilitate the identification of pharmacodynamic biomarkers that can be translated into clinical application.

Drug combinations

Currently, it is very difficult to optimize scheduling and dosing of drug combinations without using large number of animals in long-term efficacy studies and these are often not rigorously carried out in pre-clinical studies. The longitudinal readouts and high-resolution assessment of drug pharmacokinetics and pharmacodynamics provided by intravital imaging may facilitate more accurate scheduling and dosing, requiring fewer animals for multiple drug combination studies [140,158–160]. A unique advantage of high-resolution intravital imaging is the assessment of drug uptake and retention at cellular and subcellular levels, allowing us to determine how long each component of a combination therapy needs to be present in the tumour cells and/or present together to provide optimal efficacy.

Finally, the application of intravital imaging also supports the evaluation of drug combination strategies targeting multiple cellular types and paracrine signalling mechanisms. Improved drug delivery of a sequential therapy after drug-induced modification of tissue vascularization (e.g. quantum dots/fluorescent dextrans) or tissue architecture (using SHG imaging) is also possible using this approach, which is difficult to replicate within *in vitro* systems [161,162]. We therefore propose that intravital *in vivo* profiling technologies will support the development of more innovative, effective and adaptive clinical trial designs where drug combination scheduling can be guided by *in vivo* pre-clinical imaging (Table 1).

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Conflicts of interest

There are no conflicts of interest.

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