Quantification of Protein-Protein Interactions and Activation Dynamics: A New Path to Predictive Biomarkers

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Abbreviations:

TR-FRET – Time-Resolved Förster Resonance Energy Transfer
FLIM – Fluorescence Lifetime Imaging Microscopy
PKC – Protein Kinase C
PDK1 – phosphoinositide dependant kinase 1
Akt/PKB – Protein Kinase B
TMA – Tumour Microarray
HER2/3 – Human Epidermal Growth Factor Receptor 2/3
FISH – Fluorescence *in situ* hybridisation
CISH – chromogenic *in situ* hybridisation
PD-1 – Programmed death receptor 1
PD-L1 – Programmed death ligand 1
Rac1 - Ras-related C3 botulinum toxin substrate 1

Abstract

Oncogene dysregulation is a driver of neoplasia development and progression. The use of quantitative molecular imaging to quantify oncogene activation will be crucial in developing companion diagnostics which can identify personalised patient regimens. However, the evaluation of oncogene activation does not necessarily correlate with oncoprotein activation. Post-translational modifications, such as phosphorylation, lipidation and methylation, may enhance oncoprotein functionality. It is this functionality that progresses neoplasia and may correlate with patient outcome. Advanced molecular imaging may be used to directly quantify oncoprotein, as opposed to oncogene, activation. Time-resolved Förster Resonance Energy Transfer (TR-FRET) involves the non-radiative transfer of energy from one chromophore to another over distances of 1-10nm; allowing FRET to be used as a "chemical ruler". TR-FRET can be utilised to directly elucidate spatial oncoprotein activation in single cells and patient tissues. In single cells, TR-FRET has uncovered the mechanisms by which PKCβ1 is trafficked to the nucleus and cleaved. Additionally it has revealed the mechanism of activation of Akt/PKB, whereby Akt/PKB undergoes a conformational change, allowing the Thr308 site to be phosphorylated by PDK1. Moreover TR-FRET has been utilised to quantify HER2-HER heterodimerisation and Akt/PKB activation states in patient biopsies, where it is shown to be predictive of outcome/relapse. The role of TR-FRET is not solely limited to intracellular signalling events. A study has used TR-FRET to measure intercellular immune-checkpoint receptor-ligand interactions. Within this study it was seen that PD-L1 expression was not indicative of PD-1/PD-L1 interaction states in a range of solid tumours. Crucially, in melanoma and NSCLC, PD-1/PD-L1 interaction was a predictive of an improved patient outcome. PD-L1 expression did not predict patient outcome. Several groups have worked to improve Fluorescence lifetime imaging microscopy (FLIM) acquisition times, including the use of: window-galvanometers; multifocal multiphoton FLIM and parallel pixel excitation coupled with wide-field time-gated FLIM. The development of novel quantitative molecular imaging will be critical in the development of personalised patient therapies in the future.

Introduction

Whilst recent advances in cancer treatments have prolonged patient outcomes and improved patient responses to therapy, we still preside in an age where one in two people are diagnosed with cancer in their lifetime (1). A cross-disciplinary approach will be required to continue make significant advances in prolonging patient survival. To facilitate this, a focus must remain on early cancer detection and diagnosis (2). This will invariably start to shape more developments in precision medicine.

A major driving force in neoplasia formation and neoplastic progression is the activation of proto-oncogenes to oncogenes (3). A proto-oncogene is an oncogene which has derived from a wild-type cellular gene. This activation is usually facilitated by a gain-of-function mutation rising from three events: a point mutation in a proto-oncogene may result in the coding of a constitutively-active oncoprotein; localised reduplication may occur where a proto-oncogene is amplified, creating an oncogene which leads to an overexpression of an oncoprotein;

chromosomal translocations may cause a proto-oncogene to be controlled by the wrong promoter, thus resulting in abhorrent expression of the corresponding oncoprotein (4-6). Whilst the use of sequencing can detect and characterise chromosomal irregularities, these lack spatiotemporal depth. Imaging would be one way to underpin the spatial understanding of these genomic and proteomic aberrations, within cells and tissues.

One of the tools for spatial quantification, in early diagnostics and quantitative prognostics of patient biopsies, is advanced molecular imaging. This type of methodology can quantify patient oncogene expression in single cells and tissue samples, which may lead to identifying personalised treatment regimens for patients.

This review will focus on current quantitative oncogene imaging, largely based on time-resolved Förster Resonance Energy Transfer (TR-FRET) assays resolved by Fluorescence Lifetime Imaging Microscopy. Chiefly, the use of FRET/FLIM to uncover the signalling dynamics of major oncoproteins and translating this knowledge to the clinic will be discussed.

Advances in the Quantification of Oncogene Expression

As previously stated, the quantification of oncogene expression profiles is imperative for the advancement of cancer diagnostics and prognostics. To achieve this, novel imaging techniques or a multidisciplinary approach utilising basic microscopy will be critical in advancing the quantification of oncogene expression.

Fluorescence *in-situ* hybridisation (FISH) was routinely used to determine human epidermal growth factor receptor 2 (HER2) gene amplification in breast cancer tumour biopsies (7). However, this method is more costly than immunohistochemistry assays (nearly 3 times more expensive) and takes up to 9 times longer to perform (8). Moreover, this was coupled with an imaging platform that, whilst accurately determining gene amplification, failed to simultaneously assess tumour morphology (9). This led to the development of chromogenic *in-situ* hybridisation (CISH). This assay utilised a peroxidase-based chromogenic reaction to detect HER2 gene amplification, however, unlike FISH this was visualised on a standard brightfield microscope, allowing for the simultaneous assessment of tumour morphology (9). Whilst this advancement of gene amplification detection was welcomed, there was still an unmet need for rapid oncogene profiling coupled with quantitative imaging.

This unmet need was ultimately addressed by NanoString Technology. Formed in 2003, NanoString is a life science biotechnology company who have greatly advanced the quantification of oncogene expression. Their nCounter platform allows for the accurate detection of multiplexed gene expression without the need for sample amplification (10). The assay is capable of detecting up to 800 different gene expressions and less than a single transcript copy per cell (11). Briefly, the NanoString nCounter platform couples the quantification of differentially-targeted mRNA molecules with a pair of specific colour-coded probes with high resolution single molecule imaging (12). This results in the generation of “fluorescence barcodes” with each specific gene transcript being labelled with a different barcode. These barcodes are then imaged and quantified to indicate the expression of the probed genes (13).

Whilst these progresses in quantitative imaging of oncogene expression and activation have advanced our understanding of neoplasia development and progression, the assessment of oncogenes does not necessarily correlate with post-translational cellular events. The oncoprotein that results from oncogene activation will often undergo several post-translational modifications such as; phosphorylation, lipidation, acetylation, methylation or glycosylation (14). When correctly regulated, most of these post-translational modifications are reversible and act as a series of molecular switches, responsible for transitioning the cell from a resting to proliferative state (15). However, when tight regulation is not present, these post-translational modifications often confer oncoprotein functionality, as it is the increased functionality of oncoproteins which fully drive neoplastic progression. Moreover, the interaction of multiple oncoproteins, through dimerisation and oligomerisation events may also progress tumour survival and proliferation. Therefore, it is imperative not to solely assess the expression of oncogenes, but to utilise methods such as quantitative imaging platforms to assess oncoprotein functional states.

Förster Resonance Energy Transfer as a Method of Uncovering Cellular Signalling Dynamics

Förster Resonance Energy Transfer (FRET) is a photophysical event whereby energy is non-radiatively transferred, via dipole-dipole interactions, from a chromophore in an electronic excited state. This excited chromophore, termed the donor, transfers the aforementioned energy to an acceptor which is often, but not necessarily, fluorescent (16-18). This event happens over a range of 1-10nm (19). This range allows the spatiotemporal information of molecular interactions and conformational changes to be quantified; at a resolution exceeding that of colocalisation assays and conventional optical microscopy (Figure 1) (16).
There are two methods for the determination of FRET. Intensity-based (steady-state) FRET which is calculated by measuring variations in either donor or acceptor fluorescence intensities. Time-resolved FRET is determined by measuring the relative change of the lifetime of a donor molecule in the presence of an acceptor molecule. The latter is concentration independent as lifetime is an intrinsic characteristic of a chromophore. Conversely, steady-state measurements should only be performed when the stoichiometry of the donor and acceptor chromophores is one. Current techniques that aim to measure protein-protein, lipid-protein or lipid-lipid interactions, such as proximity ligation assay (PLA) or colocalisation assays claim to read out on protein functionality. However, these techniques operate at a much higher working distance (30nm for PLA and over 70nm for colocalisation assays) and therefore can only report on proximity and fluorescence intensity (rather than lifetime measurements), which fail to accurately report on protein functionality. (Figure 1) (20). FRET can be utilised as, a “chemical ruler”, via the lifetime measurements which are translated into FRET Efficiency read-outs. Although FRET efficiencies may be measured both by steady-state and time-resolved methods, the time-resolved method provides increased precision and flexibility as the stoichiometry of the donor and acceptor may exceed one. Due to the enhanced dynamic range of FRET efficiency, the precision of molecular interactive states on spatiotemporal cellular signalling events can be quantitatively reported.

A 1996 study carried out by Thomas Jovin and Phillipe Bastiaens utilised FRET, resolved by Fluorescence Lifetime Imaging Microscopy (FLIM) to elucidate the intracellular processing of

protein kinase C (PCK) β1. PKC is a serine/threonine kinase belonging to the classical PKC family and its activation is induced by diacylglycerol (DAG) and calcium. It was known that PKC isozymes underwent nuclear translocation in response to tumour promoters and growth factors, but what remined unknown was the state in which the PKC molecules arrived at the nucleus (21). In this study, PKCβ1 was labelled with a donor chromophore on the N-terminus (Cy3) and an acceptor chromophore on the C-terminus (Cy5). It was observed that upon stimulation, PKCβ1 translocated to the nucleus intact after 5 minutes. However, after prolonged stimulation, FRET signal was abolished, whilst fluorescence intensity images maintained that both the N and C termini were colocalised in the nucleus. This inferred that whilst the termini were colocalised, a cleavage event occurred whereby the N-terminus and C-terminus fragments diffused apart, thus reducing the FRET signal (Figure 2). This study gave an early indication on the suitability of using FRET to assess protein activation as well as translocation and cleavage dynamics (21).

Three years later, a study, also utilising FRET determined by FLIM was used to more precisely quantify PKCα state *in-situ.* Prior to this, the preferred means of analysing PKC activation state was to monitor membrane association and the formation of DAG-associated complexes (22). However, PKC is able to associate with the plasma membrane without prior activation, thus making this an insufficient criterion for activation state (23). The work carried out by Tony Ng et al., 1999 sought to utilise FRET/FLIM, combined with a two-site labelling assay which was, for the first time, used to assess PKCα activation state.

Here, an anti- PKCα antibody (MC5) was conjugated to Cy3, which acted as a donor chromophore, and T(P)250 was conjugated to Cy5, which acted as the acceptor chromophore. FRET readouts here reported on the proximity of the two chromophores, and thus conformational state of the kinase. Therefore, FRET was a readout of PKCα activation state. The largest activation states were observed in regions of the plasma membranes alongside cytoplasmic vesicular structures. The authors validated this novel activation state assay in fixed tissue samples. Using 23 formalin-fixed paraffin-embedded breast tumours, 11 samples were observed to have a significant PKCα activation state, whereas PKCα activation state was not detected in the remaining 12 patients. These results would be undetectable using classical immunohistochemistry methods.

The early work carried out here was able to provide a deeper insight into the mechanisms by which PKC signalling transduction takes place. Similarly, this quantitative TR-FRET imaging platform holds the ability to elucidate the signalling mechanisms of other poorly understood AGC kinases.

The Role of FRET in Elucidating PKB/Akt Activation Mechanisms

Another serine/threonine kinase, protein kinase B (Akt/PKB), also a member of the AGC kinases, had a poorly understood mechanism of activation in single cells. Often abhorrently regulated in a range of cancers, Akt/PKB signal transduction is responsible for cell proliferation, growth, survival, malignant transformations and chemoresistance (24, 25). It was thought that Akt/PKB activation was achieved through recruitment to the plasma membrane, where its pleckstrin-homology (PH) domain interacted with PtdIns(3,4,5)P_3. The

membrane-bound kinase was then thought to be phosphorylated by phosphoinositide-dependant protein kinase 1 (PDK1), also recruited to PtdIns(3,4,5)P_3 at the plasma membrane. This phosphorylation event occurs at threonine-308 (T308) in the T-loop of the protein. A secondary phosphorylation event, located on serine-473 (S473) within the C-terminus of the kinase, leads to full kinase activation (26).

Whilst the aforementioned signalling events were understood, the dynamics of the PDK1 and Akt/PKB interaction, which had not been observed *in-situ* or *in-vivo*, were not. Work carried out by Véronique Calleja et al., in 2007 utilised time-resolved FRET determined by FLIM to fully elucidate the mechanisms by which Akt/PKB interacts with PDK1 to gain activation (26). The authors constructed two fusion proteins by encoding enhanced green fluorescent protein (EGFP) to the N-terminus of PDK1 and by encoding red fluorescent protein (RFP) to the N terminus of PKBα. The authors observed that under basal conditions, Akt/PKB and PDK1 formed a complex in the cytoplasm. Upon cell stimulation with platelet-derived growth factor (PDGF), Akt/PKB-PDK1 complexes translocated to the plasma membrane, where an increased FRET efficiency was observed (7.1%) compared to that of the cytoplasm (4.9%).

The authors pursued further, seeking to elucidate the mechanism by which Akt/PKB makes its T308 residue accessible to phosphorylation by PDK1. Two reporter-Akt/PKB constructs were created: Akt/PKB tagged with GFP on the N-terminus and Akt/PKB tagged with GFP on the N-terminus and RFP on the C-terminus. Under basal conditions, the two chromophores were held within 1-10nm, indicating the protein was in a “closed, PH-in” conformation. Upon stimulation with PDGF, the authors observed a larger decrease of donor lifetime through resonance energy transfer, indicating that the GFP and RFP molecules had moved closer together (Figure 3). This was termed the “open, PH-out” confirmation, rendering T308 accessible for phosphorylation by PDK1.

Whilst the above examples are only a short highlight of the capabilities of time-resolved FRET to uncover mechanistic pathways; in the current age of precision medicine, it would be poignant to include examples of how time-resolved FRET can be utilised with clinical relevance.

The Quantification of Human Epidermal Growth Factor Receptor (HER) Dimerisation State: Resolved by FRET/FLIM

FRET/FLIM has allowed for the elucidation of intracellular signalling mechanisms and dynamics, which would have otherwise been missed without the application of quantitative molecular imaging. FRET/FLIM has also quantified the functional state of an important receptor family who are dysregulated in a range of cancers. The human epidermal growth factor receptor 2 (HER2) is a receptor tyrosine kinase belonging to the human epidermal receptor (HER) family. Like other oncoproteins, it is dysregulated in a range of solid cancers, notably breast cancers, and this dysregulation is associated with disease progression, relapse and poor prognosis (27). Activation of the HER receptor family leads to activation of the MAPK and PI3K/Akt signalling pathways (28).

Due to this, HER2 has long been a target of cancer therapeutics, with drugs such as trastuzumab (Herceptin®) aimed at blocking the receptor (29). In order to stratify patients for these lines of treatment, HER2 status must be accurately assessed. Unfortunately such tests do not exist. It is true that immunohistochemistry may be utilised to detect HER2 expression, however the reliability and subjectivity of this method is questionable as conflicting results may arise from different clinical laboratories (30). The aforementioned FISH assay may be used to assess HER2 gene amplification, however it has increase time and finance costs, and is an indirect measurement of HER2 expression (8). In 2012 Alexandre Ho-Pun-Cheung et al., utilised a two-site TR-FRET assay to simultaneously quantify HER2 expression and dimerisation with HER2 (as a homodimer) and EGFR (as a heterodimer) (31). The authors successfully analysed the expression of HER2 and EGFR on 18 breast tumour samples and found; EGFR-EGFR homodimerisation in 5 patient samples, HER2-HER2 homodimerisation in 12 patients, and HER2-EGFR heterodimerisation in 4 patients (31). Crucially, the sensitivity of the TR-FRET assay was assured as there was no overlap in TR-FRET expression levels between HER2 positive and HER2 negative tumours (as analysed by HercepTest™ and qPCR analyses) (31).

These studies were taken further and given a more clinical application by Gregory Weitsman et al., in 2016. Here, FRET/FLIM was utilised to measure HER2/HER3 heterodimerisation. This is clinically significant as HER3 has been identified as the preferred binding partner of HER2, and this heterodimer is one of the most stable and most mitogenic (32, 33). Crucially, only HER2 expression, as determined by HercepTest™ or IHC, is approved to stratify patients for pertuzumab, an antibody designed to block HER2-HER3 dimerisation. Clearly, a more stringent method of patient stratification is required to identify patients for these therapies. Several groups have aimed to measure HER2-HER3 heterodimerisation, using the proximity ligation assay or VeraTag. However, these assays work at distances of >30nm and up to 300nm respectively (Figure 1) (20, 34). This infers proximity but is unable to confer direct dimerisation (20). Weitsman et al., performed FRET/FLIM analysis on 131 primary breast cancer tumour cores, measuring HER2-HER3 heterodimerisation. FRET efficiencies (which report on heterodimerisation) ranged from 0% to 22% (34). Contrastingly to the study discussed above, no correlation was found between HER2-HER3 heterodimerisation and HER2 expression levels (analysed by HercepTest™ or IHC). To assess the clinical validity of HER2-HER3 dimerisation as a biomarker for disease progression, the authors correlated FRET efficiency with metastasis-free survival. Patients with a higher dimerisation state (a FRET efficiency above 8.56%) experienced a poorer metastasis-free survival during a 10 year follow up period. This correlation was not detected when assessing HER2 expression and metastasis-free survival (34).

Moreover, authors from the same group have utilised histological FLIM imaging to assess HER2-HER3 dimerisation, coupled to Bayesian statistics, to identify a small (<10%) subset of colorectal patients who benefit from the addition of cetuximab to standard chemotherapy (35). It was also shown that these patients have an increased survival. This application of "histological FLIM imaging" could allow for the routine spatiotemporal quantification of biomarker functionality across patient samples in order to better identify patient subsets for treatment stratification.

In addition to the clinical validation of HER2-HER3 dimerisation, FRET/FLIM has also proven useful in identifying epigenetic biomarkers in oestrogen receptor positive (ER^+) cancer. A study by Liu et al., 2019 has used time-resolved FRET coupled with FLIM to uncover an interaction between H3K27 acetylation (H3K27ac) and H4K12 acetylation (H4K12ac) interact with ERα at a nanoscopic resolution (36). The authors also sought to identify whether this could serve as a potential therapeutic target in breast cancer. It was shown that the combination of tamoxifen (antioestrogen) and anacardic acid (inhibits histone acetyltransferases) resulted in significant growth suppression of MCF7 cells in cell culture and mouse xenografts (36). As over 80% of breast cancers are ER^+, and long-term hormonal therapy against this target may result in tumour recurrence and resistance to hormonal therapies, this study is of high interest in identifying novel mechanisms by which to target this subset of tumours. This not only identifies a possible use for anacardic acid in breast cancer treatment, but also identifies a method by which epigenetic targets can be screened in an efficient manner to generate potential therapeutic targets in cancer using FRET/FLIM.

Whilst HER2-HER3 heterodimerisation, assessed by FRET/FLIM, has been clinically validated, it is not the only biomarker to achieve this milestone. The aforementioned work in the field of Akt/PKB carried out by Calleja et al., has also been translated into a clinical setting.

Translating Novel Akt/PKB Signalling Dynamics to the Clinic

Once the detailed activation of events of Akt/PKB were discovered above, a focus was directed towards investigating the clinical utility of Akt/PKB activation state, determined by FRET/FLIM. A study carried out in 2014 by Veeriah et al., endeavoured to correlate the activation state of Akt/PKB with patient outcome in breast cancer (37).

The study carried use an improved two-site labelling assay to assess the activation state of Akt/PKB. The authors labelled pan-Akt and pT308-Akt with species specific primary antibodies. These were in turn labelled with species-specific Fab fragments. The Fab fragment detecting the pan-Akt antibody was conjugated to Org488, acting as a donor chromophore. The Fab fragment detecting the pT308 antibody was conjugated to horseradish peroxidase. Tyramide signal amplification was then carried out to conjugate the acceptor chromophore, Alexa594 to the Fab-HRP (37). This amplification step, outlined in Figure 4, allows for an increase signal-to-noise ratio. In this step, Alexa594 conjugated tyramide (a phenol ring with a chromophore at the para position) forms a free radical in the presence of Horseradish peroxidase (conjugated to the sample) and hydrogen peroxide. A covalent bond is formed between the tyramide and nearby tyrosine residues (on the secondary Fab fragments labelling the acceptor site of the sample). This conjugates the acceptor chromophore to the sample and increases the signal to noise ratio of the sample.

In a bid to improve this quantitative imaging platform, the cross-disciplinary approach of the authors led to the creation of a high-throughput semi-automated system. This system allowed a user to select regions of interest and save these in a mapping file. The platform was able to use multiple-frequency domain FLIM (mfFLIM) to automatically analyse each region of interest. Crucially, the automation applied an automatically calculated signal-to-noise

threshold, excluding areas of the sample where the signal-to-noise ratio was not at least 4-fold (37).

In this study, FRET efficiency was a direct readout of Akt/PKB activation state. Kaplan-Meier analysis indicated that patients with a higher FRET efficiency, and therefore higher Akt/PKB activation state, had a statistically significantly worsened patient outcome (37). Crucially, whilst Akt/PKB activation state, predicted patient outcome, Akt/PKB expression levels, determined by standard immunofluorescence, were unable to do so.

The same Akt/PKB activation assay was utilised by Miles et al., 2017 to identify Akt/PKB as a prognostic biomarker in clear cell renal cell carcinoma (ccRCC) (38). As was observed with Veeriah et al., higher Akt/PKB activation state correlated with a worsened overall survival. pT308 Akt expression levels failed to predict patient outcome (38).

In the above studies by Weitsman et al., Veeriah et al., and Miles et al., it has been seen that oncoprotein activation states are potent predictors of patient relapse and/or outcome. However, current immunohistochemistry assays, which are utilised to stratify patients for treatment and aim to predict relapse and response, are insensitive and insufficient to predict these patient parameters (Figure 5).

Therefore, it can be stated with some confidence, that the use of quantitative imaging will be a key player in the development of novel companion diagnostics. The approaches discussed above led to the improvement of an already sensitive and powerful imaging platform. However, a focus on improving the methodology, either optically or biologically, whilst simultaneously seeking for novel applications of the technique are imperative.

The Application of Quantitative Imaging to Immune-Checkpoint Interactions

The use of FRET to analyse biomarker functional states should not solely be limited to determining oncoprotein activation. A study by Sánchez-Magraner et al., 2020 utilised the same two-site amplified FRET assays discussed above, coupled to mfFLIM, to examine PD-1/PD-L1 interaction states in a range of solid tumours. In this study, programmed death receptor 1 (PD-1) and its complimentary ligand PD-L1, were labelled with primary antibodies and further labelled with ATTO488 on PD-1, and Alexa594 on PD-L1. The determination of FRET between the two chromophores was able to report on receptor-ligand distances *in-vitro* and *in-situ.* The assay was immune-FRET (iFRET). The iFRET assay was compared with the Roche Ventana SP142 assay which is currently used to assess clinical PD-L1 expression. iFRET was able to detect a PD-1/PD-L1 interaction in 10 out of 11 PD-L1 negative clear-cell renal cell carcinoma (ccRCC) patients. This demonstrated that the confirmation of ligand expression does not corroborate receptor engagement, thus direct PD-1/PD-L1 interactions must be quantified. The authors then applied iFRET to 176 malignant melanoma patients. Of these patients, 159 were assessed by IHC to determine their PD-L1 expression. Of these patients, 117 were PD-L1 negative and 42 were PD-L1 positive. In the PD-L1 negative group, 58 patients demonstrated checkpoint interaction and 19 of 42 PD-L1 positive group showed no interaction state. The study highlighted that the expression of PD-L1, a parameter often subjectively quantified by pathologists did not correlate to patient outcome. However, the

iFRET assay, measuring PD-1/PD-L1 interaction was directly correlated with overall survival in melanoma and NSCLC immunotherapy-treated patients (39). The authors then applied the iFRET assay to 40 non-small cell lung carcinoma (NSCLC) patients. Again, iFRET was able to predict patient outcomes, with patients with a higher PD-1/PD-L1 interaction state experiencing a better outcome. As with the melanoma cohort, PD-L1 expression did not predict patient outcome in NSCLC patients.

This study therefore confirmed that FRET/FLIM quantification of biomarkers is not solely limited to protein activation dynamics and intracellular protein-protein interactions but can also successfully be applied to intercellular protein-protein interactions, a field which holds great potential in uncovering unknown signalling mechanisms between cells. However, these applications must be coupled to rapid and user-friendly imaging platforms.

Future Advancements of FLIM Assays – A Reduction of Acquisition Times

The aforementioned examples of the applications of FRET/FLIM systems make a compelling case for their use for future research and clinical applications. For the highest precision, time-correlated single-photon counting (TCSPC) offers the highest accuracy of FLIM measurements. Currently, typical FLIM acquisitions, with conventional laser scanning, are achieved in the order of high minutes (40). Advancements in the reduction of acquisition times, whilst preserving the accuracy and precision of lifetime measurements, will be key in enhancing the integration of FLIM into future biomedical research efforts. A range of techniques may be employed to enhance FLIM acquisition times, such as innovations in FLIM instrumentation, analysis and applications (41).

A range of time-domain FLIM microscopes rely on raster scanning to generate images. However this leads to high image acquisition times. In order to utilise time-domain FLIM for short-lived measurements, such as live-cell imaging, an increased acquisition speed must be achieved, whilst still maintaining a high spatiotemporal resolution. The majority of time-domain FLIM set-ups use TCSPC (42, 43). Whilst frequency domain FLIM offers faster image acquisitions, in the magnitude of seconds, the spatial resolution is lower. The key differences between time-domain FLIM and frequency-domain FLIM are outlined in Table 1. Simply increasing the raster scan speed is insufficient as this is limited by a minimal exposure time required to collect a suitable number of photons.

Table 1: Comparison of time-domain and frequency-domain FLIM

Time-Domain FLIM	**Frequency-Domain FLIM**
Pulsed laser light source	Acousto-optically modulated CW laser light source creates sinusoidally modulated incident light

Advantageous for measuring multiexponential decays	Less suitable for measuring multiexponential decays
Longer acquisition times are poor for the measuring of live-cellular events	Quick calculation of phase or modulation lifetimes allows for the measurement of short-lived events
Acquisition time in the magnitude of high seconds or minutes	Acquisition time in the magnitude of milli-seconds

To combat this, in 2012, Tsikouras et al., demonstrated a square multiple foci array which utilises a streak camera to carry out FLIM measurements (44). Here, a 2D foci array is generated using a lenslet array which is subsequently projected onto the sample plane. The generated fluorescence signal is then channelled back through the dichroic and collected by a matching 2D optical fibre array (44). The fibre array is rearranged from a 2D to a 1D configuration, suitable to enter the slit of the streak camera. This was coupled with a high-precision motorised stage. However, speed was still limiting acquisitions, and vibration perturbations from the stage make this platform less suitable to live-cell imaging (44). Mirror galvanometers could be utilised to scan an array of foci, but divergence from linear approximations can cause issues for high resolution scanning (45). The use of f-theta scanning lenses may combat this but they substantially increase the cost and complexity of the system whilst simultaneously reducing the transmission efficiency (46). This increased alignment complexity and reduction of transmission efficiency is not desirable for high-speed applications such as high content screening (HCS) and live-cell imaging.

In 2015, Tsikouras et al., designed a window tilt scanner system. This system reduces the cost, complexity and number of optical components of the previous set-ups. Briefly, this system achieves array scanning by using galvanometers mounted with flat windows which shift incident beams by refraction. These windows do not require the foci array to be shifted into the Fourier domain (45). As this scan relies on small angle approximations, it is vulnerable to the same large-angle nonlinearities encountered before. All of the members of the foci array undergo the same deflection nonlinearity, which can be corrected by adapting the angle step size at large angles (45).

This resulted in a system which can measure a FLIM data point in 0.1ms. The limiting factor is the camera read-out time, which for these authors was 10ms per frame. Here, a 30x30 scan of the 10x10 foci takes up to 9 seconds to complete, or 0.1ms per FLIM data point. A comparative, TCSPC image would require approximately 45 seconds. The authors have thus created a scanning FLIM system which is limited by camera read-out time as opposed to scan speed (45). This advancement of scanning speed, whilst maintaining a high spatiotemporal resolution could be key in transitioning FLIM to higher throughput applications such as HCS and drug discovery programs.

Another attempt to decrease FLIM acquisition times was devised by Poland et al. In 2018, these authors used a modified multifocal multiphoton FLIM system (MM-FLIM) which enables the acquisition of four individual planes simultaneously. This system acquires both

fluorescence intensity and chromophore lifetime information, which inherently gathers volumetric data without the requirement of multiple scans at different depths. Briefly, a Ti:Sapphire laser is projected onto a spatial light modulator (SLM). A phase pattern is applied which generates a 2D array of beamlets which are raster scanned using galvanometer scanners and projected onto the sample. The fluorescence each beamlet generates is then collected, de-scanned and projected onto the camera (47). Each beamlet is aligned to its corresponding detector, which matches the angular orientation and spacing of the array; thus enabling a high collection efficiency. The generated fluorescence beamlets then each generate a sub-image from a raster scan, which are stitched together to create the final image.

The authors verified this modified system by imaging live human epithelial cells which expressed a RhoA GTPase mTFP/Venus FRET biosensor. The new platform allowed for a novel spatiotemporal resolution of RhoA activation state relative to its position within the cell (48).

However, to truly be applied to high-content screening and drug discovery, these advancements in speed must be coupled with user friendliness. Garcia et al., 2020 devised an automated mutli-well plate FLIM system capable of performing fast and unsupervised acquisition (48). This system utilised parallel pixel excitation coupled with wide-field time-gated FLIM to reduce acquisition times. This system is able to acquire data from live cells at 10fps and can also determine AMPK activity in HEK293T spheroids (48, 49).

Karpf et al., have developed the spectro-temporal laser imaging by diffracted excitation (SLIDE) microscope. This is capable of high speed FLIM imaging by utilising an electro-optic modulator coupled to a high-speed sweep source Fourier-domain mode-locked (FDML) laser, whose output is passed through a diffraction element. This leads to the spectro-temporal encoding of each pulse to the extent that the wavelength provides point scanning, and the temporal information is used for signal to pixel mapping. The fluorescence is subsequently demodulated using a fast digitiser. The authors have reported FLIM acquisition speeds in the order of a million pixels per second (50).

These examples of improving the current FLIM platforms available will be critical in increasing their implementation into clinical research settings. A large research effort will be required to reduce FLIM acquisition times and the need for operator input, in a manner that still allows it to be implemented into a range of research applications. Moreover, the generation of novel and informative spatio-temporal datasets will have to be correlated to clinical parameters in order to impact patient outcomes and prognostics. However, even without these advancements, FRET/FLIM imaging platforms offer a unique manner for quantifying oncoprotein, and therefore oncogene, activation.

Discussion

The examples highlighted in this review sought to stress the crucial roles Förster Resonance Energy Transfer, couple to Fluorescence Lifetime Imaging Microscopy, have played in the field of signal transduction and oncogene activation. Particularly in an age where precision medicine is the gold-standard that clinicians and researchers are striving to achieve, the use

of FRET/FLIM will be a major player in the development of novel companion diagnostics. A recent study by Floerchinger et al., 2021, has demonstrated the use of single-cell intravital imaging *in-vivo*, using a Rac1 FRET biosensor to follow intratumoral movement. The study assesses drug response in live native tissue permitting to minimise off-target effects (51).

Whilst improvements in the quantitative imaging of oncogenes have been important in the field of cancer research, these do not report on post-translational modifications which result in the abhorrent signalling of oncoproteins. It is therefore imperative that oncoprotein activation state is assessed alongside oncogene expression profiles.

A further application of FRET/FLIM in the future of cancer prognostics would be its application to tumour-derived exosomes. Exosomes are small (30-100nm) vesicular bodies which are secreted by the majority of cells in the body (52). Tumours are known to excrete these bodies which travel throughout the body and interact with distant microenvironments (53). Felix Wong et al., in 2017 have shown that it is possible to apply their HER2-HER3 heterodimerisation assay to these circulating exosomes (52). It could, therefore, be of high interest to utilise these vesicular bodies, which are easily obtained from liquid biopsies, to offer routine cancer monitoring of patients. These could report on tumour sites from around the body, as opposed to the limited information that arises from single solid tumour biopsies (52). The ability to combine; a fast and operator independent FLIM assay, capable of generating a patient-specific oncoprotein activation signature, which can be applied to exosomes from routine liquid biopsies, could aid the C2c (cancer to chronic disease) approach first proposed by Ng and Beck in 2014 (54).

Lastly, whilst the use of FRET/FLIM is resulting in improved diagnostics and prognostics, continuous improvements to the accessibility and useability of FLIM systems should be maintained so as to increase the availability of quantitative biomarker imaging.

Perspectives

- **Importance:** the expression of oncogenes does not correlate to the functional states of their corresponding oncoproteins. Moreover, the expression of these proteins does not correlate with cellular function of patient response.
- **Current Thinking:** The evolution of time-resolved FRET, determined by FLIM has been crucial in elucidating the activation state and signal transduction of major oncoproteins; PKC, Akt/PKB, PDK1 and HER2-HER3. These activation states correlate to and predict patient outcome and relapse whereas oncoprotein expression profiles do not.
- **Future work**: should aim to ameliorate the speed and accuracy of FLIM acquisitions whilst also making these platforms more accessible to clinical research establishments. This will allow the quantification of oncoprotein activation to translate to improved personalised patient therapies.

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