pAkt is a marker for poor prognosis in breast cancer

**High throughput time-resolved-FRET reveals Akt/PKB activation as a poor prognostic marker in breast cancer.**

Selvaraju Veeriah\(^1\)\(^\ddagger\)\(^\ddagger\), Pierre Leboucher\(^1\)\(^,\)\(^2\)\(^\ddagger\)\(^\ddagger\), Julien de Naurois\(^1\)\(^,\)\(^3\)\(^\ddagger\)\(^\ddagger\), Nirmal Jethwa\(^1\), Emma Nye\(^4\), Tamara Bunting\(^4\), Richard Stone\(^4\), Gordon Stamp\(^4\), Véronique Calleja\(^1\), Stefanie S. Jeffrey\(^5\), Peter J Parker\(^3\)\(^,\)\(^6\), Banafshé Larijani\(^1\)\(^,\)\(^7\)\(^\star\)

\(^1\) Cell Biophysics, \(^3\) Protein Phosphorylation \(^4\) Experimental Histopathology Laboratories, Cancer Research UK, London Research Institute, London, UK.

\(^2\) Centre Emotion, Hôpital de la Pitié Salpetrière, Paris, France

\(^5\) Stanford University School of Medicine, Stanford, USA

\(^6\) Cancer Division King’s College London, London, UK

\(^7\) Cell Biophysics Laboratory, Unidad de Biofísica (CSIC-UPV/EHU), Sarriena s/n, 48940 Leioa, Spain. Ikerbasque, Basque Foundation for Science

\(^\ddagger\) These authors contributed equally to this work.

\(^\star\) To whom correspondence should be addressed:

Prof. Banafshé Larijani
Cell Biophysics Laboratory, Unidad de Biofísica, University of the Basque Country. Sarriena s/n, 48940 Leioa, Spain. Ikerbasque, Basque Foundation for Science

Email: banafshe.larijani@ikerbasque.org

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ABSTRACT

Dysregulation of the Akt/PKB pathway has been associated with poor prognosis in several human carcinomas. Current approaches to assess Akt activation rely on intensity-based methods, which are limited by the subjectivity of manual scoring and poor specificity. Here we report the development of a novel assay using amplified, time-resolved-FRET, which is highly specific and sensitive and can be adapted to any protein. Using this approach to analyze primary breast tissue microarrays, we quantified levels of activated pAkt at a spatial resolution that revealed molecular heterogeneity within tumors. High pAkt status assessed by amplified-FRET correlated with worse disease-free survival. Our findings support the use of amplified FRET to determine pAkt status in cancer tissues as candidate biomarker for the identification of high-risk patients.
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**INTRODUCTION**

Dysregulation of Akt/PKB (protein kinase B) signaling is considered to be a hallmark of many human cancers (1). In cancer, Akt plays a central role in cell proliferation, survival, glucose metabolism, genome stability, and neo-vascularization (2, 3). Akt also contributes to tumor invasion and metastatic spread by induction of epithelial–mesenchymal transition (EMT) (4). Breast cancer is the most common malignancy diagnosed in women worldwide (1). In breast cancer, Akt activation is correlated with advanced disease, poor prognosis, reduced patient survival, and resistance to radiotherapy (5, 6). Akt is a member of the AGC family of protein serine/threonine kinases and contains an N-terminal pleckstrin homology (PH) domain which interacts with PtdIns(3,4)P2 and PtdIns(3,4,5)P3 (7). Various mechanisms contribute to activation of the Akt pathway in human tumors, including disruption of PTEN, up-regulation of phosphoinositide 3-kinase (PI3K) and mTOR (mammalian target of rapamycin), as well as mutation of Akt itself (8, 9). Stimulation of the epidermal growth factor receptor (EGFR) by epidermal growth factor (EGF) leads to activation of Akt in a PI3K-dependent manner (1, 10). Since Akt activation is both an early event in tumor progression and also a characteristic of many advanced carcinomas, Akt activation status (as opposed to Akt expression level) may represent a useful prognostic biomarker as well as a predictive biomarker in both adjuvant and metastatic settings (11). The appreciation of functional molecular heterogeneity of tumors has far reaching implications for the development of personalized medicine and improving tumor biopsy methodologies for predictive biomarkers (12). The assessment of molecular heterogeneity at the protein level has not been extensively reported, in part due to a lack of technologies that can accurately perform this task directly on histological samples. The accurate quantification of Akt activation status
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as well as its molecular heterogeneity in patient samples is expected to be highly informative.

At present, the detection of endogenous oncoproteins in fixed tumor samples faces several important challenges: i) the accurate quantification of post-translational modifications such as phosphorylation; ii) the simultaneous localization of these oncoprotein epitopes in preserved tissue architecture; and iii) the acquisition and processing of a large quantity of data in an automated, high throughput manner. Immunohistochemistry (IHC) is the most readily available method to assess activation of intracellular proteins such as Akt, however it is limited by the absence of precise quantification and the lack of a standardized scoring system. In addition, it is a “one-site” assay (including fluorophore-based confocal assays) producing results that can only be obtained from measuring the intensity of one label at a time, therefore limiting specificity (13). Here, non-specific antibody binding cannot be excluded. When using color-based (DAB, 3,3’-diaminobenzidine) immunohistochemistry the sensitivity is an issue and IHC techniques based on fluorescence benefit from sensitivity as much as 1000 times higher than DAB based IHC (14, 15). In the context of these issues, it is notable that there are conflicting results for the prognostic significance of Akt activation in primary breast carcinomas using IHC (5, 16).

In order to address these challenges we devised a novel method that would be sensitive and quantitative, and would also lend itself to analysis of any molecular pathway. The possibility of detecting molecular heterogeneity in tumors is also retained by this non-population based approach, potentially a critical parameter in prognosis (17-19). Two main types of determinations of Förster resonance energy transfer (FRET) are possible. These are steady state and time-resolved (detection of fluorophore lifetime) measurements. Time-resolved measurements yield additional
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information about FRET. One of the major advantages of measuring fluorescence lifetime is the fact that it is independent of fluorophore concentration, unlike steady-state, which is intensity based (20). In contrast to steady state measurements, where a change in intensity of the recorded emission would be observed and this method becomes prone to artifacts that lead to overestimation of FRET efficiency. We have used time-resolved FRET for sensitive analysis. FRET detected by fluorescence lifetime imaging microscopy (FLIM) has been proven to be a highly effective method in quantifying the phosphorylation status of intracellular molecules in archived formalin-fixed paraffin-embedded (FFPE) tumor tissue (21-27). Coincidence (two-site) FRET describes the method of simultaneously labeling a single protein on two distinct sites with donor- and acceptor-conjugated probes, and detecting the FRET between them. When using two-site FRET, notwithstanding that this method also relies on antigen-antibody immune complexes (IHC), Förster's energy transfer is the most efficient when the distance between the two antibodies (pAkt and panAkt) is less than 10 nm, within the same antigen. Two-site FRET is therefore necessarily more specific. The method has gained recent popularity due to its high specificity and its relative insensitivity to intensity artifacts (24, 26). To date studies of endogenous proteins using FLIM have been limited due to the lack of sensitivity. Our method solves this problem by amplifying the signal.

In this study we have developed a high throughput, quantitative coincidence amplified FRET assay. This assay combines immunofluorescence tyramide signal amplification (TSA) with labeled Fab fragment secondary antibodies in order to maximize sensitivity, specificity and portability. In parallel, we have developed an imaging platform based on a multiple frequency fluorescence lifetime imaging microscope (mfFLIM) and accompanying software to automatically map a tissue
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microarray, acquire FLIM images and distinguish between regions of interest (ROI) in cells and tumors, in a high throughput manner with minimal user supervision. We applied this assay to quantify Akt activation status in fixed SKBR3 breast tumor cells as well as FFPE human breast TMAs. We sought to explore the clinical relevance and molecular mechanisms underlying the activation of Akt in breast carcinoma. Here we identified that high Akt activation assessed by amplified FRET, but not by IHC, was correlated with poorer prognosis. We were also able to identify molecular heterogeneity of Akt activation within the same patient. The results from our study suggest that this new methodology will have direct clinical applications in guiding targeted therapeutics and in developing companion diagnostic tools.
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MATERIALS AND METHODS

Antibodies and Reagents

Monoclonal primary antibodies, mouse anti-panAkt (40D4) (#2920), rabbit anti-P-Akt (pT308) (C31E5E) (#2965) were obtained from Cell Signaling technology, USA. IRDye conjugated secondary antibodies, goat anti-mouse IgG-IRDye 800CW (#926-32210) and goat anti-rabbit IgG-IRDye 680LT (#926-68021) were purchased from LI-COR Biosciences, USA. Affinity-purified Fab fragments antibody conjugate and goat F(ab')2 anti-rabbit IgG-HRP (#A120-118P) were purchased from Bethyl laboratories, TX, USA. Affinity-purified F(ab')2 fragments antibody, AffiniPure donkey Fab fragment anti-mouse IgG (#715-006-150) were purchased from Jackson ImmunoResearch, Suffolk, UK. PVDF Immobilon-FL (#IPFL00010) transfer membrane was purchased from Millipore Corporation. Odyssey® blocking buffer (#927-40000) was purchased from LI-COR Biosciences, USA. cOmplete, mini protease inhibitor cocktail tablets (#11836153001) was from Roche, Germany. Dimethylformamide (DMF), sequencing grade (#20673), Peroxidase Suppressor (#35000) and Pierce BCA protein assay kit was purchased from Thermo Fisher Scientific, UK. Amine-reactive dye, Oregon Green® 488 NHS-ester (ORG488, #O-6147), Dulbecco's Modified Eagle Medium (DMEM) GlutaMAX (#31966021) and Tyramide Signal Amplification (TSA) kit with Alexa Fluor® 594 tyramide (#T-20925), Pre-Cast SDS-PAGE gel, NuPAGE® Novex 4-12% Bis-Tris Gel (NP0335BOX) were purchased from Invitrogen Life Technologies, USA. PI3K inhibitor, InSolution™ LY294002 (#440204) and Epidermal growth factor (EGF) (#324831-200UG) were purchased from Calbiochem®, Germany. PD-10 dye removal columns, PD MiniTrap™ G-25 (#28-9180-07) was purchased from GE Healthcare Life Sciences, UK.
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**Conjugation of Fab-fragment with ORG488-NHS dye**

Oregon Green 488-NHS (ORG488) conjugation to the anti-mouse-specific Fab secondary antibody (1mg/ml) was performed using NHS (N-Hydroxy Succinimide)-ester conjugation protocol (22). Briefly, the ORG488-NHS-ester dye (Life Technologies Ltd, Paisley, UK) was reconstituted in anhydrous DMF to a concentration of 1mg/ml and added (125µg of dye/1mg of protein) to the Fab fragment antibody solution. The reaction mixture was incubated with constant agitation at room temperature for 1 hour. Purification of labeled antibodies was performed using pre-equilibrated (PBS, pH 7.2) gravity flow PD-10 dye removal columns (GE Healthcare Life Sciences, UK). The concentrations of labeled Fab fragment-ORG488 conjugate and the degree of labeling (DOL: 4.01) were calculated based on the absorbance at 280 nm and 556 nm (ORG488), respectively.

**Co-localization of endogenous panAkt and pT308 using Fab fragment based Tyramide Signal Amplification (TSA) assay in fixed MCF7 and SKBR3 cells**

A time course (5, 15, 20 and 30min) was performed, to establish the optimum time required for the TSA reaction in MCF7 cells. A Fab fragment-based TSA assay was performed using SKBR3 cells in two 8-well glass chamber slides. The cells on the first and second chamber slides were further incubated with species-specific Fab fragment secondary antibodies, Fab anti-mouse ORG488 (20µg/ml) Fab fragment and Fab anti-rabbit-HRP (10µg/ml) conjugate, respectively for 2h at room temperature. The cells on second chamber slide were then labeled using Alexa-594 TSA system for 15min. At this time point the amplification reached a plateau and the signal was not amplified further. The stained SKBR3 cells were mounted as described in Supplementary Methods, prior to inspection using a Zeiss LSM 710 inverted laser scanning confocal microscope.
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Co-localization of endogenous panAkt and pT308 using Fab fragment based (TSA) assay in FFPE fixed human breast tumor

Two identical FFPE breast tumor tissue sections were dewaxed, rehydrated and subjected to heat-induced antigen retrieval in TRIS-EDTA (pH 9.0) buffer for 10 min. To quench the background fluorescence signal, these slides were incubated with fresh sodium borohydride (1mg/ml in PBS) for 10min at RT and blocked with 1% BSA/PBS. The first slide was incubated with primary mouse anti-panAkt (1:50); the second slide was incubated with both primary mouse anti-panAkt (1:50) and primary rabbit anti-pT308 antibodies (1:200). For both slides incubation was overnight at 4°C. Slides were washed 3x with PBS. The first slide was labeled with anti-mouse Fab ORG488 (20µg/ml) secondary antibody. The second slide was labeled with anti-mouse Fab ORG488 and anti-rabbit Fab-HRP (10µg/ml) secondary antibodies, which was then detected by using Alexa-594-TSA assay. For each immunofluorescent labeling experiment a negative control was included, by replacing the primary antibody with BSA at the same volume as the primary antibody. All tissues samples were mounted prior to observation under a confocal microscope.

High-throughput automated multiple frequency domain FLIM (mfFLIM)

The multiple frequency domain FLIM is a lifetime imaging microscope from Lambert Instruments modified in a new way to set up an automated multiple frequency high throughput lifetime imaging microscope. For details of the algorithm implementation see Supplementary Methods. **Lifetime calculation** - To improve the precision of the lifetime calculation within all tumor sectors, we developed an algorithm that automatically excludes data below a predefined and universally applied threshold, by applying the “active contours method”(36). **Data Analysis** – We determined the FRET efficiency (Ef), using the following formula: $E_f(\%_\text{F}) = \left[1 - \left(\frac{t_{DA}}{t_{D}}\right)\right] \times 100$; where
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(upon FRET $t_{\text{D/A}} \ll t_0$; $t_0$ is donor lifetime and $t_{\text{D/A}}$ is the donor plus acceptor lifetime.

**Coincidence amplified FRET assay for quantification of pAkt (pT308) in human breast FFPE tissue**

Amplified-FRET assay for quantification of pT308 was performed on 4-μm sections of two identical FFPE-fixed breast cancer tissue samples. Following de-waxing and rehydration, sections were subjected to heat antigen retrieval by microwaving in TRIS-EDTA (pH 9.0) buffer, for 10 minutes at 800W. Sections were then incubated in freshly prepared sodium borohydride (1mg/ml in PBS) buffer for 10min at RT, followed by blocking with 1% BSA/PBS. Tissues sections were incubated with peroxidase suppressor (Thermo Scientific Pierce) for 15min. For the amplified FRET assay, the first slide was incubated with mouse anti-panAkt (1:50), and the second slide with mouse anti-panAkt (1:50) *and* rabbit anti-pT308 (1:200) primary antibodies, overnight at 4°C. The first slide was further immunolabeled with ORG488-conjugated anti-mouse Fab fragment secondary antibody (20μg/ml). The second slide was immunolabeled with ORG488-conjugated anti-mouse Fab fragment (20μg/ml) *and* HRP-conjugated anti-rabbit Fab fragment secondary antibody (10μg/ml), which was detected by using Alexa-594-TSA assay. As a control to address the specificity of the phosphorylation signal, calf intestinal alkaline phosphatase (CIP) was incubated with the tissue sections. CIP (10 units/slide) was diluted in 1X NEB buffer 3 and incubated for 30min at room temperature. Also, the substitution of the specific primary antibodies by 1% BSA in tissue sections was used as a negative control. These control slides were also prepared as described above before mounting all tissues sections with ProLong® Gold anti-fade. The donor lifetimes of ORG488 were determined from at least 10 regions of the tumors, each
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performed in triplicate unless otherwise indicated. The FRET efficiency was calculated as described above.

**High-throughput quantification of pAkt (pT308) in human breast TMAs by amplified coincidence FRET assay**

TMAs containing 230 cores of breast tissue, originating from biopsies taken from a large case mix of ER positive and ER negative breast cancer patients from the tumor bank at Guys Hospital, London UK. All biopsies had been taken prior to treatment, and linked to histological and clinical data comprehensively stored in a database. These were a consecutive series of breast cancers from patients diagnosed between 1993-94. Prior to immunofluorescence labeling, two identical TMA sections were processed as described above. The coincidence amplified FRET assay was also performed as above. Lifetime measurements were performed using both donor-labeled and donor plus acceptor-labeled TMAs, for each core. Only samples with donor intensity of at least four times higher than the background intensity were included for FRET efficiency calculations. The maximum FRET efficiency of four sectors within each core was calculated, as described above.

**Statistical Analysis**

Statistical analysis was performed using the Graphpad Prism software (GraphPad Prism software, CA, USA). Results are shown as mean values ± SEM. Statistical significance between the groups was calculated with the Mann-Whitney test (values are indicated in the Box and Whiskers plots) (24, 37). Differences were considered statistically significant when \( p \leq 0.05 \). Disease-free survival (DFS) was measured as the time from diagnosis to event or last seen if the patient had no event. Overall survival (OS) was calculated by the time from diagnosis to death or last seen if the patient lived. Potential prognostic factors (for e.g., systemic therapy, radiation,
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mastectomy, lumpectomy) were analyzed by univariate and multivariate analysis for prognostic significance for DFS and OS. DFS and OS rates were estimated using the Kaplan-Meier method (24). The differences in the survival curves were compared by the log-rank-test and this test was used to assess the hazard ratios. An unpaired t-test was used to compare the average FRET efficiency between ER-negative and ER-positive. Comparing the patients in the lower two tertiles with those in the upper tertile.
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RESULTS

Development and validation of a novel amplified FRET imaging assay for quantification of endogenous Akt activation status in SKBR3 cells

We evaluated the binding specificity of human anti-Akt antibodies (panAkt, pT308) by two-color Li-COR Western blot detecting endogenous Akt in SKBR3 cells (Supplementary Fig. S1). SKBR3 cells were treated with or without the pan PI3K inhibitor, LY294002 (50µM) for 30 minutes, +/- EGF (100ng/ml) for 5 minutes. Cells were lysed and total protein lysates evaluated by Li-COR Western blot (Supplementary Methods). Single bands demonstrated specificity of the anti-Akt (panAkt, pT308) antibodies (Supplementary Fig. S1A, B). Incubating both panAkt and pT308 antibodies followed by the addition of species-specific NIR-dye-conjugated secondary antibodies, on a single blot, did not affect the specificity of either primary antibody (Supplementary Fig. S1C). The Li-COR Western showed that Akt phosphorylation was enhanced by EGF and inhibited by LY294002 in SKBR3 cells (Supplementary Fig. S1D). These results confirmed the specificity of anti-Akt antibodies.

Our assay uses Fab fragments combined with TSA signal amplification; hence we compared the localization of pT308 in starved SKBR3 cells, with and without TSA amplification, using labeled Fab fragments. Although an increased plasma membrane localization of pT308 was visible upon EGF stimulation in non-amplified cells, the signal was greatly enhanced by TSA amplification. In both cases the signal was significantly reduced in EGF+LY treated cells (Supplementary Fig. S2A, B). Evidently TSA amplification increases sensitivity without a corresponding increase in background signal.
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Since the Fab fragment-based TSA assay resulted in a higher signal-to-noise, we used this method to investigate endogenous expression and co-localization of panAkt and pT308 in FFPE human breast tumor sections. Confocal images showed a clear co-localization of panAkt and pT308 at the plasma membrane (Supplementary Fig. S 2C). The pT308 was predominantly at the plasma membrane, indicating specificity of TSA labeling in tissue. Control experiments (Supplementary Fig. S2C) omitting primary antibodies produced a very weak signal, thus confirming the specificity of the Fab fragment-based TSA system in tissues. In order to establish the optimum time required for the TSA reaction to occur, time courses were performed in stimulated MCF7 cells. After 5 minutes, the pT308 signal was very weak, from 10 to 15 minutes the pT308 signal increased, before signal saturation was reached at 30 minutes. 15 minutes was therefore chosen as the optimum time for TSA amplification (Supplementary Fig. S3). These results demonstrate that, combined with labeled Fab fragments, TSA amplification can be exploited routinely in samples where the target proteins and their phospho-sites are poorly expressed.

We next exploited the Fab fragment based TSA assay to develop a more sensitive and generic coincidence FRET assay, in order to measure Akt activation in cells and tumor sections. Results are presented as FRET efficiency (E_f) (Methods). Higher E_f indicates increased Akt phosphorylation. Fig. 1A shows a schematic of our multiple frequency domain FLIM (mfFLIM), which we use to detect time resolved FRET. Fig. 1B shows the principle of the coincidence amplified FRET assay using Fab fragment secondary antibodies.

Cells were starved or EGF-stimulated with or without LY, and fixed according to the amplified FRET protocol. Figure. 1C (first panel) shows that the lifetime map of cells labeled with donor (panAkt) alone did not change upon EGF stimulation.
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However, the lifetime of the cells labeled with both donor and acceptor (pT308) decreased and hence the average FRET efficiency of cells increased upon EGF stimulation (Fig. 1C, second panel). The detected FRET efficiency was only (E_f =7%) (Fig. 1C, second panel). The same coincidence FRET experiment but with TSA amplification showed an increase in FRET efficiency (E_f >16%) with EGF, clearly visible at the plasma membrane, as seen from the lifetime map images. This is indicative of a significant increase in pT308 at the plasma membrane (Fig. 1D), demonstrating that TSA amplification significantly improved the dynamic range of the FRET efficiency. These results demonstrate that the amplified FRET assay can quantify the activation status of endogenous Akt in fixed cells with a high sensitivity and specificity.

Quantification of Akt activation status by amplified FRET in fixed FFPE human breast tumors

After optimizing amplified FRET in SKBR3 cells, we assessed activated Akt in FFPE breast cancer patient samples. For these experiments we used the high-throughput mfFLIM platform to acquire images and analyze automatically the average FRET efficiencies. The average FRET efficiency varied from 2.5 to 6.0% suggesting that our amplified-FRET assay is able to detect the variation in the activation status of Akt between patients (Fig. 2A, right panel). Control experiments were performed with calf intestinal alkaline phosphatase (CIP) to non-specifically dephosphorylate any phospho-proteins present (Fig. 2B). The FRET efficiency was significantly reduced from 6.0 to 1.5%, confirming the specificity of the amplified FRET assay towards phosphorylated proteins in fixed tissue.
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**Molecular heterogeneity of pAkt (pT308) expression in human breast tumors by amplified FRET**

To map and quantify the molecular heterogeneity of the Akt activation status within patient tumors we prepared tumor microarrays (TMAs) from breast tumor biopsies obtained from 10 patients. These patients had a variable ER and HER2 expression status. For each patient, four cores were selected from different regions within each biopsy. H&E staining confirmed that each core contained tumor tissue (Fig. 3A). In total there were 40 tumor cores per TMA (i.e. 4 cores x 10 patients). TMAs were labeled with anti-Akt (panAkt, pT308) primary antibodies, followed by tyramide signal amplification (TSA) amplification.

We used the mfFLIM platform to map the position of each core on the TMA, automatically acquire images, and analyze the FRET efficiencies. The software randomly segments each tumor core into 4 sectors (shown in Fig. 3A). The intensity and the corresponding FLIM images are shown for all cores in Figures 3B and 3C. The maximum FRET efficiency value of the 4 sectors is shown in Figure 3D. We detected that there was significant variability in Akt activation between patients (for example, between patient 1 and patient 5); and also between cores from the same patient, representing different regions of the same tumor (for example, patient 8, Fig. 3D).

Averaging the FRET efficiency of the 4 cores resulted in a loss of localized information reporting on the activation status of Akt (Fig. 3D, inset). These results demonstrate the coexistence of high and low FRET efficiency in different regions of the same patient biopsy, demonstrating molecular heterogeneity of the activation status of Akt within breast tumors. Hence analysis of localized FRET efficiency is critical in breast tumors in order to avoid loss of potentially valuable information.
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**Prognostic value of pAkt expression in human breast cancers assessed by high-throughput amplified FRET versus intensity based IHC**

The main objective here in exploiting this analytical platform was to assess the prognostic value of pAkt in primary breast carcinoma and compare the results with intensity based IHC (calculated as the intensity ratio of pT308 divided by panAkt). A broad case mix of tumor samples representing 230 patients was obtained from King’s Health Partners Tumor Bank in TMA format, with linked 15-year follow-up clinical data. The case mix consisted of 76% of tumors ER+ and 24% ER(-) with a mix of grade-1, grade-2, and grade-3 tumors (Table 1). The presence of tumor tissue (>50%) was confirmed by H&E staining for each breast tumor core (Fig. 4A). The TMAs were fixed and stained according to the amplified FRET protocol. Using the automated FLIM platform we mapped and acquired images from each tumor core position. It is important to note that our software enabled the rapid acquisition and analysis of 230 tumor cores with minimal intervention, thereby removing a large amount of human error and subjectivity from the sample analysis. The differences in Akt activation status of histologically homogeneous and heterogeneous samples can be appreciated in Figure 4B (right panel). We used normal breast tissue to assess the basal FRET efficiency as a negative control. The basal FRET efficiency values ranged from 2.3% to 5.7% with median average FRET efficiency of 4% (Supplementary Fig. S7). Figure 4B (bar graphs) shows the variability of FRET efficiency between the 4 sectors from the same patient, as well as between different patient cores, ranging from low (3.9%) to a high (25.1%) FRET efficiency. Figure 4C illustrates the maximum FRET efficiency of each tumor core, arranged from high (27.8%) to low (0.5%), indicating the high dynamic range of the amplified FRET assay. We investigated whether there was a relationship between pAkt status assessed by amplified FRET
pAkt is a marker for poor prognosis in breast cancer (black dots) and by intensity ratio (gray dots). The linear regression showed there was no correlation between the two methods.

We then evaluated the prognostic value of Akt activation assessed by amplified FRET compared to intensity ratio in 164 cases, consisting of 125 ER+ and 39 ER (-) tumors. Patients were ranked according to their FRET efficiency or intensity ratio, and split into two groups for comparison, upper tertile (high pAkt) and lower two tertiles (low pAkt). 15 years of clinical follow-up data was used to generate Kaplan-Meier plots for DFS and OS, in order to compare the two groups (Figure 5). When assessed by amplified FRET, high pAkt significantly correlated with reduced DFS (p=0.036, HR=0.634, 95% CI [0.385—0.694]) and OS (p=0.013, HR=0.570, 95% CI [0.331—0.876]) compared to low pAkt (Fig. 5A, B). Importantly, when assessed by intensity ratio, high pAkt was not associated with reduced DFS (p=0.890, HR=0.699, 95% CI [0.616—1.521]) or OS (p=0.746, HR=1.082, 95% [0.670—1.750]) compared to low pAkt (Fig. 5C and D).

We studied the ER+ subgroup separately (n=125), splitting this cohort into high and low pAkt groups as before. As shown in Figure 6A and B, when assessed by amplified FRET, there was a significant association between high pAkt and reduced DFS (p=0.029, HR=0.566, 95% CI [0.299—0.936]) and OS (p=0.033, HR=0.284, 95% CI [0.284—0.946]). In contrast, using the intensity ratio, high pAkt was not associated with reduced DFS (p=0.800, HR=0.932, 95% CI [0.535—1.618]) or OS (p=0.759, HR=1.098, 95% CI[0.607—1.983]) (Fig. 6C and D). Moreover, patient age (mean 60 years) did not appear to correlate with the poor DFS or OS (Supplementary Fig. S 6D). Furthermore, 11 factors of potential prognostic significance were evaluated by univariate analysis (see Table 2). The data analysis revealed that increased E_{f} of pAkt associated with diminished OS (p = 0.041) but not with intensity ratio (p = 0.218,
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Table 2). In the univariate analyses, the histology grade was the most significant independent prognostic factor for DFS and OS. These findings show that FRET efficiency, as an indicator of Akt activation status, but not intensity ratio, predicts poorer disease-free and overall survival in patients with ER+ primary breast carcinoma.

In conclusion these results highlight that, using amplified-FRET (but not the IHC intensity ratio), high Akt activation in primary breast carcinoma predicts poorer outcome in these patients.
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**DISCUSSION**

The direct quantification of the activation state of endogenous proteins in cells or in tissue using a coincidence FRET assay has been a challenge (24, 28). Previously we have conjugated dyes directly to primary antibodies in order to obtain sufficient signal to monitor endogenous biomarker activation (24). However we encountered several limitations: Firstly, the conjugation process can result in the presence of multiple dye molecules at the antigen recognition site, with adverse consequences on antibody-antigen specificity (29). Secondly, the signal obtained is limited by the maximum number of dye molecules that can be bound to each antibody molecule while maintaining epitope recognition (22). Thirdly, cost becomes a limiting factor due to the large amount of primary antibodies required for dye conjugation, making routine screening of optimum antibody pairs prohibitive. This is compounded by the fact that commercially available labeled primary antibodies with compatible FRET pairs are difficult to find (29).

With these considerations in mind, we designed a coincidence FRET assay compatible with labeled secondary antibodies. Using pairs of whole secondary antibodies (IgG) is not appropriate for FRET as the dimensions of the IgG (150-180 kDa) result in donor-acceptor fluorophore distances above 10 nm, with a consequent loss of FRET (Supplementary Fig. S4). In contrast to whole IgG, Fab fragments have several important advantages: (i) due to their small size (50-100kDa) they within the 10 nm FRET limit; (ii) they penetrate more easily fixed samples and bind to their targets (30); (iii) they lack the Fc region, therefore any background that results from non-specific binding to endogenous Fc receptors is abolished (31). The use of Fab fragments permits us to use any commercially available primary antibodies,
pAkt is a marker for poor prognosis in breast cancer potentially extending the use of our assay to assess the activation of any signaling pathway in the cell.

Additionally, we exploited TSA to increase the detection threshold for very weakly-expressed proteins (32). We first confirmed that TSA amplification resulted in an increase in intensity when detecting pT308 in SKBR3 cells. The increased intensity was not accompanied by an increase in background (Supplementary Fig. S2). Refinements to our original FRET assay (24) allowed us to quantify the low levels of endogenous pAkt in breast cancer tissue (Fig. 2).

Other non-FRET coincidence assays that quantify protein complex formation or post-translational protein modifications, such as the in situ proximity ligation assay (PLA), are potentially able to detect molecules within 30-40 nm of one another (33). However, due to the 10 nm range, FRET will only occur between directly interacting proteins or a post-translational modification on the same protein (34). We tested the in situ PLA in FFPE breast tissue but we were unable to quantify the pT308 due to the low dynamic range in the context of the non-specific background observed with PLA in these Akt assays (unpublished data).

Breast cancer is heterogeneous at both the histological and molecular levels (17). A better understanding of tumor heterogeneity at the molecular level among a large cohort of patients will aid to identify the subgroups who respond more favorably to chemotherapy or targeted therapy (18). We prepared TMAs from breast tumor biopsies. We also tested the capability of the amplified-FRET assay to detect the molecular heterogeneity of Akt activation in colon TMAs prepared from 7 patients (Supplementary Fig. S5). In both tissues we were able to detect molecular heterogeneity of Akt activation between different regions of the same tumor. In addition, we observed the heterogeneity of Akt activation in some breast tumor cores.
pAkt is a marker for poor prognosis in breast cancer when comparing the FRET efficiencies of adjacent sectors (Fig. 4B), highlighting the spatial resolution of our imaging platform.

Several studies have attempted to determine the prognostic value of Akt activation in breast cancer using IHC, with varying results (5, 16). We obtained TMAs representing 164 primary human breast tumors representative of breast cancer subtypes treated in tertiary referral centers. We used amplified-FRET/FLIM platform and also the IHC intensity ratio to investigate whether pAkt might be associated with poor prognosis in order to demonstrate the validity of both methods. In a recent study, Tokunaga et al demonstrated that high levels of pAkt assessed by IHC failed to demonstrate prognostic value in breast cancer patients before hormone therapy (16). Consistent with these findings, our results using the IHC intensity ratio in breast TMAs did not show an association between Akt activation and DFS or OS (Fig. 5C, D and Fig. 6C, D). However, contrary to these limited methodologies, we show by coincidence amplified-FRET that in primary breast TMAs there was a significant difference between high and low pAkt groups in terms of DFS and OS. This applies to the total group of 164 patients (comprising ER(+) and ER(-)) (Fig. 5A, B), as well as for the 125 ER(+) patients (Fig. 6A, B). The coincidence amplified-FRET assay is therefore a promising approach for assessing protein activation compared to traditional IHC. Our data confirms that enhanced Akt activation in primary breast carcinoma patients is associated with worse prognosis. Since we have shown that high pAkt is associated with reduced survival, we postulate that patients with high pAkt might be suitable candidates for targeted PI3K/Akt pathway inhibitors.

We observed that Akt activation was significantly higher in ER (-) than in ER (+) patients when assessed by coincidence amplified-FRET but not by IHC intensity ratio (Supplementary Fig. S 6E, F). A higher proportion of ER (-) patients had high
pAkt is a marker for poor prognosis in breast cancer

pAkt compared to the ER (+) patients (Supplementary Fig. S6G). This result suggests that ER-independent signaling pathways are involved in the activation of Akt. Finally, we observed that amongst ER (-) patients, more than half exhibited high Akt activation, compared to one third of ER (+) patients (Supplementary Fig. S6H). We suggest that the ER+ population may benefit from stratification based on a measured value of Akt activation. Currently ER (+) breast tumors are treated with hormone-based therapy – this could be supplemented with targeted Akt inhibition for those patients with high Akt activity. In contrast, stratification of ER (-) patients would allow the introduction of Akt inhibition to a subset of patients who, until now, have not had many targeted therapeutic options.

In summary we have designed and evaluated an original coincidence FRET assay, which combines signal amplification with the flexibility of labeled secondary antibodies. This provides a highly sensitive, specific and portable methodology for the quantification of low levels of endogenous protein activation in a wide range of signaling pathways. In parallel we have developed a high-throughput FRET/FLIM imaging platform capable of mapping TMAs, automatically acquiring images and processing FRET data, requiring minimal intervention from a non-specialist user. The instrument is suitable for further development in a clinical setting. Moreover, the TMA sample preparation was two days (identical to classical IHC), which involves antigen retrieval, antibody incubation, and rinsing/washing. Acquisition of lifetime in order to calculate FRET using the FLIM involves a day’s work, in our case for 230 samples in TMA. Steps include mapping of samples, followed by calculation of the FRET efficiency, which is performed by the automated algorithm. In our case, for 230 samples, the interpretation time took an hour. This compares very favorably with the
pAkt is a marker for poor prognosis in breast cancer

interpretation time required by a pathologist to manually score a similar number of samples labeled with classical IHC techniques.

We have shown that, in primary breast carcinoma, high Akt activation measured by amplified FRET (but importantly, not by IHC intensity ratio) was correlated with poorer disease-free and overall survival. The method applied was able to quantify heterogeneity of Akt activation at two levels: i) between multiple cores taken from different regions of the same tumor (>2mm range), and ii) between sectors of a single tumor core (<1mm range). The methodology worked equally well when used to analyze other tissue types such as colon carcinoma. This assay is able to directly monitor Akt pT308 as a read-out of protein activation in both cells and FFPE breast tissue, and could be used more generally to monitor post-translational modifications or protein-protein interactions in any signaling pathway. The ability to accurately quantify oncoprotein activation has several major implications for translational medicine, in particular: drug screening in tissue culture; the discovery and validation of prognostic and predictive biomarkers; patient stratification based on oncoprotein activation; and validating the mode of action of drug inhibition, for example during neo-adjuvant therapy or window trials.
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Acknowledgments

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Reference

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**TABLES**

Table 1: Patient characteristics for breast TMA samples and mean FRET efficiency.

<table>
<thead>
<tr>
<th>Characteristic</th>
<th>Value</th>
</tr>
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<tbody>
<tr>
<td>Median Age</td>
<td>57.0</td>
</tr>
<tr>
<td>Median FRET efficiency</td>
<td>11.8</td>
</tr>
<tr>
<td>Median Intensity ratio (pT308/panAkt)</td>
<td>1.7</td>
</tr>
<tr>
<td><strong>Grade</strong></td>
<td>%</td>
</tr>
<tr>
<td>Grade 1</td>
<td>17.1</td>
</tr>
<tr>
<td>Grade 2</td>
<td>31.7</td>
</tr>
<tr>
<td>Grade 3</td>
<td>42.7</td>
</tr>
<tr>
<td>Unknown</td>
<td>7.9</td>
</tr>
<tr>
<td><strong>ER</strong></td>
<td>%</td>
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<tr>
<td>ER+</td>
<td>76.2</td>
</tr>
<tr>
<td>ER-</td>
<td>22.0</td>
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<tr>
<td>Unknown</td>
<td>1.8</td>
</tr>
<tr>
<td><strong>PR</strong></td>
<td>%</td>
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<tr>
<td>PR+</td>
<td>55.5</td>
</tr>
<tr>
<td>PR-</td>
<td>42.7</td>
</tr>
<tr>
<td>Unknown</td>
<td>1.8</td>
</tr>
<tr>
<td><strong>HER2</strong></td>
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<tr>
<td>HER2+</td>
<td>9.1</td>
</tr>
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<td>HER2-</td>
<td>37.2</td>
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<tr>
<td>Unknown</td>
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<td><strong>Adjuvant Therapy</strong></td>
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<td>Adjuvant therapy (total)</td>
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<tr>
<td>Tamoxifen</td>
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<td>CMF+Tamoxifen</td>
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<td>None</td>
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<tr>
<td>EFC</td>
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<tr>
<td>Ov Abl+Tamoxifen</td>
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<tr>
<td>FEC+Tamoxifen</td>
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<tr>
<td>Ov abl</td>
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<tr>
<td>APD</td>
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<td><strong>Surgery</strong></td>
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<tr>
<td><strong>Radiation therapy</strong></td>
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<tr>
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<td>55.5</td>
</tr>
<tr>
<td>NO</td>
<td>44.5</td>
</tr>
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</table>

**Abbreviation:**
epirubicin, cisplatin and continuous infusion 5-fluorouracil (ECF)
cyclophosphamide, methotrexate and 5-fluorouracil (CMF)
fluorouracil, epirubicin and cyclophosphamide (FEC)
aprepitant, palonosetron and dexamethasone (APD)
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**Table 2: Univariate analysis of factors associated with prognostic significance**

<table>
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<tr>
<th>Variable</th>
<th>p values (OS)</th>
<th>p values (DFS)</th>
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</thead>
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<tr>
<td>Histology Grade</td>
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<td>0.000</td>
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<tr>
<td>DIAG No Path Nodes</td>
<td>0.701</td>
<td>0.702</td>
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<tr>
<td>ER Status</td>
<td>0.066</td>
<td>0.116</td>
</tr>
<tr>
<td>PR Status</td>
<td>0.006</td>
<td>0.011</td>
</tr>
<tr>
<td>HER2 Status</td>
<td>0.327</td>
<td>0.634</td>
</tr>
<tr>
<td>Tumour Size</td>
<td>0.010</td>
<td>0.005</td>
</tr>
<tr>
<td>Adjuvant Therapy</td>
<td>0.009</td>
<td>0.003</td>
</tr>
<tr>
<td>Surgery</td>
<td>0.104</td>
<td>0.131</td>
</tr>
<tr>
<td>Radiotherapy</td>
<td>0.028</td>
<td>0.040</td>
</tr>
<tr>
<td>Int.Ratio (A/D)</td>
<td>0.218</td>
<td>0.248</td>
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<tr>
<td>FRET tertiles</td>
<td>0.041</td>
<td>0.149</td>
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</tbody>
</table>
pAkt is a marker for poor prognosis in breast cancer

FIGURE LEGENDS

Figure 1: Quantification of endogenous pAkt (pT308) in fixed SKBR3 cells shows significantly higher dynamic range of FRET efficiency by amplified FRET.

(A) Schematic diagram of the mfFLIM experimental setup (B) Schematic diagram showing the principle of the amplified FRET assay. Akt is labeled with primary antibodies on site 1 (panAkt) and site 2 (pT308). Fab fragment are used as secondary antibodies, conjugated to ORG488 (donor) or HRP/TSA-ALX594 (acceptor). (C) SKBR3 cells were pretreated with LY294002 (50µM) for 30 min prior to EGF stimulation (100ng/ml) for 6 min as indicated. The panels show the intensity images and lifetime maps of the donor alone (panAkt) or donor+acceptor (panAkt+pT308). Fab-ALX495 was used as acceptor. (D) Same experiment as in (C) but with TSA signal amplification (blue, low FRET efficiency; red, high FRET efficiency). FRET efficiencies are also shown as box and whiskers plots representing the mean ±SEM for at least 20 different cells. (***, p<0.0001; ns, not significant). Box and whiskers plots represent the mean ±SEM of the FRET efficiency (***, p<0.0001; ns, not significant).

Figure 2: Quantification of endogenous pAkt (pT308) in fixed FFPE human breast tumor using amplified FRET.

(A) Intensity images and lifetime maps (scales are relative and in pseudo-color) of FFPE human breast tumors from three different patients labeled with donor alone (panAkt) or donor+acceptor (panAkt+pT308). FRET efficiencies are shown as box and whiskers plots representing the mean ±SEM for at least 10 different regions from the same tissue section (***, p<0.0001). The increased FRET efficiency represents the phosphorylation status of endogenous Akt and shows the variability from patient to patient. (B) Tissue sections were treated with CIP (10 units/slide) for 30min, then labeled with panAkt or panAkt+pT308. FRET efficiencies are shown as a box and whiskers plot representing the mean ±SEM for at least 10 different regions from the same tissue section (***, p<0.0001).

Figure 3: Molecular heterogeneity of Akt activation in human breast TMA revealed by amplified FRET.

(A) H&E staining of breast TMAs prepared from breast tumor biopsies obtained from 10 patients. For each patient, 4 cores (circles 1 to 4) were selected from different regions within each biopsy. In total 40 tumor cores (4 x 10 patients) were spotted to make the TMA. The lower image shows an expanded view of one core divided into 4 sectors for further analysis. The panels on the right show 3 different magnifications of sector 4. Intensity images and lifetime maps (scales are relative and in pseudo-colors) of the TMAs stained with (B) panAkt Or (C) panAkt+pT308, followed by TSA- amplification. TMAs were mapped and images automatically acquired using our FRET/FLIM platform. (D) For each patient, the maximum FRET efficiency of each core is shown; (inset) for each patient the mean FRET efficiency for the 4 cores is shown.
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**Figure 4.** High-throughput quantification of Akt activation in human breast TMA using amplified FRET/FLIM platform.

(A) H&E staining of breast TMAs prepared from breast tumor biopsies obtained from 250 patients. Each core is a representative tissue sample from a single patient. The panels on the right show 3 different magnifications of sector 4. (B) Intensity images and lifetime maps (scales are relative and in pseudo-color ns is nanoseconds) of the TMAs stained with panAkt alone or panAkt+pT308, followed by TSA-amplification. TMAs were mapped and images automatically acquired using our FRET/FLIM platform. The three graphs present the FRET efficiency of each sector of the same patient core, for 3 representative patients. (C) Patients were ordered by descending FRET efficiency. For each patient we plotted the FRET efficiency (black dots, left Y-axis) and the intensity ratio (gray dots, right Y-axis). The regression line for the intensity ratio (gray line, $R^2=0.3414$) is shown for comparison with FRET efficiency.

**Figure 5.** Kaplan–Meier survival curves for all breast carcinoma patients (ER-/ER+) comparing high and low Akt activation

Kaplan–Meier survival plots demonstrating (A) disease-free and (B) overall survival of patients with high Akt activation (upper tertile, red) or low Akt activation (lower tertiles, blue) as determined by amplified FRET. Results of log rank tests are shown. (C) and (D) show the corresponding results when Akt activation is assessed by intensity ratio (calculated as pT308 divided by panAkt intensity).

**Figure 6.** Kaplan–Meier survival curves comparing high and low Akt activation for ER+ breast carcinoma patients.

Kaplan–Meier survival plots demonstrating (A) disease-free and (B) overall survival of patients with high Akt activation (upper tertile, red) or low Akt activation (lower tertiles, blue) as determined by amplified FRET. Results of log rank tests are shown. (C) and (D) show the corresponding results when Akt activation is assessed by intensity ratio (calculated as pT308 divided by panAkt intensity).
Figure 2

A

<table>
<thead>
<tr>
<th>panAkt (donor)</th>
<th>panAkt (donor) + pT308 (acceptor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intensity</td>
<td>Lifetime Map</td>
</tr>
<tr>
<td>Patient 1</td>
<td></td>
</tr>
<tr>
<td>Patient 2</td>
<td></td>
</tr>
<tr>
<td>Patient 3</td>
<td></td>
</tr>
</tbody>
</table>

B

<table>
<thead>
<tr>
<th>panAkt (donor)</th>
<th>panAkt (donor) + pT308 (acceptor)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Intensity</td>
<td>Lifetime Map</td>
</tr>
<tr>
<td>(+) CIP</td>
<td></td>
</tr>
<tr>
<td>(-) CIP</td>
<td></td>
</tr>
<tr>
<td>(+) CIP</td>
<td></td>
</tr>
</tbody>
</table>
Figure 3

Veeriah S. et al., 2014
Figure 4: Veeriah S, et al., 2014

A

B

panAkt (donor) vs panAkt (donor) + pT308 (acceptor)

Intensity

Lifetime Map

Intensity

Lifetime Map

Heterogeneous ROI with high FRET Efficiency

Homogeneous ROI with high FRET Efficiency

Homogeneous ROI with low FRET Efficiency

C

Plot showing FRET efficiency (%) vs label ratio (dot product)
Figure 5

Veeriah S. et al., 2014

A

B

C

D

Percent survival

Disease-free survival (yrs)

Overall survival (yrs)

Disease-free survival (yrs)

Percent survival

p=0.036
HR=0.834 (95% CI, 0.385 - 0.964)

p=0.013
HR=0.570 (95% CI, 0.331 - 0.876)

p=0.890
HR=0.969 (95% CI, 0.616 - 1.521)

p=0.746
HR=1.082 (95% CI, 0.670 - 1.750)
**Figure 6**

**A**
- **ER+ Upper FRET tertile**
- **ER+ Lower FRET tertiles**

p = 0.029
HR = 0.566 (95% CI, 0.299 - 0.936)

**B**
- **ER+ Upper FRET tertile**
- **ER+ Lower FRET tertiles**

p = 0.033
HR = 0.284 (95% CI, 0.284 - 0.946)

**C**
- **ER+ Upper Int.Ratio tertile**
- **ER+ Lower Int.Ratio tertiles**

p = 0.800
HR = 0.932 (95% CI, 0.535 - 1.618)

**D**
- **ER+ Upper Int.Ratio tertile**
- **ER+ Lower Int.Ratio tertiles**

p = 0.759
HR = 1.098 (95% CI, 0.607 - 1.983)
High throughput time-resolved-FRET reveals Akt/PKB activation as a poor prognostic marker in breast cancer


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