

## REVIEW ARTICLE

# Experimental pathology by intravital microscopy and genetically encoded fluorescent biosensors

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Cdk1, cyclin-dependent kinase 1; CFP, cyan fluorescent protein; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; EPR, enhanced permeability and retention; FRET, Förster resonance energy transfer; H&E, hematoxylin-eosin; HUVECs, human umbilical vascular endothelial cells; IVM, intravital microscopy; MMTV, mouse mammary tumor virus; PKA, protein kinase A; VEGF, vascular endothelial growth factor; YFP, yellow fluorescent protein; 2P, two-photon; 2PEM, 2P excitation microscopy

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**Funding information**

Core Research for Evolutional Science and Technology, Grant/Award Number: JPMJCR1654; Precursory Research for Embryonic Science and Technology; Japan Society for the Promotion of Science, Grant/Award Numbers: 11242209, 16390078, 17012014, 19209008, 22113002

The invention of two-photon excitation microscopes widens the potential application of intravital microscopy (IVM) to the broad field of experimental pathology. Moreover, the recent development of fluorescent protein-based, genetically encoded biosensors provides an ideal tool to visualize the cell function in live animals. We start from a brief review of IVM with two-photon excitation microscopes and genetically encoded biosensors based on the principle of Förster resonance energy transfer (FRET). Then, we describe how IVM using biosensors has revealed the pathogenesis of several disease models.

**KEYWORDS**

biosensor, experimental pathology, FRET, intravital microscopy

**INTRODUCTION**

The field of modern cellular pathology began with the introduction of light microscopy and hematoxylin-eosin (H&E) staining in the late 19th century, and reached a zenith in the early 20th century, when a number of academic feats were

achieved by pathologists. Even if we restricted ourselves to the history of the Japan Society of Pathology, we can easily recall the chemically induced squamous cell carcinoma in rabbits by Katsusaburo Yamagiwa and Kōichi Ichikawa,<sup>1</sup> and the discovery of chicken sarcoma virus by Akira Fujinami,<sup>2</sup> which heralded the era of chemical carcinogenesis and viral

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oncology, respectively. Thereafter, experimental pathology has continued to evolve by adopting cutting-edge technologies in each successive period, such as electron microscopy, immunohistochemistry, *in-situ* hybridization and so on. However, such techniques can be applied only to a fixed sample. Researchers in experimental pathology have long desired to microscopically observe the tissues in live experimental animals. Moreover, the field of experimental pathology was somewhat neglected during the flourishing of biochemistry and molecular biology in the late 20th century, because these fields tend to handle tissue samples as a mass for the analysis of molecular activities rather than considering the heterogeneity at a cellular resolution in the manner of pathologists. Now, the goal of observing the molecular activities in the diseased tissue of live animals has been realized through the development of intravital microscopy (IVM) with a two-photon (2P) microscope and various fluorescent probes. Here, we summarize the uses of IVM with a 2P microscope in experimental pathology, with a particular focus on the use of genetically encoded fluorescent biosensors.

## TWO-PHOTON EXCITATION MICROSCOPY AND GENETICALLY ENCODED FLUORESCENCE BIOSENSORS

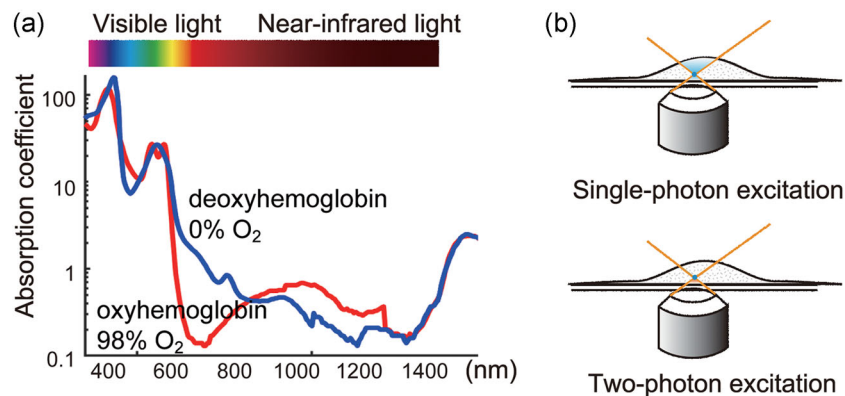
### Two-photon excitation microscopy

Although IVM has a long history, the number of studies using IVM started to increase only after the new millennium,<sup>3</sup> largely due to development of the 2P excitation microscope. The process of 2P absorption is a nonlinear optical one that depends on the square of the light intensity. Taking advantage of this property, Denk *et al.* invented 2P excitation microscopy (2PEM),<sup>4</sup> which is now widely used across the broad field of life

science. The first advantage of 2PEM is that it uses near-infrared wave length for excitation (700–1300 nm). The primary obstacle that prevents us from seeing deep tissues is light scattering – more specifically, Rayleigh scattering. The degree of Rayleigh scattering is inversely correlated with the fourth power of the wavelength. Therefore, near-infrared light can penetrate the tissues more deeply than visible light. Furthermore, the absorption of hemoglobin, one of the major light-absorbing molecules, is markedly lower in the near-infrared range than the visible light range<sup>5</sup> (Fig. 1a). The second advantage of 2PEM is related to the nonlinear optical process. In conventional single-photon excitation, all fluorescent molecules along the light path emanate fluorescence. Therefore, to obtain a clear image of the focal plane, the fluorescence light derived from out-of-focus planes must be excluded, such as by using a confocal pinhole. This process also reduces the signal from the focal plane. In contrast, only the molecules at the focal plane are excited in 2PEM (Fig. 1b), enabling maximum recovery of photons from the molecules of interest. Through these principles, 2PEM now allows us to see brain tissue to more than 1 mm depth, in contrast to traditional microscopy, which can visualize only structures closer to the surface of tissues.<sup>6</sup>

### Fluorescence labeling for 2PEM

Much like conventional fluorescence microscopy, 2PEM also requires tissues to be labeled with fluorescent molecules, except when observing autofluorescent molecules or using second harmonic generation. Fluorescent dyes have been routinely used for the staining of tissue sections or tissue culture cells; however, staining of cells or molecules of interest in live animals is not an easy task, limiting the application of fluorescent dyes in IVM. Exceptions are the vasculature, which can be labeled by intravenous injection of fluorescent



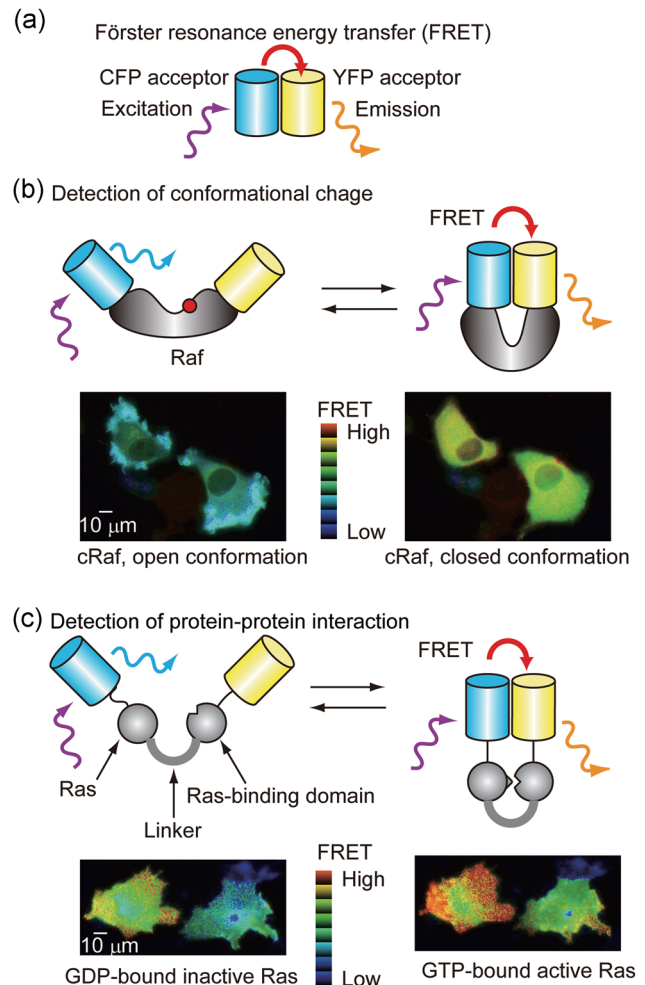
**Figure 1** Properties of two-photon excitation microscopy (2PEM) using a near-infrared high power femtosecond (fs) pulsed laser. **(a)** The light absorption of hemoglobin is the lowest, in the wavelength range of 700 to 1300 nm, which is often referred to as the near-infrared window in biological tissue. The spectrum is from Bosschaart *et al.*<sup>5</sup> **(b)** In the conventional single-photon fluorescence microscopy, all fluorescence molecules along the light path are excited. In 2PEM, two photons must reach the single fluorophore almost simultaneously, which occurs only at the focal plane, markedly reducing the background fluorescence.

dyes,<sup>7</sup> and immune cells, which can be labeled *ex vivo* and introduced back into the host.<sup>8</sup> Meanwhile, there are two advantages of fluorescence proteins, which derive from their being “genetically encoded” dyes. First, the fluorescent proteins will be produced as long as the cells carry the fluorescent protein genes. After the establishment of transgenic mice expressing the fluorescent proteins, there is no need to add anything for the visualization. Thus, fluorescence proteins are a very stable and inexpensive labeling technique. The second advantage is that the cells of interest can be labeled with fluorescent proteins expressed from a cell type-specific promoter/enhancer. For example, lymphocyte-specific expression of fluorescent proteins has visualized how immune cells communicate with each other in time- and space-specific manners.<sup>9</sup> In other studies, cells are labeled with fluorescent proteins by ubiquitously expressed promoters and distinguished by the characteristic morphology of each cell type<sup>10,11</sup> Markers for the cell membrane and nucleus are often used for this purpose.<sup>12–14</sup> Notably, a number of mouse reporter lines have been developed by Aizawa and his colleagues, who utilized the ROSA26 locus for ubiquitous expression *in vivo*,<sup>12,14</sup> providing the basis for IVM by 2P excitation microscopes. In addition, we recently established mouse lines expressing a morphology marker named NuCyM, which enables us to distinguish the nucleus, plasma membrane and cytoplasm by 2PEM.<sup>15</sup> With NuCyM, images acquired by 2PEM can be converted to H&E-like images.

### Biosensors based on the principle of Förster resonance energy transfer

The utility of fluorescent proteins has been further extended by the development of a variety of biosensors. There are a plethora of genetically encoded probes;<sup>16,17</sup> however, here we will only consider biosensors based on the principle of Förster resonance energy transfer (FRET), because we will refer to transgenic mice expressing the FRET biosensors in the following sections. FRET is a process by which a donor fluorophore in an excited state non-radiatively transfers its energy to a neighboring acceptor fluorophore.<sup>18,19</sup> Most of the currently available FRET biosensors use cyan fluorescent protein (CFP) and yellow fluorescent protein (YFP) as the donor and acceptor, respectively (Fig. 2a). By fusing the donor and acceptor proteins to the protein of interest, the activity change of the protein, which is caused by the conformational change, can be monitored by the change in FRET efficiency. As a typical example, we adduce a FRET biosensor for cRaf (Fig. 2b). cRaf adopts open active and closed inactive conformations in the manner of many protein kinases. By fusing CFP and YFP to both ends, a FRET biosensor for cRaf was generated, in which FRET efficiency was inversely correlated with the activity of cRaf.<sup>20</sup> Alternatively, protein–protein interaction, which is a key step in signal

transduction, can be monitored by measuring the FRET efficiency (Fig. 2c). For example, Ras is known to bind to cRaf in an activation-dependent manner. By fusing Ras and the Ras-binding domain of cRaf, a FRET biosensor for Ras was generated, in which FRET efficiency was correlated with the activity of Ras. Upon growth factor stimulation, guanosine diphosphate (GDP)-bound inactive Ras becomes guanosine triphosphate



**Figure 2** Typical examples of the Förster resonance energy transfer (FRET) biosensor. (a) FRET is a process by which a donor fluorophore in an excited state non-radiatively transfers its energy to a neighboring acceptor fluorophore. (b) Many protein kinases including Raf adopt an open active conformation and closed inactive conformation. The former is often induced by phosphorylation as indicated by the red circle. By fusing the donor and acceptor fluorophore, this conformational change can be detected by the efficiency of FRET. In each pixel of these FRET images, the color hue reflects the fluorescence intensity ratio of acceptor versus donor in eight grades, and the intensity of each color reflects the concentration of the probe. (c) In another FRET biosensor design, signal-induced protein–protein interaction can be detected by FRET. In this example, Ras and the Ras-binding domain of Raf are tandemly connected, and further sandwiched by the donor and acceptor fluorophores. Upon Ras activation, the guanosine triphosphate-bound active Ras binds to Raf and thereby provokes FRET.

(GTP)-bound active Ras and transmits a signal to cRaf. By the use of the probe, it was found that this GDP-GTP exchange reaction occurs primarily at the free-edge of epithelial cells, solving the enigma known as contact inhibition of cell growth.<sup>21</sup>

## EXPERIMENTAL PATHOLOGY BY IVM WITH GENETICALLY ENCODED BIOSENSORS

Currently, the field of neuroscience has seen by far the most applications of IVM with 2PEM. Because the refractive index of the brain is relatively homogenous, light scattering occurs much less in the brain than in the other organs, allowing deeper penetration of excitation light, up to 1 to 2 mm from the brain surface. Moreover, a glass window implanted to the skull allows researchers to observe neurons without motion artifacts and to revisit the same neuron for several months. Because there are a huge number of articles on the intravital imaging of mouse brains, here we will refer only to several excellent review papers,<sup>22–24</sup> and proceed to IVM in the other tissues.

What are the events that we can see by IVM but not by conventional microscopy? A good example is cell migration, such as that in leukocyte extravasation or epithelial cell wound healing. We know that leukocytes extravasate from the blood vessels and we may imagine how they seek bacteria or diseased cells, but without seeing live images, we can hardly predict with any precision how cells move around in the tissues. Other examples are those events which fluctuate with time. For example, endothelial cell damage induces both pro- and anti-thrombotic signaling cascades, leading to the cycles of thrombus formation and resolution. This phenomenon cannot be foreseen by static images. Similarly, temporal changes of signaling molecule activities are also hard to predict by conventional immunohistochemical techniques. Below, we will consider some key applications of IVM in detail.

### Visualization of neutrophil extravasation

Next to neuroscience, immunology is the second largest research area capitalizing on the advantages of IVM.<sup>9,25</sup> This is probably because a period of several hours of observation under anesthesia is sufficient to study the dynamic interaction between immune cells and target cells. Two-photon microscopy analysis of lymphocytes has already been reviewed.<sup>26</sup> Here we focus on the migration of neutrophils.

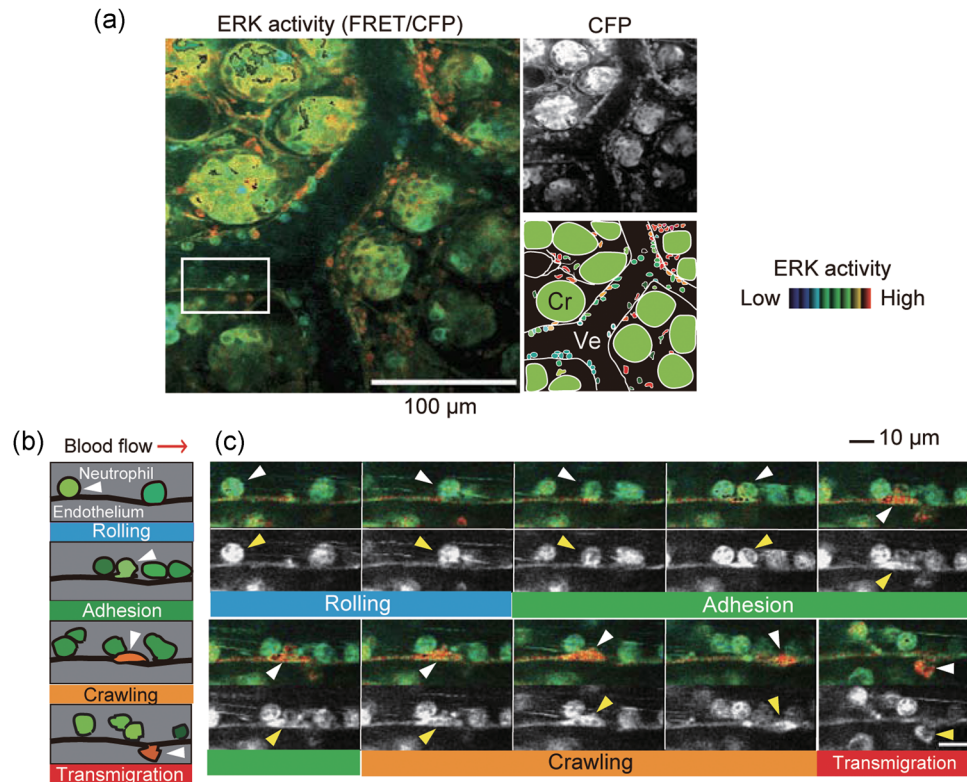
Extravasation of neutrophils is the hallmark of acute inflammation. IVM has revealed that the neutrophil recruitment involves four steps: rolling, adhesion, crawling and transmigration.<sup>27–29</sup> It has been shown *in vitro*, but not *in vivo*, that various intracellular signaling molecules are activated in neutrophils and endothelial cells during extravasation of neutrophils. We previously developed transgenic mice expressing

FRET biosensors for protein kinase A (PKA) and extracellular signal-regulated kinase (ERK).<sup>30</sup> By observing the inflamed intestines of these transgenic mice by 2PEM, we visualized the activity change of PKA and ERK in neutrophils.<sup>31,32</sup> First, neutrophils roll on and adhere firmly to the endothelial cells (Fig. 3). Neutrophils then crawl for a few minutes, and transmigrate to the interstitial tissue. ERK is activated during the adhesion process, supporting the positive role of ERK in the extravasation of neutrophils. Pharmacological perturbation experiments have shown that this ERK activation is dependent on leukotriene B (LTB). Meanwhile, it was found that prostaglandin E<sub>2</sub> (PGE<sub>2</sub>) activates PKA and suppresses ERK. Thus, LTB<sub>4</sub> recruits neutrophils to the inflamed lesion by ERK activation, and PGE<sub>2</sub> stops neutrophils from functioning as scavengers of damaged tissues by PKA-mediated ERK inactivation, highlighting the antagonistic action of these two chemical mediators derived from arachidonic acid.

Let us consider one other example of a secret of life that was uncovered by IVM. For many years, people believed that neutrophils recruited to the inflamed tissue live only for a short period of time, long enough to conduct their missions as tissue destroyers and scavengers, and then are doomed to die. This dogma has now been challenged based on observations made using IVM. It is reported that, upon sterile injury of the liver, the recruited neutrophils create new vasculature, through which they return back to the blood.<sup>33,34</sup> Under conditions of ischemia-reperfusion as well, neutrophils return to the vasculature<sup>35</sup> as do the lymphocytes. These phenomena, called either reverse migration or disrupted transendothelial migration, contribute to systemic inflammation, particularly in the lung.<sup>36</sup>

### Dynamics of thrombosis

Formation of stable thrombus has been visualized with conventional wide-field microscopy or confocal microscopy,<sup>37–40</sup> and high-resolution imaging has further revealed that the platelet aggregation could be divided into two phases.<sup>41–43</sup> First, the discoid platelets are tethered loosely to the blood vessels. Second, the platelets change their shape to form a core of fully activated platelets. Meanwhile, *in vitro* biochemical studies have delineated the intracellular signal transduction cascades that promote or inhibit thrombus formation.<sup>44</sup> One of the key accelerators of thrombus formation is ERK, which mediates adenosine diphosphate-induced TxA<sub>2</sub> generation and activation of integrin  $\alpha_{11b}\beta_3$ .<sup>45,46</sup> On the other hand, PKA is the major brake of thrombus formation.<sup>44,47–50</sup> Both of the major anti-thrombotic molecules, prostacyclin I<sub>2</sub> and nitric oxide, are known to suppress thrombus formation via PKA activation.<sup>48,51</sup> However, spatio-temporal regulation of ERK or PKA during thrombus formation has not been observed due to technical difficulties. By using transgenic mice expressing the FRET biosensor for ERK or PKA, Hiratsuka *et al.*



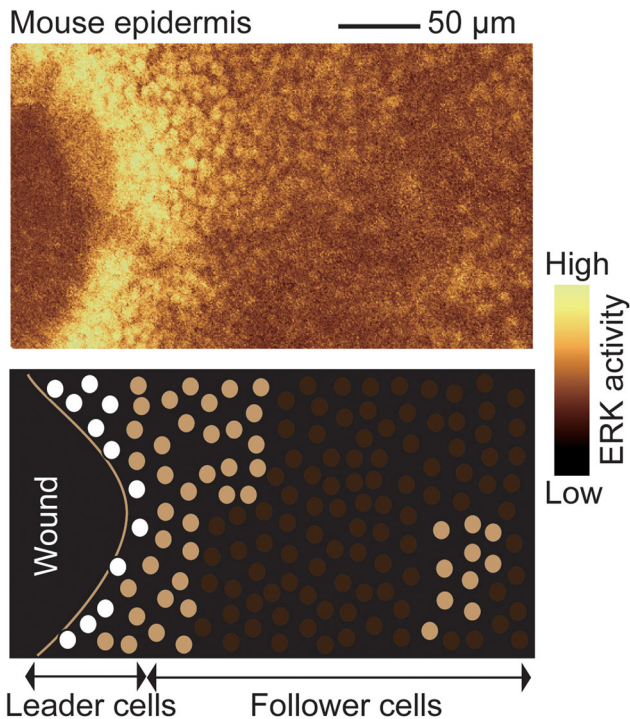
**Figure 3** An example of intravital imaging of molecular activity during neutrophil recruitment to the lamina propria of the intestinal mucosa. (a) A representative Förster resonance energy transfer (FRET)/cyan fluorescent protein (CFP) image of the inflamed intestine of a mouse expressing the FRET biosensor for extracellular signal-regulated kinase (ERK). Images are shown in intensity-modulated display (IMD) mode using eight ratios with 32 intensities and a CFP image in grayscale, with a schematic view of this region. Cr, crypt; Ve, venule. (b) Schematic of the four steps of extravasation. White arrowheads indicate the same neutrophil at different time points. (c) Time-lapse FRET/CFP and CFP images of neutrophil extravasation. The boxed region (a) was magnified and shown in a time series. Reproduced from Mizuno *et al.*<sup>31</sup>

visualized the activity change of ERK and PKA during thrombus formation in platelets.<sup>52</sup> As anticipated, ERK is activated rapidly at the core of contracting platelet aggregates. However, unexpectedly it was found that PKA is also activated almost simultaneously with ERK. Thus, by stimulating both pro- and anti-thrombotic signaling molecules, fine tuning of the thrombus formation could be accomplished. In fact, the thrombus formation is not a straightforward process, but is the outcome of repeated growth and resolution phases. This is another good example of the visualization of signaling activity in live tissues helping us to understand pathogenesis in the real world.

### Wound healing of epithelial cells

In many textbooks of basic pathology, wound healing is described as a typical example of a tissue repair process, wherein inflammation, cell migration, cell proliferation and tissue remodeling occur in an organized fashion.<sup>53</sup> Meanwhile, in cell biology, wound healing has been studied extensively as a representative form of collective cell migration

of epithelial cells, wherein a number of mechanisms, such as chemotaxis, haptotaxis, durotaxis, and so on, underlie the organized cell movement.<sup>54</sup> One of the key questions in regard to collective cell migration is how the moving cue is propagated from the leader cells to the follower cells.<sup>55</sup> In previous studies, we noticed that the activation of epidermal growth factor receptors (EGFRs) and their canonical downstream signaling protein ERK could be propagated between epithelial cells both in tissue culture cells and in the epidermis of living mice.<sup>56,57</sup> Interestingly, big waves of ERK activation are generated at the wound edge of the epidermis and propagated to the follower cells up to several millimeters distance<sup>56,58</sup> (Fig. 4). Pharmacological inhibition of A disintegrin and metalloprotease (ADAM), EGFR or ERK, perturbed the ERK activation wave and also collective cell migration during epidermal wound healing.<sup>56</sup> ADAM is required for shedding of the epidermal growth factor (EGF)-family protein; collectively, therefore, these observations clearly indicate that the ERK activation wave is propagated by activation of the EGF-signaling cascade and that the ERK activation wave drives the follower cells to go forward to chase the leading cells and to efficiently fill the defect.



**Figure 4** Wound healing and propagation of extracellular signal regulated kinase (ERK) activation. The epidermis of a transgenic mouse expressing a Förster resonance energy transfer (FRET) biosensor for ERK was subjected to epithelial wounding 12 h before imaging. The FRET/cyan fluorescent protein (CFP) ratio is shown by gold pseudocolors (top panel). The cartoon schematically shows the ERK activation. The white circles indicate nuclei of the leader cells showing highest ERK activity, whereas gray circles indicate nuclei of follower cells consisting of ERK activation waves.

Is such an ERK activation wave observed in other tissues? We noticed a similar phenomenon within intestinal epithelial cells, but its role in migration has not been addressed.<sup>59</sup> What about urothelium? Urothelial cells exhibit ERK activation upon stretching;<sup>60</sup> however, no ERK activation wave is observed at the wound.<sup>61</sup> Unexpectedly, a critical difference between the epidermis and urothelium was revealed by 2PEM. The urothelium, but not the epidermis, can glide over the interstitial tissues, which appears to be the reason why an ERK activation wave is not required for the wound healing of urothelium.

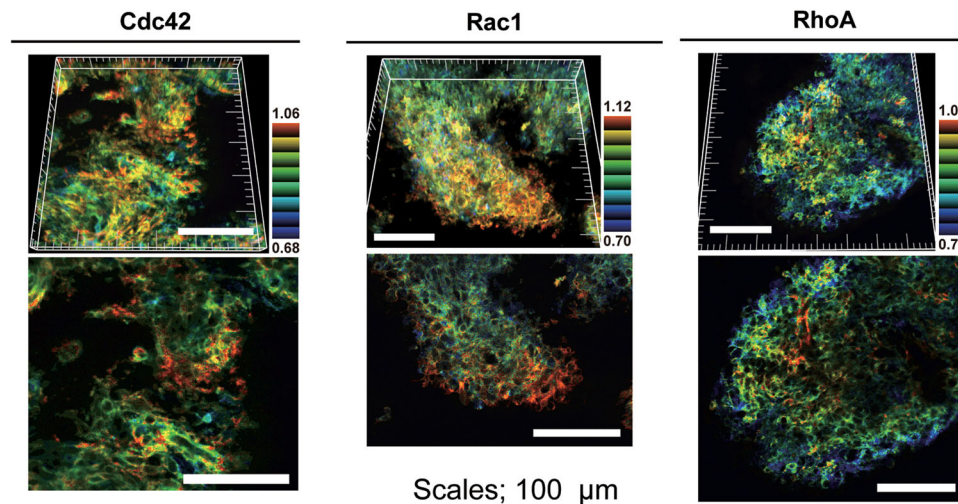
#### CANCER RESEARCH BY IVM AND GENETICALLY ENCODED FLUORESCENT BIOSENSORS

For many years, it has been known that cancer tissues consist of heterogeneous cell populations. Cancer cells themselves are heterogeneous both in phenotypes and genotypes. Furthermore, growing cancer cells always recruit vascular cells, interstitial cells and immune cells. Microscopy is the best approach to study the heterogeneity of

cancer tissues, and, therefore, the gold standard for the diagnosis of cancer. IVM further provides great opportunities to decipher the dynamic interaction among cancer cells, interstitial cells, immune cells, vascular cells, and so on. There are several excellent review papers on the contribution of IVM to the understanding of cancer cell intravasation, metastasis and sensitivity to anticancer drugs.<sup>62–66</sup> Here we focus on cancer research by IVM with genetically encoded fluorescent biosensors. In the pre-green fluorescent protein (GFP) era, IVM in cancer research was mostly focused on the visualization of tumor vasculature.<sup>62</sup> GFP technology enabled researchers to observe live tumor cells for a long period sufficient to analyze the mode of invasion.<sup>67</sup> Introduction of 2PEM further accelerated IVM of tumor tissues because of its superiority for deep tissue imaging.<sup>68</sup> Moreover, the development of peripheral devices such as the imaging window has created ideal conditions for observing the pathophysiology of tumor environments by 2PEM.<sup>69,70</sup> As already stated, one advantage of GFP is that it can be used not only to label tumor cells, but also to supply a variety of biosensors that monitor the function of cancer cells and the interaction between cancer cells and host cells. For example, by using a FRET biosensor for the stress-responsive kinase Tak1, it has been shown that the Tak1 activity is high at the periphery of tumors, suggesting that tumor cells at the periphery of the tumor mass are exposed to higher levels of stress than those in the central region of the tumor mass.<sup>71</sup> Below, we will consider additional examples of the benefit of genetically encoded fluorescent biosensors used in combination with IVM.

#### Regulation of glioblastoma invasion by Rho-family GTPases

As stated earlier, brain tissue has a great advantage for use in intravital imaging because the skull can be firmly stabilized with a fixing device. By 2PEM, it has been visualized that glioblastoma cells move much faster in the perivascular space than in the brain parenchyma in mouse or rat glioblastoma models.<sup>72,73</sup> Cell migration is generally regulated by Rho-family GTPases, which dictate actin polymerization and actomyosin contraction.<sup>74–76</sup> Hirata *et al.* studied the role of Rho-family GTPases in glioblastoma invasion by using FRET biosensors for Rho-family GTPases (Fig. 5).<sup>73</sup> Rac1 and Cdc42, which promote actin polymerization, are activated in glioblastoma cells invading into the brain parenchyma. Meanwhile, RhoA, which induces actomyosin contraction, is activated primarily in the center of tumor tissue, particularly at the perivascular region, suggesting that the balance of GTPase activities may control the migratory property of glioblastoma cells. How, then, is such heterogeneity of GTPase activity generated in growing glioblastoma cells? Time-lapse



**Figure 5** Activity of Rho-family GTPases in glioblastoma invading into the brain parenchyma. C6 glioblastoma cells stably expressing Förster resonance energy transfer (FRET) biosensors for Cdc42, Rac1 and RhoA were injected into rat brain. After 7 days, the invasion front of the tumor was imaged under a two-photon excitation microscope. The three-dimensional reconstructed images are shown in intensity-modulated display (IMD) mode with 32-intensity in eight-ratio. Reproduced from Hirata E *et al.*<sup>73</sup>

imaging of Rac1 activity over several days has revealed that Rac1 activity in glioblastoma cells fluctuates over a timescale, substantially longer than that of the replication cycle.<sup>77</sup> When glioblastoma cells were embedded in gel, the high Rac1 activity cell population was found to invade into the gels, leading the other cells.<sup>73</sup> RNA-Seq analysis of Rac1-hi and Rac1-lo cells revealed a signaling network that comprises both positive and negative feedback loops, which may be sufficient to cause oscillation of the activity change of Rho-family GTPases. Such an intrinsic mechanism of generating heterogeneity may be beneficial to adapt to variations in the tissue environment.

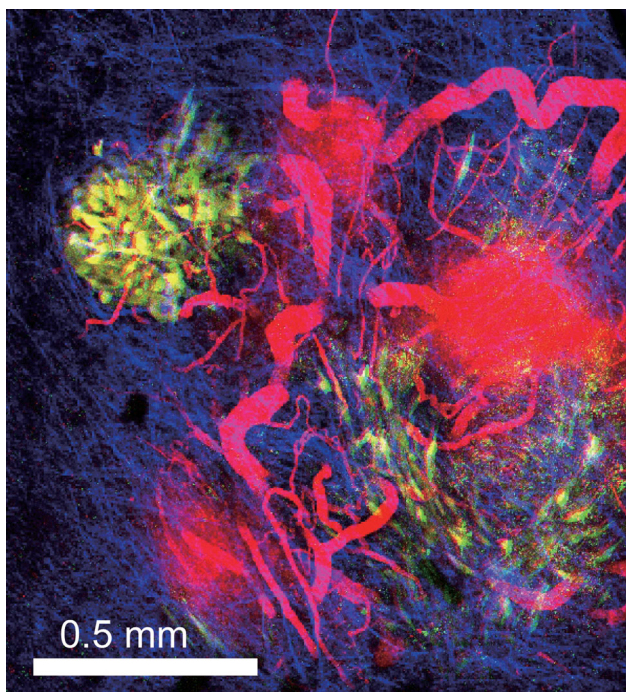
### Stem cell property of breast cancer

Transgenic mice expressing fluorescent proteins or genetically encoded fluorescent biosensors are also frequently used to improve our understanding of naturally occurring cancers. For example, the roles of oncogene and tumor suppressor genes in oncogenesis have been extensively studied in a variety of transgenic mice, in which fluorescent proteins are expressed in the cell types of interest.<sup>78</sup> We crossed transgenic mice expressing a FRET biosensor for ERK with mouse mammary tumor virus (MMTV)-Neu transgenic mice, which are widely used as a mouse model of HER2/Neu-positive luminal-type breast cancer.<sup>79</sup> By 2PEM, significant heterogeneity of ERK activity was observed among the breast cancer cells. Intriguingly, the ERK activity was inversely correlated with the efficiency of tumor sphere generation *in vitro* and the expression of the cancer stem cell markers CD49f, CD24 and CD61, suggesting that ERK

negatively regulates the stem cell property. Meanwhile, the role of ERK activity in the prognosis of human breast cancer patients is controversial. One report claimed that high ERK activity in breast cancer tissues predicts poor prognosis of patients,<sup>80</sup> whereas others reports proposed that high ERK activity in breast cancer tissues is associated with improved survival.<sup>81–83</sup> ERK is a major signaling output of receptor-type tyrosine kinases including HER2/Neu/ErbB2. Thus, our observation in mice suggests that high ERK activity accelerates tumor growth and at the same time suppresses the stemness of cancer cells. It is likely that the ERK activity varies significantly among the different tumor regions, which renders the correlation of ERK activity to prognosis controversial.

### Enhanced permeability of tumor vessels

IVM is particularly useful to visualize the dynamic properties of cancer tissues, such as the enhanced permeability and retention (EPR) effect. EPR is a hallmark of cancer tissue and is closely related to angiogenesis and metastasis<sup>84–86</sup> (Fig. 6). Harney *et al.* have proposed the presence of a tumor microenvironment of metastasis, where transient vascular permeability and tumor cell intravasation simultaneously occur.<sup>87</sup> Vascular endothelial growth factor (VEGF) is known as the key molecule controlling the vascular hyperpermeability. Within cells, many intracellular signaling molecules, such as Src-family tyrosine kinases, PI 3-kinases and phospholipase C, have been shown to mediate the actions of VEGF.<sup>88</sup> However, signaling cascades that may compete with the VEGF signaling cascade have not received much attention in cancer research. For example, the cAMP



**Figure 6** Increased vascular permeability. Panc-02 cancer cells (yellow) implanted subcutaneously into a C57BL/6N Jcl mouse were observed by two-photon excitation microscopy (2PM). Texas red-conjugated dextran was injected to visualize blood vessels (red). Collagen fibers were visualized by second harmonic generation (blue).

signaling cascade was shown to maintain normal endothelial barrier function,<sup>89–91</sup> but its role in the vascular hyperpermeability in tumor tissue remained elusive. Using a combination of FRET biosensors and 2PEM, a more recent study revealed that the activity of PKA is significantly lower in tumor endothelial cells than in normal endothelial cells.<sup>92</sup> Further experiments showed that VEGF decreased PKA activity in normal endothelial cells, but that a VEGF inhibitor, Motesanib, increased PKA activity in tumor blood vessels, indicating that the decreased PKA activity causes vascular hyperpermeability. Importantly, in human umbilical vascular endothelial cells (HUVECs), which are the most widely used endothelial cells for *in vitro* experiments, VEGF does not decrease, but rather increases PKA activity,<sup>93</sup> highlighting the critical difference between *in vivo* and *in vitro* conditions. Since the EPR is often utilized to deliver anticancer drugs, any knowledge pertaining to control of the permeability of the tumor vasculature would be of great clinical significance.<sup>94</sup>

### Anticancer drug actions

Recent developments in IVM that have shed light on the actions of anticancer drugs have been extensively reviewed already.<sup>64</sup> Here, we refer to the mechanisms underlying the

remittance after anticancer drug treatment. Genetic heterogeneity in cancer cell populations is of course one of the primary reasons for such remittance, but it has also been shown that the microenvironment of cancer cells is a critical factor for the drug response of cancer cells.<sup>95</sup> Using 2PEM and a FRET biosensor for ERK, Hirata *et al.* showed that a BRAF inhibitor activates ERK in melanoma-associated fibroblasts by a mechanism known as ‘paradoxical activation’, and that the activated fibroblasts provide a “safe haven” for melanoma cells to tolerate BRAF inhibition.<sup>96</sup> This and other studies have highlighted the importance of the activated fibroblasts, as described in a recent review.<sup>97</sup>

The development of an effective anticancer drug for inoperable pancreatic cancer patients is urgently needed. Combination therapy with gemcitabine and nab-paclitaxel is the current gold standard, but the overall survival is still less than 1 year.<sup>98</sup> By the use of a FRET biosensor for RhoA, Timpson and colleagues showed that RhoA activation at the invading front of the pancreatic cancer is critical for the invading capacity of pancreatic cancer.<sup>99</sup> They further examined the role of the principal effector of RhoA, ROCK, on the sensitivity to gemcitabine and nab-paclitaxel. Using a FRET biosensor for cyclin-dependent kinase 1 (Cdk1) as a surrogate marker of M-phase cell cycle arrest induced by gemcitabine/nab-paclitaxel, they found that the ROCK inhibitor Fasudil can improve the effect of the combination therapy in patient-derived xenografts.<sup>100</sup> Because the RhoA signaling cascade is activated in pancreatic cancer,<sup>101</sup> inhibitors of the RhoA signaling cascade may provide a promising molecular target for anticancer drugs. Another potential target molecule is Src, which is highly expressed in pancreatic cancer.<sup>102,103</sup> However, the clinical trials of anti-Src drugs have failed to show effectiveness in combination with gemcitabine in advanced pancreatic cancer.<sup>104,105</sup> This may be due to the poor delivery of the Src inhibitors. In fact, using a FRET biosensor for Src, it has been shown that the pancreatic cells distal to the vasculature respond poorly to the Src inhibitor dasatinib.<sup>106</sup> Thus, the genetically encoded biosensors could help to reveal the discrepancy between the *in vitro* and *in vivo* observations.

### FUTURE PROSPECTIVES

The recent success of IVM does not come from a single invention. Indeed, the handling of very large 4D images has become possible only with the advent of information technology. Introduction of adaptive optics, software to alleviate motion artefacts during imaging,<sup>107,108</sup> and instruments for organ fixation have also improved the quality of images. Moreover, the introduction of artificial intelligence will greatly help in analyzing the vast amounts of imaging data generated by IVM. Thus, IVM will continue to expand its role in



experimental pathology. However, what should be emphasized here is the continued importance of investigation by the human eye. Artificial intelligence (AI) may be better at answering labor-intensive questions than humans, but AI cannot answer unasked questions. The questions hidden in the images can be mined only by scientists with profound knowledge of the histology. IVM will provide unlimited opportunities to identify clues to understand the pathogenesis and the keys to developing new remedies.

### ACKNOWLEDGMENTS

Michiyuki Matsuda has been announced as the winner of the Japan Pathology Award in 2019. This review article was prepared based on the data presented at the 108th Annual Meeting of the Japanese Society of Pathology, Tokyo, 29 April 2019. The authors would like to thank Naoki Mochizuki (National Cerebral and Cardiovascular Center), Yusuke Ohba (Hokkaido University), Kazuo Kurokawa (RIKEN), Takeshi Nakamura (Tokyo University of Science), Etsuko Kiyokawa (Kanazawa Medical University), Yuji Kamioka (Kansai Medical University), Masamichi Imajo (Hokkaido University), Takuya Hiratsuka (Kyoto University), Kazuhiro Aoki (National Institute of Basic Biology), Eishu Hirata (Kanazawa University) and all collaborators who kindly supported the research. The experimental work presented here was supported in part by JSPS KAKENHI Grants Numbers 11242209, 16390078, 17012014, 19209008, 22113002, 15H02397 and 15H05949, by a JST PRESTO grant, by grants from the program Innovative Cell Biology by Innovative Technology (Cell Innovation Program) and the Platform Project for Supporting Drug Discovery and Life Science Research (Platform for Dynamic Approaches to Living System) from the Japan Agency for Medical Research and development (AMED), by a CREST grant (JPMJCR1654) and by the Nakatani Foundation.

### DISCLOSURE STATEMENT

None declared.

### AUTHOR CONTRIBUTIONS

Drafting the manuscript and figures, MM and KT.

### REFERENCES

- 1 Yamagiwa K, Ichikawa K. Über die atypische Epithelwucherung. *Gann* 1914; **8**: 11–15.
- 2 Fujinami A, Inamoto K. Ueber Geschwülste bei japanischen Haushühnern, insbesondere über einen transplantablen Tumor. *J Cancer Res Clin Oncol* 1914; **14**: 94–119.
- 3 Secklehner J, Lo Celso C, Carlin LM. Intravital microscopy in historic and contemporary immunology. *Immunol Cell Biol* 2017; **95**: 506–13.
- 4 Denk W, Strickler JH, Webb WW. Two-photon laser scanning fluorescence microscopy. *Science* 1990; **248**: 73–76.
- 5 Bosschaart N, Edelman GJ, Aalders MC, van Leeuwen TG, Faber DJ. A literature review and novel theoretical approach on the optical properties of whole blood. *Lasers Med Sci* 2014; **29**: 453–79.
- 6 Helmchen F, Denk W. Deep tissue two-photon microscopy. *Nat Methods* 2005; **2**: 932–40.
- 7 Kleinfeld D, Mitra PP, Helmchen F, Denk W. Fluctuations and stimulus-induced changes in blood flow observed in individual capillaries in layers 2 through 4 of rat neocortex. *Proc Natl Acad Sci U S A* 1998; **95**: 15741–46.
- 8 Miller MJ, Wei SH, Parker I, Cahalan MD. Two-photon imaging of lymphocyte motility and antigen response in intact lymph node. *Science* 2002; **296**: 1869–73.
- 9 Germain RN, Robey EA, Cahalan MD. A decade of imaging cellular motility and interaction dynamics in the immune system. *Science* 2012; **336**: 1676–81.
- 10 Giel-Moloney M, Krause DS, Chen G, Van Etten RA, Leiter AB. Ubiquitous and uniform in vivo fluorescence in ROSA26-EGFP BAC transgenic mice. *Genesis* 2007; **45**: 83–89.
- 11 Hasegawa Y, Daitoku Y, Sekiguchi K *et al.* Novel ROSA26 Cre-reporter knock-in C57BL/6N mice exhibiting green emission before and red emission after Cre-mediated recombination. *Exp Anim* 2013; **62**: 295–304.
- 12 Abe T, Kiyonari H, Shioi G *et al.* Establishment of conditional reporter mouse lines at ROSA26 locus for live cell imaging. *Genesis* 2011; **49**: 579–90.
- 13 Rhee JM, Purity MK, Lackan CS *et al.* In vivo imaging and differential localization of lipid-modified GFP-variant fusions in embryonic stem cells and mice. *Genesis* 2006; **44**: 202–18.
- 14 Shioi G, Kiyonari H, Abe T *et al.* A mouse reporter line to conditionally mark nuclei and cell membranes for in vivo live-imaging. *Genesis* 2011; **49**: 570–78.
- 15 Imanishi A, Murata T, Sato M *et al.* A novel morphological marker for the analysis of molecular activities at the single-cell level. *Cell Struct Funct* 2018; **43**: 129–40.
- 16 Miyawaki A, Niino Y. Molecular spies for bioimaging—fluorescent protein-based probes. *Mol Cell* 2015; **58**: 632–43.
- 17 Rodriguez EA, Campbell RE, Lin JY *et al.* The growing and glowing toolbox of fluorescent and photoactive proteins. *Trends Biochem Sci* 2017; **42**: 111–29.
- 18 Förster T. Energiewanderung und Fluoreszenz. *Naturwissenschaften* 1946; **33**: 166–75.
- 19 Jares-Erijman EA, Jovin TM. FRET imaging. *Nat Biotechnol* 2003; **21**: 1387–95.
- 20 Terai K, Matsuda M. Ras binding opens c-Raf to expose the docking site for mitogen-activated protein kinase kinase. *EMBO Rep* 2005; **6**: 251–55.
- 21 Mochizuki N, Yamashita S, Kurokawa K *et al.* Spacio-temporal images of growth factor-induced activation of Ras and Rap1. *Nature* 2001; **411**: 1065–68.
- 22 Lecoq J, Orlova N, Grewe BF. Wide. Fast. Deep: Recent advances in multiphoton microscopy of in vivo neuronal activity. *J Neurosci* 2019; **39**: 9042–52.
- 23 Peron S, Chen T-W, Svoboda K. Comprehensive imaging of cortical networks. *Curr Opin Neurobiol* 2015; **32**: 115–23.
- 24 Yang W, Yuste R. In vivo imaging of neural activity. *Nat Methods* 2017; **14**: 349–59.
- 25 Cyster JG. B cell follicles and antigen encounters of the third kind. *Nat Immunol* 2010; **11**: 989–96.

- 26 Okada T. Two-photon microscopy analysis of leukocyte trafficking and motility. *Semin Immunopathol* 2010; **32**: 215–25.
- 27 Borregaard N. Neutrophils, from marrow to microbes. *Immunity* 2010; **33**: 657–70.
- 28 Megens RTA, Kemmerich K, Pyta J, Weber C, Soehnlein O. Intravital imaging of phagocyte recruitment. *Thromb Haemost* 2011; **105**: 802–10.
- 29 Kolaczowska E, Kubes P. Neutrophil recruitment and function in health and inflammation. *Nat Rev Immunol* 2013; **13**: 159–75.
- 30 Kamioka Y, Sumiyama K, Mizuno R *et al*. Live imaging of protein kinase activities in transgenic mice expressing FRET biosensors. *Cell Struct Funct* 2012; **37**: 65–73.
- 31 Mizuno R, Kamioka Y, Kabashima K *et al*. In vivo imaging reveals PKA regulation of ERK activity during neutrophil recruitment to inflamed intestines. *J Exp Med* 2014; **211**: 1123–36.
- 32 Mizuno R, Kamioka Y, Sakai Y, Matsuda M. Visualization of signaling molecules during neutrophil recruitment in transgenic mice expressing FRET biosensors. *Methods Mol Biol* 2016; **1422**: 149–60.
- 33 McDonald B, Pittman K, Menezes GB *et al*. Intravascular danger signals guide neutrophils to sites of sterile inflammation. *Science* 2010; **330**: 362–66.
- 34 Wang J, Hossain M, Thanabalasuriar A, Gunzer M, Meininger C, Kubes P. Visualizing the function and fate of neutrophils in sterile injury and repair. *Science* 2017; **358**: 111–16.
- 35 Woodfin A, Voisin MB, Beyrau M *et al*. The junctional adhesion molecule JAM-C regulates polarized transendothelial migration of neutrophils in vivo. *Nat Immunol* 2011; **12**: 761–69.
- 36 David BA, Kubes P. Exploring the complex role of chemokines and chemoattractants in vivo on leukocyte dynamics. *Immunol Rev* 2019; **289**: 9–30.
- 37 Denis C, Methia N, Frenette PS *et al*. A mouse model of severe von Willebrand disease: Defects in hemostasis and thrombosis. *Proc Natl Acad Sci U S A* 1998; **95**: 9524–29.
- 38 Kulkarni S, Dopheide SM, Yap CL *et al*. A revised model of platelet aggregation. *J Clin Invest* 2000; **105**: 783–91.
- 39 Falati S, Gross P, Merrill-Skoloff G, Furie BC, Furie B. Real-time in vivo imaging of platelets, tissue factor and fibrin during arterial thrombus formation in the mouse. *Nat Med* 2002; **8**: 1175–81.
- 40 Nishimura S, Manabe I, Nagasaki M *et al*. In vivo imaging visualizes discoid platelet aggregations without endothelium disruption and implicates contribution of inflammatory cytokine and integrin signaling. *Blood* 2012; **119**: e45–56.
- 41 Mazzucato M, Pradella P, Cozzi MR, De Marco L, Ruggeri ZM. Sequential cytoplasmic calcium signals in a 2-stage platelet activation process induced by the glycoprotein Ibalph mechanoreceptor. *Blood* 2002; **100**: 2793–800.
- 42 Maxwell MJ, Westein E, Nesbitt WS, Giuliano S, Dopheide SM, Jackson SP. Identification of a 2-stage platelet aggregation process mediating shear-dependent thrombus formation. *Blood* 2007; **109**: 566–76.
- 43 Stalker TJ, Traxler EA, Wu J *et al*. Hierarchical organization in the hemostatic response and its relationship to the platelet-signaling network. *Blood* 2013; **121**: 1875–85.
- 44 Bye AP, Unsworth AJ, Gibbins JM. Platelet signaling: A complex interplay between inhibitory and activatory networks. *J Thromb Haemost* 2016; **14**: 918–30.
- 45 Li Z, Zhang G, Feil R, Han J, Du X. Sequential activation of p38 and ERK pathways by cGMP-dependent protein kinase leading to activation of the platelet integrin alphaIIb beta3. *Blood* 2006; **107**: 965–72.
- 46 Garcia A, Shankar H, Murugappan S, Kim S, Kunapuli SP. Regulation and functional consequences of ADP receptor-mediated ERK2 activation in platelets. *Biochem J* 2007; **404**: 299–308.
- 47 Salzman EW, Kensler PC, Levine L. Cyclic 3',5'-adenosine monophosphate in human blood platelets. IV. Regulatory role of cyclic amp in platelet function. *Ann N Y Acad Sci* 1972; **201**: 61–71.
- 48 Smolenski A. Novel roles of cAMP/cGMP-dependent signaling in platelets. *J Thromb Haemost* 2012; **10**: 167–76.
- 49 Raslan Z, Naseem KM. The control of blood platelets by cAMP signalling. *Biochem Soc Trans* 2014; **42**: 289–94.
- 50 Versteeg HH, Heemskerk JW, Levi M, Reitsma PH. New fundamentals in hemostasis. *Physiol Rev* 2013; **93**: 327–58.
- 51 Jensen BO, Selheim F, Doskeland SO, Gear AR, Holmsen H. Protein kinase A mediates inhibition of the thrombin-induced platelet shape change by nitric oxide. *Blood* 2004; **104**: 2775–82.
- 52 Hiratsuka T, Sano T, Kato H *et al*. Live imaging of extracellular signal-regulated kinase and protein kinase A activities during thrombus formation in mice expressing biosensors based on Förster resonance energy transfer. *J Thromb Haemost* 2017; **15**: 1487–99.
- 53 Shaw TJ, Martin P. Wound repair: A showcase for cell plasticity and migration. *Curr Opin Cell Biol* 2016; **42**: 29–37.
- 54 Haeger A, Wolf K, Zegers MM, Friedl P. Collective cell migration: Guidance principles and hierarchies. *Trends Cell Biol* 2015; **25**: 556–66.
- 55 Rorth P. Fellow travellers: Emergent properties of collective cell migration. *EMBO Rep* 2012; **13**: 984–91.
- 56 Hiratsuka T, Fujita Y, Naoki H, Aoki K, Kamioka Y, Matsuda M. Intercellular propagation of extracellular signal-regulated kinase activation revealed by in vivo imaging of mouse skin. *eLife* 2015; **4**: e05178.
- 57 Aoki K, Kumagai Y, Sakurai A *et al*. Stochastic ERK activation induced by noise and cell-to-cell propagation regulates cell density-dependent proliferation. *Mol Cell* 2013; **52**: 529–40.
- 58 Aoki K, Kondo Y, Naoki H, Hiratsuka T, Itoh RE, Matsuda M. Propagating wave of ERK activation orients collective cell migration. *Dev Cell* 2017; **43**: 305–17.
- 59 Muta Y, Fujita Y, Sumiyama K *et al*. Composite regulation of ERK activity dynamics underlying tumour-specific traits in the intestine. *Nat Commun* 2018; **9**: 2174.
- 60 Sano T, Kobayashi T, Negoro H *et al*. Intravital imaging of mouse urothelium reveals activation of extracellular signal-regulated kinase by stretch-induced intravesical release of ATP. *Physiol Rep* 2016; **4**: e13033.
- 61 Sano T, Kobayashi T, Ogawa O, Matsuda M. Gliding basal cell migration of the urothelium during wound healing. *Am J Pathol* 2018; **188**: 2564–73.
- 62 Condeelis JS, Segall JE. Intravital imaging of cell movement in tumours. *Nat Rev Cancer* 2003; **3**: 921–30.
- 63 Ellenbroek SI, van Rheenen J. Imaging hallmarks of cancer in living mice. *Nat Rev Cancer* 2014; **14**: 406–18.
- 64 Miller MA, Weissleder R. Imaging of anticancer drug action in single cells. *Nat Rev Cancer* 2017; **17**: 399–414.
- 65 Margarido AS, Bornes L, Vennin C, van Rheenen J. Cellular plasticity during metastasis: New insights provided by intravital microscopy. *Cold Spring Harbor Perspect Med* 2019; a037267. <https://doi.org/10.1101/cshperspect.a037267>
- 66 Hirata E, Kiyokawa E. Future perspective of single-molecule FRET biosensors and intravital FRET microscopy. *Biophys J* 2016; **111**: 1103–11.
- 67 Farina KL, Wyckoff JB, Rivera J *et al*. Cell motility of tumor cells visualized in living intact primary tumors using green fluorescent protein. *Cancer Res* 1998; **58**: 2528–32.
- 68 Wang W, Wyckoff JB, Frohlich VC *et al*. Single cell behavior in metastatic primary mammary tumors correlated with gene expression patterns revealed by molecular profiling. *Cancer Res* 2002; **62**: 6278–88.

- 69 Brown EB, Campbell RB, Tsuzuki Y *et al.* In vivo measurement of gene expression, angiogenesis and physiological function in tumors using multiphoton laser scanning microscopy. *Nat Med* 2001; **7**: 864–68.
- 70 Imamura T, Saitou T, Kawakami R. In vivo optical imaging of cancer cell function and tumor microenvironment. *Cancer Sci* 2018; **109**: 912–18.
- 71 Takaoka S, Kamioka Y, Takakura K *et al.* Live imaging of TAK1 activation in Lewis lung carcinoma 3LL cells implanted into syngeneic mice and treated with polyI:C. *Cancer Sci* 2016; **107**: 644–52.
- 72 Winkler F, Kienast Y, Fuhrmann M *et al.* Imaging glioma cell invasion in vivo reveals mechanisms of dissemination and peritumoral angiogenesis. *GLIA* 2009; **57**: 1306–15.
- 73 Hirata E, Yukinaga H, Kamioka Y *et al.* In vivo fluorescence resonance energy transfer imaging reveals differential activation of Rho-family GTPases in glioblastoma cell invasion. *J Cell Sci* 2012; **125**: 858–68.
- 74 Sahai E. Illuminating the metastatic process. *Nat Rev Cancer* 2007; **7**: 737–49.
- 75 Croft DR, Olson MF. Regulating the conversion between rounded and elongated modes of cancer cell movement. *Cancer Cell* 2008; **14**: 349–51.
- 76 Sanz-Moreno V, Gadea G, Ahn J *et al.* Rac activation and inactivation control plasticity of tumor cell movement. *Cell* 2008; **135**: 510–23.
- 77 Yukinaga H, Shionyu C, Hirata E *et al.* Fluctuation of Rac1 activity is associated with the phenotypic and transcriptional heterogeneity of glioma cells. *J Cell Sci* 2014; **127**: 1805–15.
- 78 Hutchinson JN, Muller WJ. Transgenic mouse models of human breast cancer. *Oncogene* 2000; **19**: 6130–37.
- 79 Kumagai Y, Naoki H, Nakasho E, Kamioka Y, Kiyokawa E, Matsuda M. Heterogeneity in ERK activity as visualized by in vivo FRET imaging of mammary tumor cells developed in MMTV-Neu mice. *Oncogene* 2015; **34**: 1051–57.
- 80 Mueller H, Flury N, Eppenberger-Castori S, Kueng W, David F, Eppenberger U. Potential prognostic value of mitogen-activated protein kinase activity for disease-free survival of primary breast cancer patients. *Int J Cancer* 2000; **89**: 384–88.
- 81 Milde-Langosch K, Bamberger AM, Rieck G *et al.* Expression and prognostic relevance of activated extracellular-regulated kinases (ERK1/2) in breast cancer. *Br J Cancer* 2005; **92**: 2206–15.
- 82 Svensson S, Jirstrom K, Ryden L *et al.* ERK phosphorylation is linked to VEGFR2 expression and Ets-2 phosphorylation in breast cancer and is associated with tamoxifen treatment resistance and small tumors with good prognosis. *Oncogene* 2005; **24**: 4370–79.
- 83 Bergqvist J, Elmberger G, Ohd J *et al.* Activated ERK1/2 and phosphorylated oestrogen receptor alpha are associated with improved breast cancer survival in women treated with tamoxifen. *Eur J Cancer* 2006; **42**: 1104–12.
- 84 Matsumura Y, Maeda H. A new concept for macromolecular therapeutics in cancer chemotherapy: Mechanism of tumor-tropic accumulation of proteins and the antitumor agent smancs. *Cancer Res* 1986; **46**: 6387–92.
- 85 Yuan F, Dellian M, Fukumura D *et al.* Vascular permeability in a human tumor xenograft: Molecular size dependence and cutoff size. *Cancer Res* 1995; **55**: 3752–56.
- 86 Gerlowski LE, Jain RK. Microvascular permeability of normal and neoplastic tissues. *Microvasc Res* 1986; **31**: 288–305.
- 87 Harney AS, Arwert EN, Entenberg D *et al.* Real-time imaging reveals local, transient vascular permeability, and tumor cell intravasation stimulated by TIE2hi macrophage-derived VEGFA. *Cancer Discovery* 2015; **5**: 932–43.
- 88 Weis SM, Cheresh DA. Pathophysiological consequences of VEGF-induced vascular permeability. *Nature* 2005; **437**: 497–504.
- 89 Irie K, Fujii E, Ishida H *et al.* Inhibitory effects of cyclic AMP elevating agents on lipopolysaccharide (LPS)-induced microvascular permeability change in mouse skin. *Br J Pharmacol* 2001; **133**: 237–42.
- 90 Lum H, Jaffe HA, Schulz IT, Masood A, RayChaudhury A, Green RD. Expression of PKA inhibitor (PKI) gene abolishes cAMP-mediated protection to endothelial barrier dysfunction. *Am J Physiol* 1999; **277**: C580–88.
- 91 Fukuhara S, Sakurai A, Sano H *et al.* Cyclic AMP potentiates vascular endothelial cadherin-mediated cell-cell contact to enhance endothelial barrier function through an Epac-Rap1 signaling pathway. *Mol Cell Biol* 2005; **25**: 136–46.
- 92 Yamauchi F, Kamioka Y, Yano T, Matsuda M. In vivo FRET imaging of tumor endothelial cells highlights a role of low PKA activity in vascular hyperpermeability. *Cancer Res* 2016; **76**: 5266–76.
- 93 Xiong Y, Huo Y, Chen C *et al.* Vascular endothelial growth factor (VEGF) receptor-2 tyrosine 1175 signaling controls VEGF-induced von Willebrand factor release from endothelial cells via phospholipase C-gamma 1- and protein kinase A-dependent pathways. *J Biol Chem* 2009; **284**: 23217–24.
- 94 Maeda H, Fang J, Inutsuka T, Kitamoto Y. Vascular permeability enhancement in solid tumor: Various factors, mechanisms involved and its implications. *Int Immunopharmacol* 2003; **3**: 319–28.
- 95 Hirata E, Sahai E. Tumor microenvironment and differential responses to therapy. *Cold Spring Harbor Perspect Med* 2017; **7**: a026781.
- 96 Hirata E, Girotti MR, Viros A *et al.* Intravital imaging reveals how BRAF inhibition generates drug-tolerant microenvironments with high integrin beta1/FAK signaling. *Cancer Cell* 2015; **27**: 574–88.
- 97 Sahai E, Astsaturov I, Cukierman E *et al.* A framework for advancing our understanding of cancer-associated fibroblasts. *Nat Rev Cancer* 2020; **20**: 174–86.
- 98 Blomstrand H, Scheibling U, Brattahl C, Green H, Elander NO. Real world evidence on gemcitabine and nab-paclitaxel combination chemotherapy in advanced pancreatic cancer. *BMC Cancer* 2019; **19**: 40.
- 99 Timpson P, McGhee EJ, Morton JP *et al.* Spatial regulation of RhoA activity during pancreatic cancer cell invasion driven by mutant p53. *Cancer Res* 2011; **71**: 747–57.
- 100 Vennin C, Chin VT, Warren SC *et al.* Transient tissue priming via ROCK inhibition uncouples pancreatic cancer progression, sensitivity to chemotherapy, and metastasis. *Sci Transl Med* 2017; **9**: eaai8504.
- 101 Joseph J, Radulovich N, Wang T, Raghavan V, Zhu CQ, Tsao MS. Rho guanine nucleotide exchange factor ARHGEF10 is a putative tumor suppressor in pancreatic ductal adenocarcinoma. *Oncogene* 2020; **39**: 308–21.
- 102 Lutz MP, Eßer IBS, Flossmann-Kast BBM *et al.* Overexpression and activation of the tyrosine kinase Src in human pancreatic carcinoma. *Biochem Biophys Res Commun* 1998; **243**: 503–08.
- 103 Donahue TR, Tran LM, Hill R *et al.* Integrative survival-based molecular profiling of human pancreatic cancer. *Clin Cancer Res* 2012; **18**: 1352–63.
- 104 Renouf DJ, Moore MJ, Hedley D *et al.* A phase I/II study of the Src inhibitor saracatinib (AZD0530) in combination with gemcitabine in advanced pancreatic cancer. *Invest New Drugs* 2012; **30**: 779–86.

- 105 Evans TRJ, Van Cutsem E, Moore MJ *et al.* Phase 2 placebo-controlled, double-blind trial of dasatinib added to gemcitabine for patients with locally-advanced pancreatic cancer. *Ann Oncol* 2016; **28**: 354–61.
- 106 Nobis M, McGhee EJ, Morton JP *et al.* Intravital FLIM-FRET imaging reveals dasatinib-induced spatial control of Src in pancreatic cancer. *Cancer Res* 2013; **73**: 4674–86.
- 107 Lee S, Vinegoni C, Sebas M, Weissleder R. Automated motion artifact removal for intravital microscopy, without a priori information. *Sci Rep* 2014; **4**: 4507.
- 108 Warren SC, Nobis M, Magenau A *et al.* Removing physiological motion from intravital and clinical functional imaging data. *eLife* 2018; **7**: e35800.