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Real-Time Multiplex Kinase Phosphorylation Sensors in Living Cells

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ABSTRACT: Phosphorylation is an important post-translational modification implicated in cellular signaling and regulation. However, current methods to study protein phosphorylation by various kinases lack spatiotemporal resolution or the ability to simultaneously observe in real-time the activity of multiple kinases in live cells. We present a peptide biosensor strategy with Time Correlated Single Photon Counting-Fluorescence Lifetime Imaging (TCSPC-FLIM) to interrogate the spatial and temporal dynamics of VEGFR-2 and AKT phosphorylation activity in real-time in live 2D and 3D cell culture models at single cell resolution. By recording the increase in fluorescence lifetime due to a change in the solvatochromic environment of the sensor upon phosphorylation, we demonstrate that spatio-temporal maps of protein kinase activity can be obtained. Our results suggest that fluorescence lifetime imaging of peptide biosensors can be effectively and specifically used to monitor and quantify phosphorylation of multiple kinases in live cells.

KEYWORDS: Peptide biosensor, FLIM, kinase phosphorylation, live 2D and 3D cultures, zebrafish, multiplex singe cell monitoring.

Pivotal cellular decisions comprising of cell proliferation, migration, apoptosis, and other diverse physiological functions rely on precise control of the cells' response to extracellular stimulation[1]. The specificity of a cell's response to a receptor stimuli involves transmission of the signal from the receptor to its downstream signaling network[2]. Kinase phosphorylation plays an important role as a signal transmitter in the intracellular signaling cascade[3]. Upon phosphorylation, the protein substrate is subjected to conformational changes[4], altered subcellular localization[5], or interaction with adaptor protein to elicit a change in its activity as a response. This phosphorylation event triggers subsequent biochemical events, including succeeding phosphorylation cascade, that eventually results in a cellular response constituting cell metabolism, survival, proliferation, morphological change and alteration in gene expression[6]. In many cases, multiple kinases are involved in the regulation of downstream proteins in the signaling network, allowing complex, combinatorial regulation at the post-translational level. In addition, the heterogeneity of protein kinase subcellular localization and its differential activity based on its location and the type of extracellular signal reveals the intricate nature of kinase signaling network. This complex regulatory system poses a challenge in real-time monitoring of kinase signaling. Therefore, identification of the activated kinase within the signaling cascade, and monitoring its differential activity at single molecule sensitivity in live cells will be crucial to understanding the interaction of small molecule inhibitors, delineating disease etiology, or uncovering drug resistant mechanism. Conventional methods to measure protein phosphorylation involves the use of radioactive labeling[7], western blot[8], and mass spectrometry[9]. Despite their extensive utility, these are ensemble techniques requiring millions of cells and do not consider intra-population heterogeneity of kinase activity. Real-time monitoring of kinase activity in live cells, on the other hand, allows direct and specific measurement of kinase activity in a specific signaling pathway in live single cells in response to an external stimulus in a physiological setting.

Past efforts[10-13] using genetically engineered probes or peptide biosensor have successfully measured the activity of protein kinases and have noted the subcellular heterogeneity of the kinases involved. However, some of the methods still rely on cell lysates and utilize Förster Resonance Energy Transfer (FRET), or intensity-based techniques which may pose a challenge for multiplex evaluation. FLIM is superior to fluorescence intensity-based methods because it is less susceptible to excitation power and relatively independent of probe concentration. In this work, we propose a biosensing methodology based on TCSPC-FLIM[14-16] utilizing multiple peptide biosensors in live cells, a key novelty of our work. Our approach only requires a single reporter for each sensor. In addition, TCSPC-FLIM enables discrimination of specific species based on its lifetime at picosecond resolution. Taken together, these features make TCSPC-FLIM a practical platform for multiplex monitoring of kinase phosphorylation in real time. VEGFR-2 (Vascular Endothelial Growth Factor Receptor-2), signaling pathway plays a key role indiverse cellular functions, especially in the formation of blood vessels such vasculogenesis and angiogenesis[17]. Protein kinase B, also known as AKT, is a downstream signaling protein of VEGFR-2, which is shared by

multiple pathways[18]. In the past, immunoblotting[19], RNA interference[20-21], and pharmacological inhibition[22] have shown that differential response of VEGFR-2-AKT signaling could result in diverse cellular response. Therefore, developing a strategy to monitor the kinase signaling between VEGFR-2 and its downstream kinase, AKT will be of broad interest to researchers, especially in drug development, oncology, synthetic biology, biomaterials, and toxicology, both from a basic and applied perspective.

MATERIALS AND METHODS

Peptide Biosensor. Peptide sensors were designed based on a well-defined preferential substrate motif for each kinase. A delivery sequence is included in the main kinase-recognition sequence of each sensor. VEGFR-2 sensor, VSOR, contains a VEGFR-2 kinase domain with phosphorylatable Tyrosine site. The complete sequence for VSOR is: GRRRAAPEDLYK(5-FAM) DFLTGRKKRRQRRRQ). The Akt sensor, ASOR, contains the phosphorylatable serine site. The amino acids sequence for ASOR is: ARKRERAYSFK(5-FAM) HHARKKRRQRRRPQ. The excitation/emission for 5-FAM is 492/518 nm. To facilitate multiplex detection, Cy5 fluorophore with excitation/emission at 649/670 nm was used in ASOR. The sequence for non-phosphorylatable ASOR is: ARKRERAYCFK(5-FAM)HHARKKRRQRRRPQ, and phosphorylated ASOR is ARKRERAY(Serinephospho)FK(5-FAM)HHARKKRRQRR RPQ. All peptides were prepared by Alpha Diagnostic at 90 % purity. Characterization of labeled materials is provided in the Supporting Information (SI).

***In-vitro* 2D Cell Culture and *In-vitro* 3D Vasculogenesis Model.** Human endothelial colony forming cells (ECFC) were obtained as a kind gift from Dr. Mervin Yoder (Indiana University School of Medicine, Indianapolis, IN) and cultured as previously described[29]. Details of the 2D cell culture and treatments are provided
In SI. The 3D vasculogenesis model was built in a multi-tissue interface format as previously described[23] with slight modifications. Details are provided in SI Figure 14.

Sensor Delivery and Imaging in 2D and 3D Cultures. Live cells in 2D and 3D cultures were incubated with the respective biosensors in serum free medium for 20 minutes prior to FLIM imaging experiments. Cells were washed and maintained in the culture medium and live cell imaging chamber with controlled temperature at 37°C.

TCSPC-FLIM. FLIM experiments utilized a scanning confocal time-resolved microscope system (MicroTime 200, PicoQuant, Germany)[15-16, 24-27] that allows precise recording of the arrival time of the at the detector. The instrument Response Function (IRF)[27] was taken into consideration during measurement and data analysis. Detailed schematic of the instrumentation and analysis steps are provided in SI Fig. 9 and 10.

RESULTS AND DISCUSSION

Sensor Design and Delivery

The biosensor is a peptide substrate, targeting the specific protein kinase, VEGFR-2 (VSOR, Fig. 1a top) and AKT (ASOR, Fig. 1a bottom) designed from known

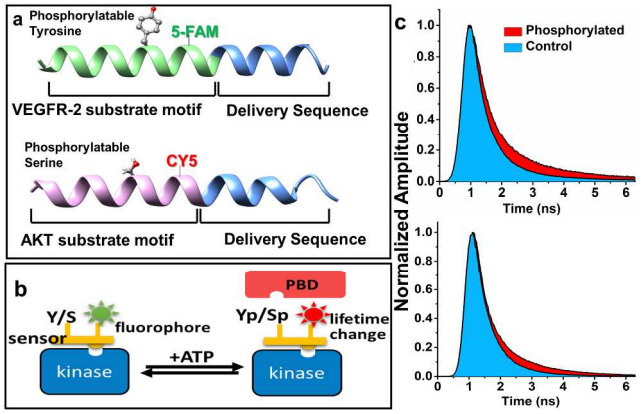
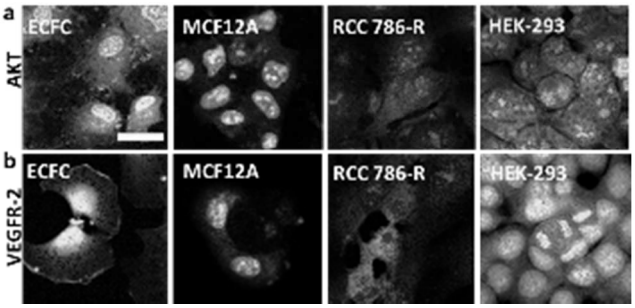


Figure 1. (a) Schematic of VEGFR-2 and AKT peptide sensor design. (b) Working principle of (a) (VEGFR-2 (i) and (a) AKT (ii) peptide kinase biosensors for targeting the respective kinase due to its sequence specificity. Upon phosphorylation, the phosphotyrosine (Yp) or phosphoserine (Sp) will bind to the intracellular phosphobinding domain (PBD). The binding of phosphotyrosine or phosphoserine causes solvatochromic changes in the microenvironment of the sensor that increase the fluorescence lifetime of the reporter labeled sensor. (c) TCSPC of VSOR (i) and ASOR (ii) shows longer fluorescence decay (red) upon kinase phosphorylation in stimulated cells compared to the control (blue) in non-stimulated cells.

consensus motif of the respective kinases. In this design, the respective peptide substrates contain known preferential substrate motif for; AKT[28] (Fig. 1a bottom, light red segment) and VEGFR-2[29] (Fig. 1a top, light green segment). Each biosensor also contains a phosphorylatable site; serine for AKT, and tyrosine for VEGFR-2. For live-cell kinase monitoring, a cell-penetrating peptide was linked to the consensus substrate motif of the sensor (Fig. 1a (top and bottom, blue segment)) to aid in sensor uptake by live cells[30]. A fluorophore was conjugated to the lysine residue adjacent (+2 or +3 amino acids) to the phosphorylation site (Fig. 1a, VSOR fluorophore is 5-FAM, ASOR fluorophore is Cy5) to report on a phosphorylation event. In Figure 1b we summarize the working principle of the biosensor. Live cells were incubated with the peptide sensors. After the sensor was internalized by the cells, each peptide sensor can be preferentially recognized by the respective target kinase due to the presence of a consensus motif. In our work, ASOR is recognized by AKT and VSOR is recognized by VEGFR-2.

Upon phosphorylation by the target kinase, phosphoserine of ASOR will bind to the phosphoserine-binding domain of AKT, while phosphortyrosine of the VSOR will bind to the phosphotyrosine-binding domain of VEGFR-2, due to their sequence specificity. The binding of the phosphorylated peptide sensor to the cellular phosphobinding domain will elicit a change in the solvatochromic environment of the sensor fluorophore reporter. This change alters the fluorescence lifetime of the fluorophore reporter in a phosphorylation dependent manner (Fig. 1b)[14]. As a proof of above mentioned concept, both sensors demonstrated longer fluorescence decay curves (Fig. 1c), after kinase phosphorylation stimulation for 20

minutes (VEGF for VSOR, Insulin for ASOR) in ECFC, resulting in an increase in the lifetime of the respective fluorophore reporters (28% increase for VSOR and 32% increase for ASOR). The efficacy of cellular uptake of both the sensors, ASOR (Fig. 2a) and VSOR (Fig. 2b) was tested in five different cell types. Experiments indicated that the sensors were rapidly internalized within 20 minutes. Interestingly, each biosensor entered the cells through distinct mechanisms (Supplementary Fig. 2a-d). VSOR was internalized via an energy independent process while ASOR uptake was mediated through an energy dependent process[31]. Immunofluorescence validated the inherent



subcellular location of VSOR and ASOR, as depicted in SI (Supplementary Fig. 3a, b).

Figure 2. (a) ASOR and (b) VSOR are internalized by different cell lines. Fluorescence images after 20 minutes of incubation with ASOR-Cy5 (a) and VSOR-5-FAM (b) (20 μM each) in different cell types cultured on 2D glass coverslips at 37°C. Scale bar=20 μm.

Real-Time Phosphorylation Sensing

To assess whether the shift in fluorescence lifetime of ASOR corresponds to AKT phosphorylation activity, five positive controls were developed: (i) phosphorylated ASOR peptide, (ii) AKT-specific small molecule activator (Fumonisin B), (iii) cell surface receptor agonist (insulin), (iv) MCF12a p53 (-/-) mutant cell with constitutively active AKT (Supplementary Fig. 4a-e, k), and (v) TGF-β stimulated MCF10A (Supplementary Fig. 5a-c). All positive controls exhibited a longer fluorescence lifetime compared to controls (Supplementary Fig. 4b, c). Two negative controls were implemented: (i) an AKT-specific inhibitor, Honokiol, and (ii) a non-phosphorylatable mutant ASOR peptide (Supplementary Fig. 4 f-j). Compared to insulin stimulated cells, negative controls for ASOR demonstrated a 40% decrease in fluorescence lifetime with Honokiol at 2.44 ns and the mutant ASOR at 2.33 ns (Supplementary Fig. 4.f-j, k). A similar approach was carried out for VSOR with the VEGFR-2 agonist, VEGF, as the positive control (Supplementary Fig. 6b, i), and VEGFR specific small molecule inhibitor, Axitinib, as the negative control (Supplementary Fig. 6c-i). Real-time tracking was performed for each sensor in human endothelial colony forming cells (ECFC) where either ASOR (Supplementary Fig. 7a, b) or VSOR (Supplementary Fig.8 a, b) were internalized, and they both exhibited a 35% increase in fluorescence lifetime within 20 minutes after stimulation with the respective kinase activator.

In addition to monitoring the temporal dynamics of the VSOR signal, distinct VSOR spatial profiles were obtained, showing a nuclear localization (Supplementary Fig.8a) after 20 minutes of stimulation with 20 ng/mL VEGF. Our findings are in agreement with previous reports on VEGFR-2 nuclear localization upon phosphorylation[32].

Multiplex Kinase Phosphorylation in 2D Cell Cultures

After validating the performance of each sensor, we next evaluate its potential in multiplex monitoring of ASOR and VSOR phosphorylation in live ECFC.

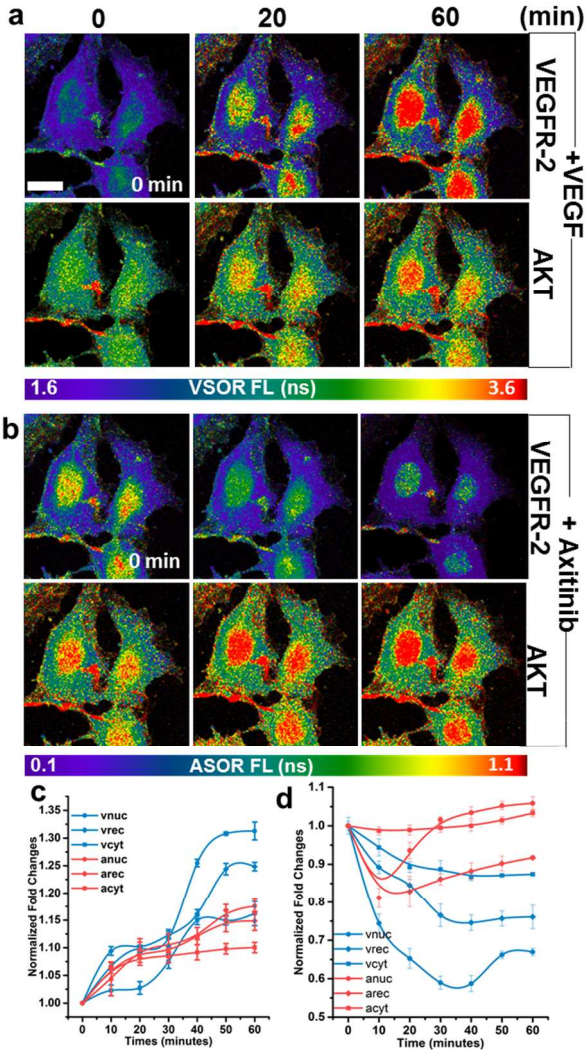


Figure 3. Real-time multiplex monitoring of VEGFR-2 and AKT phosphorylation in 2D culture. (a) FLIM images and (c) quantitative analysis of VSOR and ASOR upon 20 ng/mL of VEGF stimulation (b) FLIM images and quantitative analysis (d) of VSOR and ASOR treated for 60 minutes with 10 nM of the inhibitor, Axitinib in ECFC. Quantitative analysis was conducted on three cellular compartments: cell nucleus (nuc), receptor(rec) and cytoplasm (cyt) compared to control (top). Scale bar = 20 um. n = 3 biological replicates.

We replaced the fluorophore reporter 5-FAM (Ex/Em 492/518 nm) of the ASOR with Cy5 (Ex/Em 649/670 nm) so that the emission signals of both the ASOR and VSOR can be detected simultaneously with two separate highly sensitive single photon avalanche diode (SPAD)

detectors (Supplementary Fig. 9). VEGFR-2–AKT signaling cascade was observed during real-time multiplex monitoring of VSOR and ASOR (Fig. 3a-d). Within 20 minutes of VEGF stimulation, VSOR exhibited an increase in fluorescence lifetime in all three major cellular compartments; cell receptor (membrane area) (25% increase compared to t=0), cytoplasm (30% increase from t=0) and nucleus (35% increase compared to t=0) (Fig 3a, b, Supplementary Fig. 10). Subsequently, ASOR also exhibited an increase in fluorescence lifetime indicating an increase in AKT activity at the receptor, cytoplasm and nucleus (Fig.3a, c). Increase in the lifetime signal of both VSOR and ASOR following VEGF stimulation Fig.3a, c) demonstrates the signaling cascade between VEGFR-2 and its downstream AKT. We also show that the stimulated signals can be inhibited and the rate of inhibition can be observed (Fig. 3b, d). After 60 minutes of VEGF stimulation (Fig.3c, d), Axitinib, a potent VEGFR-2 small molecule inhibitor, was introduced and the signals from both the sensors were continuously monitored for another 60 minutes (Fig. 3c, d). Both VSOR and ASOR signals initially demonstrated a 10% decrease during the first 10 minutes following Axitinib administration. However, ASOR signal increased after 20 minutes while VSOR signal was 45% lower than the initial state (t=0, Fig. 3c, d). To further demonstrate the versatility of our approach, small molecule inhibitor, Sunitinib was utilized in a Sunitinib resistant cell line, 786-R[33] (Supplementary Fig.11a, b), and similar AKT activation was observed. This result is expected, given the fact that AKT is a common signaling protein shared by distinct signaling pathways[34-35] and activated by many extracellular signals[36,37]. Therefore, inhibition of VEGFR-2 alone is not sufficient to inhibit AKT activation.

To further test our multiplex sensors, we tested the inhibition of AKT alone with a potent AKT small molecule inhibitor, Honokiol. We monitored both VSOR and ASOR signal for 60 minutes (Supplementary Fig.12 a, b). ASOR signal continuously decreased up to 10% during 60 minutes while VSOR signal remained relatively constant (P>0.05). Further testing of the sensors was demonstrated using insulin stimulation since insulin and VEGFR-2 signaling pathways share AKT as a common downstream kinase in their otherwise distinct signaling cascades[38-39]. We stimulated ECFC with insulin, an agonist for insulin receptor signaling pathway, in a VEGF free medium and observed the ASOR and VSOR signal for 60 minutes (Supplementary Fig. 13 a, b). As expected, ASOR signal increased 35% within 20 minutes of insulin stimulation (Supplementary Fig.13 a, b). On the other hand, VSOR signal also showed a 10% increase from the initial state, as expected from AKT feedback[40]. These results demonstrate the ability of our multiplex sensing methodology to monitor the direct activity of multiple kinases in real-time.

Multiplex Kinase Phosphorylation monitoring in a 3D Vasculature Model

After validating the VSOR and ASOR sensors to monitor VEGFR-2–AKT signaling cascade in 2D cultures of ECFC, we extended our study to 3D cultures of ECFC. In our tissue-like 3D vasculogenesis model (Fig. 4, Supplementary Fig.14a-c), ECFCs form a vascular 3D networkstructure[23] after 24 hours (Supplementary Fig. 14) of culturing. After 30 minutes of incubation both sensors were internalized inside the 3D vasculature structure (Supplementary Fig. 15a-c). The strategy used in 2D cultures to monitor VEGFR-2 and AKT signaling cascade by VEGF stimulation was utilized in 3D culture.

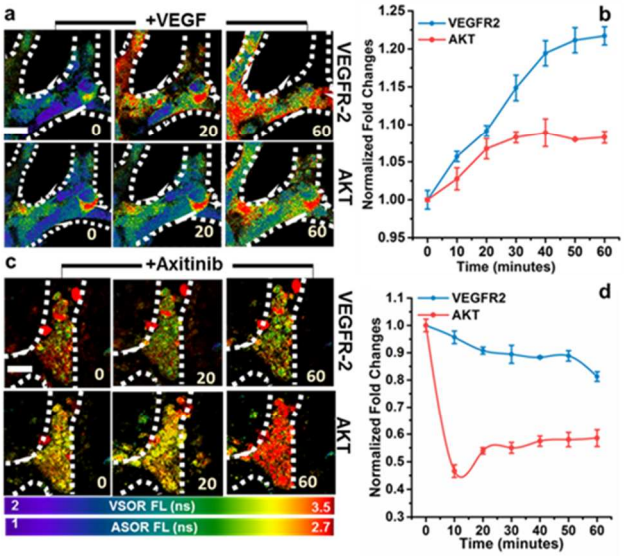


Figure 4. Real-time monitoring of VEGFR-2 and AKT phosphorylation in 3D culture. FLIM images (a, c), and quantitative analysis ((b, d)) for VSOR and ASOR in 3D vasculature during 60 minutes of observation: (a, b) Fluorescence lifetime of VSOR and ASOR increases upon 10 ng/mL VEGF stimulation, (c, d) show that fluorescence lifetime of VSOR and ASOR decreases within 10 minutes upon treatment with the inhibitor, Axitinib at 20 nM. Scale bar = 20 um. n = 3 biological replicates.

Consistent with our results from 2D cultures, upon VEGF stimulation, the fluorescence lifetime of both VSOR and ASOR signal increased (Fig. 4a, b). Different regions of the vasculature structure exhibited distinct amplitude of signal response upon VEGF stimulation (Fig. 4a). As in our 2D culture, we also observed a decrease in the fluorescence lifetime of VSOR and ASOR upon treatment with VEGFR-2 inhibitor Axitinib (Fig. 4c, d), as expected.

Multiplex Kinase Phosphorylation Monitoring in Zebrafish *In-Vivo* Model

After testing the ASOR and VSOR in 2D and 3D culture systems, we then evaluated the performance of both sensors for monitoring VEGFR-2–AKT signaling *in-vivo.* Zebrafish was used as an *in-vivo* model to further demonstrate our sensors and the imaging strategy. The uptake of sensors was highly efficient in the vascular system of anesthetized zebrafish (Fig. 5a, Supplementary Fig. 16 a-f) via intravenous microinjection through caudal vein[41]. Biodistribution assay was also carried out in older 7 days' post fertilization (dpf) larvae (Supplementary Fig. 16c, f) and younger embryo as early as 3 hpf (Supplementary Fig. 16a, d). Notably, rapid sensor uptake was observed within 5 minutes after injection in both ages of the fish and the sensors did not disrupt embryo development into larvae

(Supplementary Fig. 17). The choice of 5 dpf larvae was based on our based on our observation that larvae has a more developed organ than embryo and is more durable

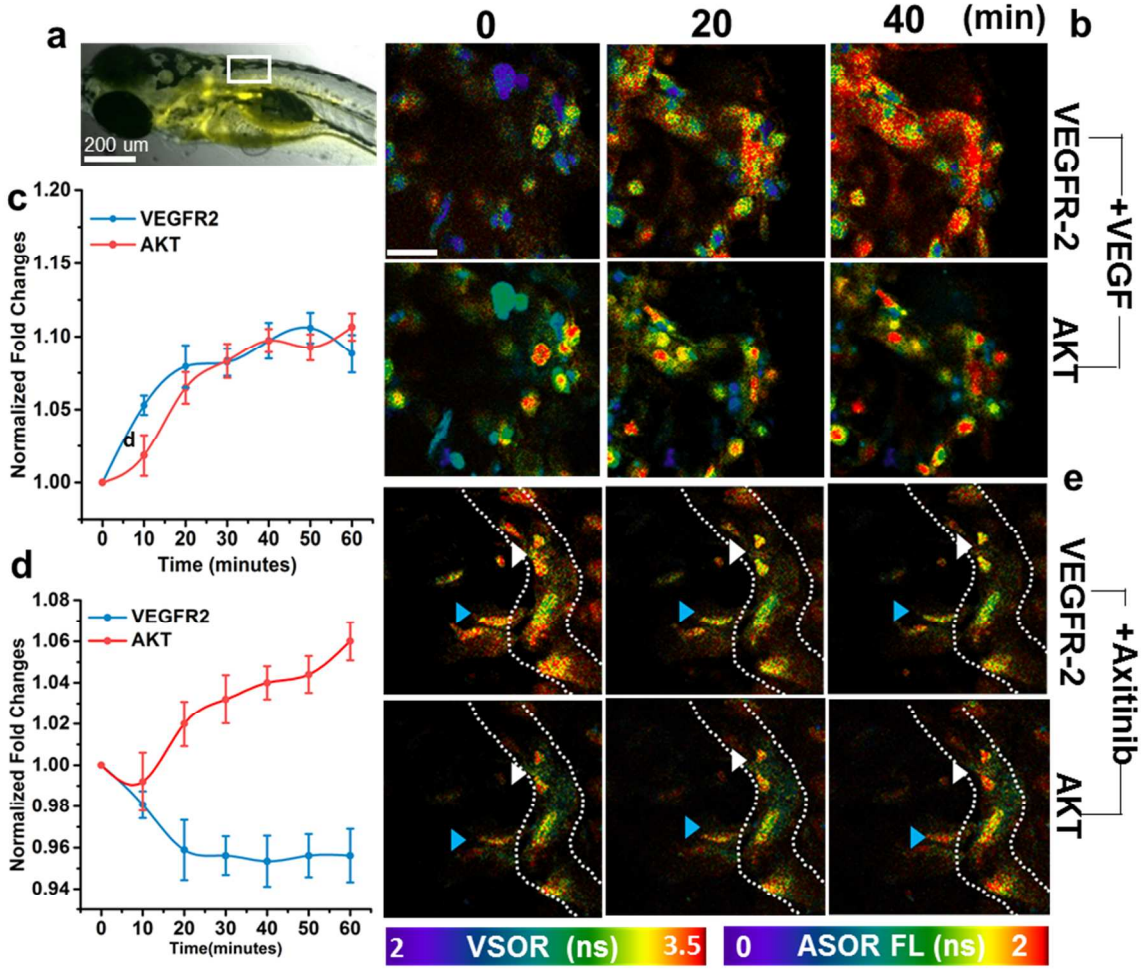


Figure 5. Real-time monitoring of VEGFR-2 and AKT phosphorylation in live zebrafish (a) Sensor distribution in live 5 dpf zebrafish larvae. FLIM images (b, e) and quantitative analysis (c, d) of VSOR and ASOR in the vasculature (white box) of zebrafish depicted in (a) during 60 minutes of observation: (b, c) show that the fluorescence lifetime of VSOR and ASOR increases upon treatment with 100 ng/mL of the VEGF stimulant; (d, e) show a decrease in fluorescence lifetime of VSOR and ASOR within 10 minutes upon treatment with 100 nM of Axitinib. Scale bar = 20 um. n = 3 biological replicates.

when transferring it from the microinjection platform to the FLIM system. Consistent with *in-vitro* 2D and 3D results, VSOR signal within the vasculature area of live zebrafish increased within 20 minutes of VEGF stimulation (Fig. 5a white box, 5b (top), c). Subsequent increase in the signal of VSOR followed by ASOR upon stimulation with 100 ng/mL VEGF (Fig. 5b (bottom), c) was observed. As observed in 2D and 3D cultures, the signal of VSOR decreased upon treatment with 200 nM of Axitinib within 60 minutes of observation (Fig 5e, white and blue arrow, Fig.5d), indicating inhibited activity of VEGFR-2. Within 10 minutes of Axitinib treatment, lower signal of ASOR activity was observed indicating a signaling cascade between VEGFR-2–AKT noting a decrease in VEGFR-2 activity to lower AKT activity. Interestingly, ASOR exhibited increased activity after 10 minutes of inhibition indicating an elevation in AKT activity, which was also observed in 2D and 3D cultures. Our results in live zebrafish are consistent with our observations in 2D and 3D cultures, demonstrating the broad applicability and translation potential of our methodology from single cells to more complex systems, such as 3D cultures and live zebrafish.

CONCLUSION

We demonstrate a sensing and imaging approach to obtain spatiotemporal maps of multiplex kinase activity in live cells, which will allow the interrogation of signaling kinetics in space and time. Our methodology can be adapted to examine multiple kinase activity by expanding the number of kinase sensors with appropriate choice of fluorophores with different dynamic range of fluorescence lifetime. The toolbox

comprising of peptide sensors and lifetime imaging and analysis modules developed in this work presents a powerful strategy to query the kinase signaling cascade and response to drugs within live intact 2D and 3D cultures as well as in zebrafish.

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Supporting Information Available: The following files are available free of charge. Supporting information. Sensor characterization, validation experiment, instrument schematic, quantification step, 3D model, 3D biodistribution. In-vivo model, in-vivo biodistribution.

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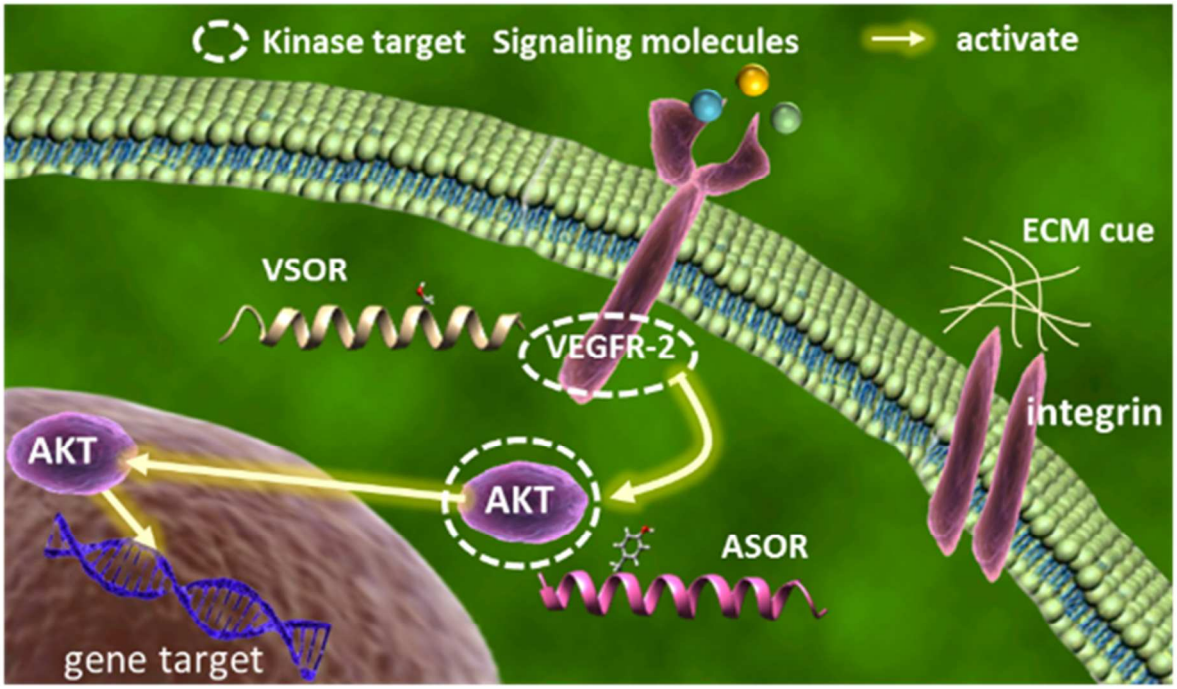
Kinase target
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activate
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ASOR
gene target