IN VIVO ASSESSMENT OF FOCAL ADHESION KINASE (FAK) ACTIVITY IN BREAST CANCER CELLS USING FLUORESCENCE RESONANCE ENERGY TRANSFER (FRET) SENSOR AND CONFOCAL LASER SCANNING MICROSCOPE (CLSM)

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ABSTRACT
Focal Adhesion Kinase (FAK) is essential for cell migration and plays an important role in tumor metastasis. However, the complex intermolecular and intramolecular interactions that regulate FAK activity at the focal adhesion remain unresolved. We have engineered a toolbox of Fluorescence Resonance Energy Transfer (FRET) sensors for the assessment of FAK activity in human breast cancer cells (MCF-7). Major activity of cancerous cells is drastically growth of the cell in an uncontrollable manner in such cases our human anatomy system normally consists of cell growth activity. The important protein involved in cell functionality in the human body is FAK, due to FAK activity, cell motility, proliferation, survival has been managed in the human body hence, it is necessary to investigate the performance of FAK activity on breast cancer becomes important.

In our study, the differences in bleed through between zoom = 1 and for zoom >1 for donor and acceptor was evaluated. There were no significant differences in Pearson correlation coefficient and bleed through coefficient for both the zooms. With recent advances in fluorescent probes, instrumentation and methodologies, FRET is sure to revolutionize scientific research in the near future.

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LIST OF ABBREVIATIONS

MCF7 – Michigan Cancer Foundation 7
FAS - Focal Adhesion site
FAK - Focal Adhesion Kinase
CLSM - Confocal Laser Scanning Microscope
BT - Bleeding Through
FRET- Fluorescence Resonance Energy Transfer
FP - Fluorescent Proteins
LUT - Look Up Tables
1. INTRODUCTION

Developments in fluorescence microscopy have enabled the study of membrane diffusion, signal transduction and cell adhesion at the molecular level in living cells grown in culture. Progresses in optical microscopy have produced a diversity of approaches suitable for the study of protein changing aspects in cultured cells. This has been accelerated by the improvement of fluorescent proteins (FPs), which allow not only selective labeling and localization of virtually any protein, but also permit engineering of functional probes that report on the activity of signaling molecules such as kinases [Su Hao Lo, 2006] and GTPases [Adid.Dubash et al., 2009].
Cell migration, both single and collective, is a vastly unified multistep process that is important in tissue homeostasis, embryonic morphogenesis and immune surveillance. While collective migration requires the movement of cohesive groups of cells [Burridge, K et al., 1988], the single migrating cell is highly polarized with complex regulatory pathways that are spatiotemporally controlled [David D. Schlaepfer et al., 1994]. Migration contributes to several important pathological processes, including cancer progression and metastasis formation.

Fluorescence resonance energy transfer (FRET) involves the excitation of a donor fluorophore by incident light within its absorption spectrum. This radiative absorption elevates the donor fluorophore to a higher-energy excited state that would normally decay (return to the ground state) radiatively with a characteristic emission spectrum. If another fluorophore molecule (the acceptor) exists in proximity to the donor with its energy state characterized by an absorption spectrum that overlaps the emission spectrum of the donor, then the possibility of non-radiative energy transfer between donor and acceptor exists.

Using a mathematical linear regression analysis, a graph can be plotted keeping donor channel as an independent variable (x-axis) and FRET channel as dependent variable (y-axis). The graph plots a point for each pixel with donor channel intensity and its corresponding FRET channel intensity. A linear regression and standard error from the predicted value of regression can be calculated, thus, predicting the FRET channel from the donor channel pixel intensities.

1.1. FOCAL ADHESIONS

Focal adhesions are major sites of interaction between a cell and its extracellular matrix environment. Thus outside mechanical signals can be sensed at FAs through transmembrane receptors, such as integrin. Focal adhesions were first discovered via electron microscopy by Abercrombie et al. (1970) as electron-dense regions of the plasma membrane that make intimate contact with the substratum in cultured cells [Abercrombie M et al., 1970]. Physical interaction between the cells and outside extracellular matrix (ECM) is achieved through transmembrane integrins [Jihye Seong et al., 2011]. Integrin’s are heterodimer receptors containing α and β subunits, they function as transmembrane linkers (or integrators), mediating the interaction between the cytoskeleton and the extracellular matrix that are required for the cell to grip the matrix. When Focal adhesion are bound to the ECM, the
transmembrane integrin receptor can recruit signaling proteins and structural proteins such as Src, focal adhesion kinase integrin linked kinase (ILK). Focal adhesions play critical role in the maintenance of cell attachment, cell shape, proliferation, migration, differentiation, death and gene expression [Soule.H.D at al., 1973].

1.2. FOCAL ADHESION KINASE

Focal adhesion kinase is a cytoplasmic tyrosine kinase that plays critical roles in integrin-mediated signal transduction. FAK integrates signals from integrin and growth factor receptors to regulate cellular responses. FAK localizes at focal adhesions upon integrin clustering to regulate cell adhesion, migration, and mechanotransduction. FAK is phosphorylated in response to integrin engagement, growth factor stimulation and the action of mitogenic neuropeptides [Marc Thiriet, 2012]. In response to growth factors FAK regulates cell migration and proliferation in the integrin mediated signaling cascades. FAK plays a critical role in regulating cell migration, adhesion, spreading, reorganization of the actin cytoskeleton, formation and disassembly of focal adhesions and cell cycle progression, cell protrusions, cell proliferation and apoptosis [Martin Alexander Schwartz et al., 2001]. To visualize subcellular molecular event in live cells, genetically encoded biosensors based on fluorescent proteins (FPs) and Fluorescence Resonance Energy Transfer (FRET) were developed.

1.3. BIOSENSORS

FRET biosensors are genetically engineered proteins containing two fluorescent protein sequences, suitable to investigate the molecular regulations in living cells. Two particular types of FRET sensor have been used in this study. The Lyn-FAK biosensor allows for the visualization of activity of the membrane bound fraction of FAK localized to detergent resistant lipid rafts, whereas Cyto-FAK allows for the visualization of activity of soluble FAK localized to the cytosol.

1.4. FRET

FRET is a physical process by which energy is transferred from an excited fluorophore, the donor, to an acceptor fluorophores without radiation. As a result the emission of the excited donor is diminished (as part of the energy is transferred to the acceptor) and the emission of the acceptor gets enhanced [Forster, T, 1965]. A fluorophore is a chemical compound that absorbs light energy at a specific wavelength and re-emits the energy absorbed upon excitation in the form of fluorescence (light). The absorbed wavelength, energy transfer
efficiency and time before emission depend on both the fluorophore structure and its chemical environment. FRET technique is widely used by biologists who study the interaction between two proteins inside cells by expressing one of the proteins of interest fused to a donor fluorophore and the other protein fused to the adequate acceptor fluorophore. Measuring FRET between two interactive proteins is noninvasive. The emission of the acceptor fluorophore, due to the FRET from the donor, is measured in the so-called FRET channel.

1.5. 3CUBE FRET AND BIOLOGICAL MATERIAL

To measure the FRET index, 3 types of treatment (i.e. cells expressing different constructs) are acquired at each confocal microscope session. (a) One treatment where cells express only the donor fluorophore (e.g.: eCFP) in order to evaluate the donor bleed through coefficient. (b) One treatment with cells expressing the acceptor fluorophores (e.g.: eYFP) only, in order to evaluate the acceptor bleed through coefficient, i.e. the (c) one sample with cells co-expressing the two fluorophores in which FRET signals will be measured. A common combination of fluorophores for studying protein-protein interaction in cell of interest is Cyan Fluorescent protein (e.g.: eCFP) and Yellow Fluorescent Protein (YFP) as donor acceptor pair. In the Donor channel Image, the Donor have been emitted upon excitation of the donor. For CFP, excitation was achieved at short wavelength (458nm) and emission detected between 477-500nm. In the acceptor channel image (denoted “A” subsequently), the light emitted by the acceptor have been emitted upon excitation of the acceptor. For YFP, the excitation with long wavelength 514nm and emission between 540nm-570nm. In the FRET channel Image, For CFP/YFP pairs: excitation with wavelength 458nm end emission wavelength between 540nm-570nm was maintained. During the acquisition period and the collection of the image for the FRET analysis the Channel settings and the PMTs settings was maintained same, the argon - laser lines are adjusted to get the maximal dynamics.

In a FRET system, the emission wavelength of a fluorophore known as donor, excites another fluorophore at proximity known as acceptor. Thus, in FRET channel the excites in the donor wavelength and records the emission in acceptor wavelength. This can occur only when the emission wavelength of donor is equal to the excitation wavelength of the acceptor and the two fluorophores are in proximity. This sort of interactions between a pair of fluorophores open an exciting opportunity in understanding the interaction between two proteins of interest in molecular level by incorporating donor and acceptor fluorophores in each protein. One of
the demerits of FRET is the false positive in FRET which occurs due to two reasons: Donor bleed through and acceptor bleed through. Due to broader and overlapping peaks during emission of donor and excitation of acceptor fluorophores, there are probability of emission or leakage of donor in FRET channel known as donor bleed through and direct excitation of acceptor in FRET channel known as acceptor bleed through. Due to either of these, there are chances of getting a false positive values in FRET channel. Hence, the calibration of donor, acceptor and FRET channel can aid in reduction of false positive values. This can be achieved by calculating the bleed through values of the pair of fluorophores and subtracting them from the raw FRET images.

Using a mathematical linear regression analysis, a graph can be plotted keeping donor channel as an independent variable (x-axis) and FRET channel as dependent variable (y-axis). The graph plots a point for each pixel with donor channel intensity and its corresponding fret channel intensity. A linear regression and standard error from the predicted value of regression can be calculated, thus, predicting the FRET channel from the donor channel pixel intensities.

2. AIM AND OBJECTIVES OF THE STUDY

- To study the in vivo assessment of FAK activity in breast cancer cells using FRET sensor and CLSM.
- To visualize subcellular molecular event in live cells, genetically encoded biosensors based on fluorescent proteins (FPs) and Fluorescence resonance energy transfer (FRET) were developed.

3. MATERIALS AND METHODS

- In this study, we systematically developed and characterized several highly sensitive and specific biosensors Cyan Fluorescent Protein (eCFP) and Yellow Fluorescent Protein (eYFP) based on Fluorescence Resonant Energy Transfer (FRET) for visualizing Human Mammary cancer cells (MCF-7) activity in live cells.
- To adapt the FRET measurements, images were collected by a Confocal Laser Scanning Microscope (CLSM) and the images were analyzed by using free Software ImageJ (1.47v).
3.1 DNA CONSTRUCTION

The plasmid DNA was prepared according to PerfectPrep™ Endofree Maxi Kit Manual from 5 PRIME.

3.2. REAGENTS

Human Mammary cancer Cell lines were purchased from the American Type Culture Collection (ATCC, Manassas, VA) and cultured at 35°C and 5% CO2. Human Mammary cancer cells (MCF-7) were cultured to ~80% confluence in T-75-cm² Flasks. Cells were maintained in Dulbecco’s modified Eagle’s Medium (DMEM) supplemented with 10% Fetal bovine serum (FBS), 2 mmole/L Glutamine, 1 Unit ml⁻¹ Pencillin, 100 µg ml⁻¹ Streptomycin and 1 mM Mmole/L of sodium pyruvate. Cell culture reagents were purchased from GIBCO BRL and SIGMA. Cells were cultured in a humidified 95% air, 5% CO2 incubator at 37°C. Medium was replenished 3 times weekly (50-90%) in cultures that had not been transferred weekly. For cells transferred weekly medium was replaced on days 3 and 6. Trypsin EDTA (Sigma Aldrich) of 0.25% for 10mins was used for transferring cultures. Free floating passages were initiated by centrifuging the cells at 500 rpm for 5mins, the supernatant was removed and cells were resuspended with Medium [Gu, Y.H et al., 2006].

3.3. TRANSFECTION

The MCF-7 DNA was transfected in a 24 well plate (ROTH). The day before transfection, the cells were trypsinized and counted. About 4 x 10⁴ cells were plated per well in 0.5 ml of complete growth medium. Cell density was maintained between 50~80% confluent on the day of transfection. 3 glass coverslip (Therma scientific) of each 6×6mm diameter was given in each well. 9µl Transfast (Pro-omega), 3µg DNA, 700µl MEM was thawed, mixed together, vortexes and kept at 10-15mins at room temperature. Medium was changed after 24hrs - 48hrs of transfection. Cells that were cultured on coverslip was mounted onto confocal microscope slides along with CO₂ independent medium containing 0.5% FBS (Sigma) at 37°C to acquire images for analysis.

3.4. IMAGE ACQUISITION BY CLSM

To adapt the FRET measurements, images were collected by a Confocal Laser Scanning Microscope (CLSM) and the images were analyzed by using free Software ImageJ (1.47v). The freely available ImageJ Plug-in called FRET and Colocalization Analyser includes
several steps of image analysis controls before and after computation of the FRET Index Image. The False FRET images are eliminated since the FRET images are displayed according to the colocalization of the pair of the fluorophores. In this study we have also described our image treatment to evaluate donor and acceptor BTs, then the plug-in and finally our data analysis.

Cells were observed with an inverted microscope (Leica) and a laser scanning confocal imaging system (Leica microsystem) using a HCX PL APO CS 63× 1.2W CORR objective. Electronic zoom was set to 3, the pinhole was 1.00 array and the resulting pixel size was 0.154μm. eYFP was excited with the 514nm laser-line of the Argon Laser and detected and amplified by one photomultiplier tube(PMT1) in the so-called eYFP channel from 540-570nm. eCFP was excited with the 458nm laser-line of the Argon laser, detected and amplified by a second photomultiplier tube(PMT2) in the so-called eCFP channel from 477-500nm. For analysis consecutive set of images were obtained to get the average of images without noise ratio.

3.5. IMAGE ANALYSIS WITH IMAGEJ SOFTWARE

ImageJ version 1.47v for windows was obtained from the NIH (National Institute of Health) website. Various number of tools were used in this software.

- The mathematical functions applied on one image can be performed by “process/Math/Add/subtract…”
- The images are edited cut, copy, copy to the system by using “EDIT”.
- The Look up tables (LUT) were chosen using “Image /Look up tables…” the color of the images are changed using this option.
- The type of the images are changed using “Image/type”
- The tool “Analyse/plot profile” was used to visualize the profile of the pixel intensities along a linear selection in the images.
- To evaluate the Bleed Through (BT) of the Donor-, the total number of control images should be entered-, the donor channel image and the FRET channel Image from the same image should be chosen .the regression control image and the regression graph are obtained for the same image . the resulted regression graph indicates the pixel on the image that follows linear regression function used to calculate the Bleed-through parameter and of the pixels that are divergent from the equation.
The similar step is followed to Evaluate Acceptor BT, by choosing acceptor channel image and FRET channel image.

The regression graph along with the regression control image allows the user to decide whether the Bleed-Through coefficient is indeed correctly defined by the linear equation that is given in the <<Results>> text file

4. RESULTS

4.1. CALIBRATION OF 3 CUBE FRET

FRET measurements in living cells using “three-cube FRET” fluorescence microscopy has become increasingly popular as the method is fast, simple, non-destructive and requires only a standard fluorescence imaging microscope. With this method, images are acquired using three different fluorescence filter cubes: (1) the donor channel (IDD, donor excitation and emission), (2) the FRET channel (IDA, donor excitation, acceptor emission), and (3) the acceptor channel (IAA, acceptor excitation and emission). Because of spectral overlap between donor and acceptor fluorescent proteins (FP), procedures are used to isolate the donor (Idd), sensitized acceptor (Fc, i.e., fraction of IDA resulting from FRET) and direct acceptor (Iaa) fluorescence intensities from the uncorrected intensity images (IDD, IDA and IAA).

4.1.1. DONOR BLEED THROUGH CALIBRATION

FRET and Colocalization analyser plug-in was used to calculate the donor and acceptor bleed through calibration. For donor bleed through evaluations, both donor channel (D) and FRET channel (F) images of the cells expressing only the donor fluorophore (d) was obtained (Dd and FD respectively). Images were acquired at zoom = 1 and at zoom > 1 (Figures 2A and 4A, digital zoom function of the CLSM). Fluorescence intensity values (ΔF, in arbitrary units) were assessed for individual pixels. ΔF_{Dd} vs ΔF_{Fd} values were plotted to obtain a linear regression function according to the following equation.

ΔF_{Fd} = a.ΔF_{Dd} + b

By linear regression four different parameters were obtained from each individual set of images (i) the donor bleed through coefficient a (a=ΔΔF_{Fd} / ΔΔF_{Dd}), (ii) the values of ΔF_{Fd} at ΔF_{Dd}=0 (b;interception ), (iii) pearson correlation coefficient of the regression (r) and (iv) the standard error (SEM) in the estimation of r up to ten sets of images for both zoom
settings 1 and >1 from an individual experiment was analysed. See, figure 1 and 3 for original images with high correlation of ΔF<sub>Fd</sub> with ΔF<sub>Dd</sub> (r ≥ 0.9) and figures 2 and 4 for poor correlation (r ≤ 0.9). The differences between zoom =1 and for zoom >1 were analysed for the four parameters obtained (a, b, r and SEM respectively). These parameters were analysed separately for all the images as well as for the images with pearson correlation coefficient ≥ 0.9.

1A

Dd  Fd  Transmission

Fig.1: Original images and correction for donor bleeding through at zoom =1 and with high correlation coefficient for ΔF<sub>Dd</sub>/ΔF<sub>Fd</sub>. Fig.1A: The sequence of channel from left to right is original Donor channel (Dd) image, FRET channel image (Fd) and transmission(for better visualization of colocalization) in the right, of which cells expressing only donor fluorophores at zoom = 1 and with high correlation coefficient r >0.9. Fig.1B: The donor

Regression ΔF<sub>Fd</sub> = a.ΔF<sub>Dd</sub> + b
a: 0.768  b: -1.873
r: 0.960   SEM: 2.557
bleeding through correction and their corresponding regression graph at zoom=1 and with high correlation coefficient ie r >0.9

2A

Dd Fd Transmission

![Image of original images and correction for donor bleeding through at zoom >1 and with high correlation coefficient](image)

2B

Dd ΔF_{Dd}

![Image of donor bleeding through correction and their corresponding regression graph at zoom >1 and with high correlation coefficient](image)

Regression:\Delta F_{Fd} = a \cdot \Delta F_{Dd} + b

\begin{align*}
a & = 0.853 \\
b & = -4.201 \\
r & = 0.985 \\
\text{SEM} & = 4.980
\end{align*}

Fig.2: The original images and correction for donor bleeding through at zoom >1 and with high correlation coefficient \( \Delta F_{Dd}/\Delta F_{Fd} \). Fig.2A: The sequence of channel from left to right is original Donor channel (Dd) image, FRET channel image (Fd) and transmission(for better visualization of colocalization) in the right, of which cells expressing only donor fluorophores at zoom >1 and with high correlation coefficient ie r >0.9. Fig.2B: The donor bleeding through correction and their corresponding regression graph at zoom >1 and with high correlation coefficient ie r >0.9

3A

Dd Fd Transmission

![Image of original images and correction for donor bleeding through at zoom >1 and with high correlation coefficient](image)
Fig. 3: The original images and correction for donor bleeding through at zoom >1 and with low correlation coefficient $\Delta F_{Dd}/\Delta F_{Fd}$. Fig. 3A: The sequence of channel from left to right is original Donor channel (Dd) image, FRET channel image (Fd) and transmission (for better visualization of colocalization) in the right, of which cells expressing only donor fluorophores at zoom $=1$ and with low correlation coefficient $r < 0.9$. Fig. 3B: The donor bleeding through correction and their corresponding regression graph at zoom $=1$ and with low correlation coefficient $r < 0.9$.

Regression: $\Delta F_{Fd} = a \cdot \Delta F_{Dd} + b$

- $a: 0.729$
- $b: 3.267$
- $r: 0.534$  SEM: $5.429$
Fig. 4: The original images and correction for donor bleeding through at zoom >1 and with low correlation coefficient $\Delta F_{Dd}/\Delta F_{Fd}$. Fig. 4A: The sequence of channel from left to right is original Donor channel (Dd) image, FRET channel image (Fd) and transmission(for better visualization of colocalization) in the right, of which cells expressing only donor fluorophores at zoom >1 and with low correlation coefficient $r < 0.9$. Fig. 4B: donor bleeding through correction and their corresponding regression graph at zoom >1 and with low correlation coefficient $r < 0.9$.

Regression: $\Delta F_{Fd} = a \cdot \Delta F_{Dd} + b$

a : 0.562 b : -6.937

SEM: 26,404 r : 0.362
Fig. 5: Acquired ten sets of images of the cells expressed at zoom =1 and zoom >1 are compared with the four different parameters, which was obtained from linear regression graph for every individual set of images. A: \( \Delta F_d / \Delta F_{d'} \); the donor bleed through coefficient of the cells expressed at zoom=1 and zoom >1 are compared. B: intersection value obtained from the cells expressed at zoom=1 and zoom >1 are compared. C: Pearson correlation coefficient of the cells expressed at zoom=1 and zoom >1 are compared. D: standard error obtained from the cells expressed at zoom=1 and zoom >1 are compared.
4.1.2 ACCEPTOR BLEED THROUGH CALIBRATION

Similar to donor bleed through calibration, acceptor bleed through coefficient was determined. In this case acceptor channel and FRET channel images were obtained from cells expressing only acceptor fluorophores and a regression graph was plotted accordingly (Figure 3) for zoom = 1 and for zoom >1. Pearson correlation coefficient and standard error were obtained for individual regression graphs for up to ten images for zoom = 1 and for zoom >1. The difference in bleed through coefficient and any compensation between zoom = 1 and for zoom >1 were analysed. Various parameters between zoom = 1 and for zoom >1 were analysed (Figure 4) for all the images as well for images with Pearson correlation coefficient greater than 0.9 (Figure 4 B). Parameters such as FRET intensity, standard error and Pearson correlation coefficient were compared between zoom = 1 and for zoom >1.
Fig. 7: Original images and correction for acceptor bleeding through at zoom = 1 and with high correlation coefficient for $\Delta F_{Aa}/\Delta F_{Fa}$. Fig. 7A: the sequence of channel from left to right is original acceptor channel (Aa) image, FRET channel image (Fa) and transmission (for better visualization of colocalization) in the right, of which cells expressing only acceptor fluorophores at zoom = 1 and with high correlation coefficient $r > 0.7$. Fig. 1B: acceptor bleeding through correction and their corresponding regression graph at zoom = 1 and with high correlation coefficient i.e. $r > 0.7$.

Regression $\Delta F_{Fa} = a \cdot \Delta F_{Aa} + b$

$a: 0.419$ $b: 4.155$

SEM: 3.709 $r: 0.762$
Fig. 8: Original images and correction for acceptor bleeding through at zoom >1 and with high correlation coefficient for $\Delta F_{Aa}/\Delta F_{Fa}$. Fig. 8A: the sequence of channel from left to right is original acceptor channel (Aa) image, FRET channel image (Fa) and transmission (for better visualization of colocalization) in the right, of which cells expressing only acceptor fluorophores at zoom > 1 and with high correlation coefficient $r > 0.7$. Fig. 1B: acceptor bleeding through correction and their corresponding regression graph at zoom > 1 and with high correlation coefficient ie $r > 0.7$. 

Regression: $\Delta F_{Fa} = a \cdot \Delta F_{Aa} + b$

$a : 0.468$  
$b : 9.106$

SEM: 6.156  
$r = 0.900$
Fig. 9: Original images and correction for acceptor bleeding through at zoom =1 and with low correlation coefficient for $\Delta F_{Aa}/\Delta F_{Fa}$. Fig. 9A: the sequence of channel from left to right is original acceptor channel (Aa) image, FRET channel image (Fa) and transmission (for better visualization of colocalization) in the right, of which cells expressing only acceptor fluorophores at zoom = 1 and with low correlation coefficient $r > 0.7$. Fig. 9B: acceptor bleeding through correction and their corresponding regression graph at zoom=1 and with low correlation coefficient ie $r > 0.7$. 

\[ \Delta F_{Aa} \]
Fig. 10: Original images and correction for acceptor bleeding through at zoom >1 and with low correlation coefficient for $\Delta F_{Aa}/\Delta F_{Fa}$. Fig. 10A: the sequence of channel from left to right is original acceptor channel (Aa) image, FRET channel image (Fa) and transmission (for better visualization of colocalization) in the right, of which cells expressing only acceptor fluorophores at zoom >1 and with low correlation coefficient $r > 0.7$. Fig. 10B: acceptor bleeding through correction and their corresponding regression graph at zoom>1 and with low correlation coefficient i.e $r > 0.7$
Fig. 11: Acquired ten sets of images of the cells expressed at zoom =1 and zoom >1 are compared with the four different parameters, which was obtained from linear regression graph for every individual set of images. A: \( \frac{\Delta F_{Fa}}{\Delta F_{Aa}} \); the acceptor bleeding through coefficient of the cells expressed at zoom=1 and zoom >1 are compared. B: intersection value obtained from the cells expressed at zoom=1 and zoom >1 are compared. C: pearson correlation coefficient of the cells expressed at zoom=1 and zoom >1 are compared. D: standard error obtained from the cells expressed at zoom=1 and zoom >1 are compared.
Fig. 12: Same as fig 11, but only images with Pearson correlation coefficient $r \geq 0.7$ of the cells expressed at zoom $=1$ and zoom $>1$ are compared.

4.2. FRET ANALYSIS

Static FRET analysis was performed for cytosolic located FRET sensor (cFAK) and for FRET sensor localized to detergent resistant domains of the plasma membrane (LynFAK).

4.2.1. cFAK
5. DISCUSSION

The necessity for *invivo* imaging has established in aggregation with the considerate that several cellular responses within a tissue niche are determined by signals. Imaging experiments in vivo differ from in vitro experiments in numerous ways. Not only do cells look and behave differently but also the questions asked are often slightly different. Moreover, imaging cells within tissues presents various technical challenges that coerce *invivo* experimental design and influence the choice of imaging approach. In this section, we consider some of the subjects that relate to *invivo* experimental design and offer suggestions how to create an experimental pipeline that promotes successful invivo imaging [Paul Timpson *et al.*, 2011].

Cancerous cell major activity is drastically growth of the cell in an uncontrollable manner in such cases our human anatomy system normally consists of cell growth activity. The important protein involved in cell functionality in the human body is FAK (Focal Adhesion kinase) located in the 8q24.3 chromosomes and in gene PtK2. Due to FAK activity cell motility, proliferation, survival has been managed in the human body hence, it is necessary to investigate the performance of FAK activity on breast cancer becomes important.
The research aim was to investigate the FAK activity on the breast cancer using FRET and CLSM. FRET provides the clear view about the interaction between the molecules within the cell further through FRET interaction between proteins can be clearly defined protein conformation. The images of breast cancer cells are collected through CLSM which is fed into FRET for analysis. This section provides the detailed description regarding the FAK activity on breast cancer which is examined through FRET.

In our study, the differences in bleed through between zoom = 1 and for zoom >1 for donor and acceptor was evaluated. There were no significant differences in Pearson correlation coefficient and bleed through coefficient for both the zooms.

A powerful imaging method to study protein-protein interactions in living cells is fluorescence resonance energy transfer (FRET). FRET is the non-radiative transfer of energy from a donor fluorophore in an excited state to a nearby acceptor fluorophore to allow energy transfer if within only 10 nm. Because this distance is in the range of protein sizes, FRET can also be used to study conformational changes of proteins tagged with a FRET donor and FRET acceptor. The most frequently used FRET methods are sensitised emission, ratio imaging and acceptor photo-bleaching FRET but the latter is not appropriate for studying rapid changes of protein interactions over time. The sensitized emission approach detects the emission of the acceptor fluorophore (often cyan fluorescent protein, CFP) while the donor fluorophore (often yellow fluorescent protein, YFP) is excited [Elangovan M et al., 2002]. Cross-talk and bleed through from one fluorophore to another makes the analysis highly dependent on control measurements of cells in which only one of the two fluorophores is present. An alternative approach to determine FRET is acceptor/donor ratio imaging (e.g. YFP/CFP) where both donor and acceptor emission are detected simultaneously when excited at the excitation wavelength of the donor. However, this method can be only applied when donor and acceptors are equally expressed in a cellsystem which is always the case when using FRET biosensors. In the study of adhesions, a few FRET biosensors have been designed to monitor in live cells the activity of a number of kinases, e.g. Src and FAK [Rajesh BS., Periasamy A., 2003]. When correctly applied, FRET is a useful tool for investigating the molecular mechanisms that regulate integrin-mediated signalling in migrating cells [Kraynov, V.S et al., 2000].

6. ETHICAL ASPECTS AND IMPACT OF RESEARCH ON THE SOCIETY
In vivo experimental design presents many challenges that are not encountered in in vitro, including surgical preparation of animals, anaesthesia, suppression of motion artefacts arising from breathing, heartbeat and muscle twitching, a limited timescale of observation, a reduced number of experimental observations, difficulty synchronizing experiments and reduced optical sensitivity and resolution.

The integration of molecular engineering and nano/micro-technology with fluorescent proteins can provide powerful tools for live cell imaging. The molecular activities and hierarchy inside live cells are largely dependent on the subcellular location. FRET biosensors can be applied to investigate the molecular regulation in live cells under mechanical stimulations.

7. CONCLUSION

Biology, imaging and spectroscopy have been recently combined to provide powerful tools for research and clinical applications. FRET technology is a powerful tool to study protein-protein interactions in living cells. As FRET efficiency is dependent upon the distance between the two fluorophores and their relative orientation, changes in FRET correspond to changes in distance and orientation between the fluorescent probes and in the case of a single protein fused to two fluorophores can reflect changes in conformation. Here, we have developed FRET biosensors to visualize changes in FAK conformation in vivo. Potential applications for tissue FRET imaging are also burgeoning. With recent advances in fluorescent probes, instrumentation and methodologies, FRET is sure to revolutionize scientific research in the near future.

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9. REFERENCES


