In Vivo Characterization of Calcium Signaling of NIH 3T3 Fibroblast Cells in Response to Growth Factors

Linda M. Nguyen
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Thesis Advisor:
Amy Palmer, Ph.D. (Department of Chemistry and Biochemistry)

Committee Members:
Rob Knight, Ph. D. (Department of Chemistry and Biochemistry)
Robert Garcea, Ph. D. (Department of Molecular Cellular and Developmental Biology)

University of Colorado at Boulder
Department of Chemistry and Biochemistry
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Abstract

Calcium is a vital ion in biological systems. It works to regulate and monitor a multitude of processes from macroscopic functions such as muscle contraction to microscopic processes such as cell proliferation through a complex signaling system. A signaling cascade is initiated when a stimulus binds to a receptor that generates calcium release which serves to activate a specific cellular function. The binding of growth factors to receptors located on the cell membrane can lead to activation of transcription factors. In addition, previous biological studies concerning a strain of polyomavirus have also found that certain transcription factors, namely *c-myc, c-jun, c-fos,* and *JE* (growth factor inducible genes) accumulate immediately after infection with the virus. As such, the focus of this thesis was to characterize the nature of calcium responses to dosages of growth factor in efforts to lay a foundation for future investigations of growth factors and infection with polyomavirus in NIH 3T3 fibroblast cells. Cells were treated with varying concentrations of growth factor and responses were monitored *in vivo* with one of two genetically-encoded sensors—D3cpv pc3' (targeted to the cytosol) or D3cpv nuc (targeted to the nucleus). Efforts were made to optimize transfection efficiency, characterize the nature of the responses, as well as to lay a basic foundation of knowledge to use in later infections with polyomavirus.

Introduction

Calcium signaling

Calcium is a ubiquitous ion responsible for a vast and vital signaling system within biology. It initiates and controls a wide range of cellular processes from small-scale functions such as cell proliferation and differentiation to large-scale processes such as muscle contractions. Other functions of calcium include its role in fertilization, transcription factor activation, cancer, and apoptosis. These varying processes are regulated by the speed, amplitude, and spatio-temporal patterning of the calcium signals[1]. Spatio-temporal patterning refers to the duality of where the signals are located within a cell and the amount of time calcium is retained in the cell or the amount of time it takes to return back to resting levels. The figure below displays the effects of spatio-temporal patterning on the regulation of familiar cellular processes.

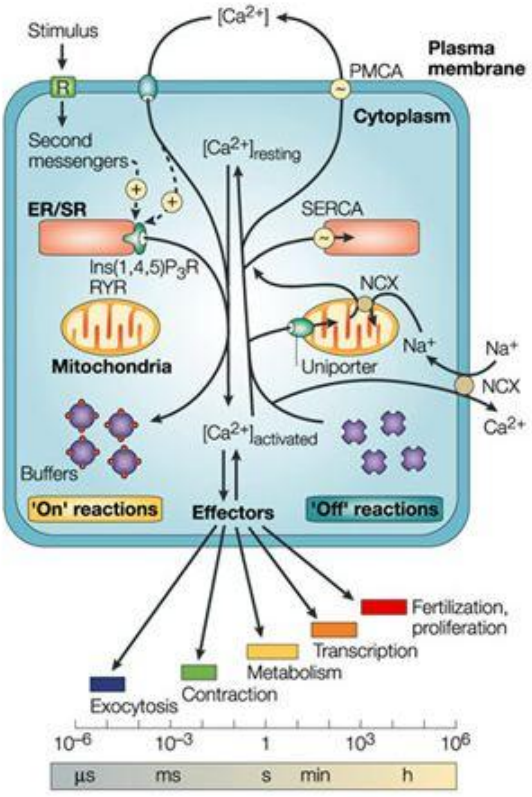

Figure 1. Regulation of cellular processes due to the spatio-temporal patterning of calcium signals. Figure reproduced from reference [1].

A cell that is not maintained within the normal boundaries of calcium levels can result in negative consequences such as high toxicity leading to cell death. In the simplest sense, a calcium signaling occurs when resting levels of calcium are elevated. A cell at rest maintains a constant calcium concentration of approximately 100 nM, whereas activated cells exhibit an increased amount of roughly 10 times the resting amount of calcium of 1000 nM[2]. The change in concentration and activity is generated by the flux between internal stores and external sources. Internal stores are encompassed by the endoplasmic reticulum (ER) or the sarcoplasmic reticulum (SR). Through the use of a diverse set of channels, calcium can be exchanged with external stores. Of the known ER/SR calcium channels, two of the most extensively studied are the inositol-1,4,5 trisphosphate receptor ($InsP_3R$) and ryanodine receptor (RYR).

Specifically for the $InsP_3R$ pathway, binding of a ligand to a receptor on the cell membrane leads to the production of $InsP_3$ which diffuses through the cell and engages $InsP_3R$ to release calcium from the ER[2]. A study conducted by Kuriyama and coworkers explored growth factor-induced changes on free calcium ion concentrations in the cytosol in human retinal pigment epithelial (RPE) cells[7]. Various stimuli were used to generate a response in the RPE cells including: epidermal growth factor (EGF), basic fibroblast growth factor (bFGF), nerve growth factor (NGF), platelet-derived growth factor (PDGF), insulin-like growth factor, fibronectin, and fetal bovine serum (FBS). However, only FBS, bFGF, and PDGF increased the production of $InsP_3$ through activation of tyrosine kinases[7]. Tyrosine kinases work through different phospholipase C (PLC) mechanisms[12] in response to extracellular signals to generate $InsP_3$, as mentioned above, which is upstream from calcium release from inner-stores located in the ER. The external store of calcium is the cytosol and is moderated by a negative feedback

loop. At low concentrations (below 300 nM), calcium acts as a stimulant, but at higher concentrations (above 300 nM) calcium is inhibitory and the channel is switched off. Though calcium is plentiful in biological systems, careful regulation of calcium within biology is crucial. Too little or too much calcium can result in health a problem as disruptions to its signaling mechanism is suggested as the cause of various diseases and cancers[1].

Detection of calcium within cells using fluorescent sensors

The use of fluorescent microscopy is widely used within the biochemical field. It has the ability to quantify the concentration of an ion of interest through approximations. However, paired with the application of mathematical formulas, the true concentration of an ion of interest can be determined as well. The basic concept of fluorescent sensors relies on the ability of a flourophore (a molecule that can fluoresce) to absorb a higher energy (shorter wavelength) and emit one at a lower energy (longer wavelength). By blocking out the excitation source light and focusing solely on the emitted light, the fluorescence of molecules can be analyzed[8].

The detection of calcium within cells and biological systems in this experiment are monitored through the use of fluorescent sensors. Fluorescent sensors have the capability to change their absorbance and/or emission spectra in response to binding to an ion of interest such as calcium. Some sensors are used to approximate the amount of calcium ions within a system through the intensity of fluorescence. However, a multitude of fluorescent sensors are available including small-molecule sensors, probes, and genetically-encoded sensor**s.** Each type of fluorescent sensor has advantages and disadvantages to its use. For example, detection of calcium with small-molecule sensors has the benefit of increased sensitivity and increased kinetics, but is paired with the large disadvantage in the low control of dye localization.

For measuring calcium levels in biological systems, genetically encoded sensors have grown in recent years due to the ability to target them to specific organelles. The sensors used in this study to monitor calcium changes in response to growth factors are genetically encoded calcium indicators (often called cameleons) that are able to be used to generate ratiometric data. Paired with a Zeiss Axiovert 200M microscope and MetaFlour software, the ratiometric data for this thesis were obtained by dividing the intensity of the FRET channel by the intensity of the CFP channel $(I_{FRET} - I_{background}) / (I_{CFP} - I_{background})$. By doing so, FRET ratios that were quantified at low intensities were represented by cooler colors such as blue while higher intensities, and therefore higher concentrations of calcium, were represented by warmer colors such as red. The indicators are comprised of two fluorescent proteins and two Ca^{2+} responsive elements[11]. Through the incorporation of a circularly permuted (cp) variant of an acceptor fluorescent protein (a variant of YFP) greater ratio metric sensitivity and larger dynamic range are achieved[6]. The acceptor fluorescent protein is often Venus or citrine. As the figure below exhibits, when Ca^{2+} binds to the two Ca^{2+} responsive elements (calmodulin, CaM, and the peptide, M13), the sensor undergoes a conformation change leading to fluorescence resonance energy transfer (FRET) from ECFP to the attached cpV[10].

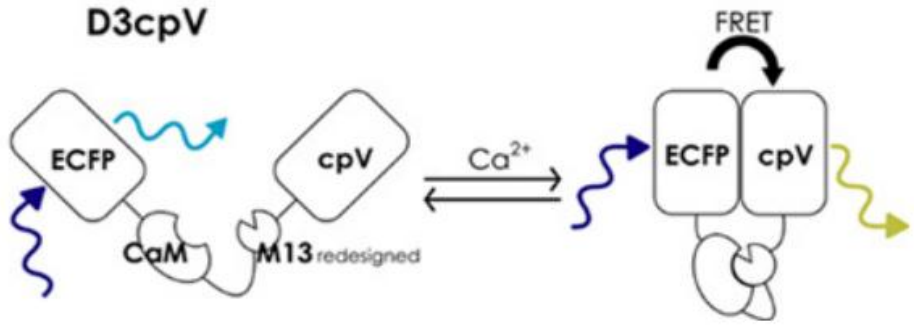

Figure 2. Schematic of calcium genetically-encoded sensors. Figure reproduced from literature[10].

The sensors utilized in this thesis are based on the overall design presented in Figure 2 and are referred to as D3cpv and D3cpv nuc, for cytosolic and nuclear-localized sensors, respectively. These sensors contain mutant versions of the Ca^{2+} responsive elements (mCam and m-smMLCKp), ECFP and cp-Venus[11].

Significance of calcium in understanding the nature of polyomavirus

Previous research[3,5,13] has indicated that elevated levels of the transcription factors *c-myc*[5,13], *c-fos*[5,13], *c-jun*[5], and *JE*[5] (PDGF-inducible genes) are characteristic in cells infected with polyomavirus[5,13] and simian virus 40[3] (a variant of polyomavirus). According to Garcea and co-workers, cellular genes associated with commitment to the cell cycle may be important in the two stages of the replicative cycle of the DNA virus[13]. The first stage of competence is regulated by platelet-derived growth factor and the second stage of progression is regulated by epidermal growth factor[13]. Detection of PDGF-inducible genes *(*specifically *c-myc, c-fos, c-jun,* and *JE)* was found in BALB/c 3T3 cells when infected with polyomavirus. Garcea and coworkers have found that with the infection of polyomavirus, *c-myc, c-fos, c-jun,* and *JE* behave in a biphasic manner. That is, the mRNA genes accumulate heavily within the first hour of infection; drop to undetectable levels at the 6 hour mark, and return to elevated levels 12 hours post-infection and remain at elevated[13]. The figure below illustrates the biphasic behavior of the early response genes as described above.

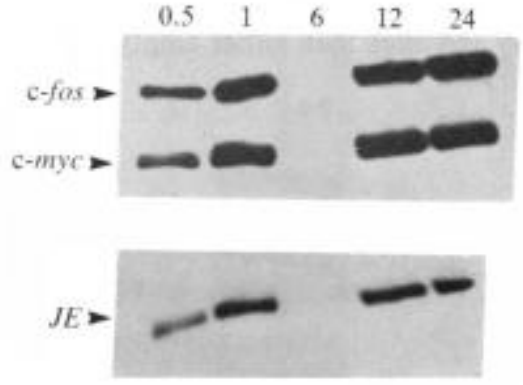

Figure 3. Time course of mRNA accumulation post infection with CsCl-purified virus. Figure reproduced from literature[13].

Therefore, research conducted by Zullo, Garcea, and Stiles suggested that the initial response at 1 hour could be attributed to viral attachment to a cell membrane receptor—thereby mimicking a growth factor.

Research performed by Glenn and Eckhart, examined the expression of early-response genes and polyomavirus through the analysis of small (sT), medium (MT), and large (LT) T-antigens by focusing on how mRNA levels affected regulation at the transcriptional level. The three T-antigens are encoded by the virus and serve different purposes. LT is required for initiation of viral DNA for replication and immortalization, MT affects phosphorylation of the major virus capsid protein VP1, and sT enhances the viral DNA replication in infected cells[5]. By varying the availability of T antigens at different stages of the virus, it was determined that the initial activation of early genes at 1 hour was due to interaction of the major viral capsid with cell receptors, whereas the second response at 12 hours post infection was due to early viral expression[5]. Also in conjunction with the findings of Glenn and Eckhart, sT antigens were found to be vital in virus regulation whereas MT and LT served smaller roles[5].

In a similar experiment performed by Dangoria and coworkers, a variant of polyomavirus named simian virus 40 was also shown to up-regulate primary response genes[3]. However, the study worked to verify the virus' dependence of the tyrosine kinase and protein kinase C (PKC) pathways[3]. It is also known that these pathways initiate a calcium signaling cascade. Therefore, through the observation of calcium, tyrosine kinase and PKC channels can be analyzed in relation to the up-regulation of early response genes. More specifically, calcium concentrations can be moderated to see if the virus uses a similar method to incorporate polyomavirus into the cell cycle.

Goals of this thesis project

The goals of this study were to determine the nature of calcium signaling in NIH 3T3 fibroblasts in response to growth factors while becoming familiar with mammalian cell culture and conducting imaging experiments with genetically-encoded sensors. This work will serve as the foundation for future efforts aimed at examining whether cells infected with polyomavirus upregulate early response genes by activating cellular Ca^{2+}. Growth factors have been shown to induce certain mRNA transcription factors such as *c-myc*, *c-fos,* and *c-jun*. However, studies conducted by Garcea, Glenn, Dangoria and coworkers have identified that within the first hour after infection of polyomavirus (or similar strains), cells accumulate early response genes in a biphasic manner[3,5,13] without growth factor stimulation. Therefore, the primary goal of this experiment was to determine if polyomavirus mimics the effect of growth factors by binding to a receptor on the cell membrane and activating a Ca^{2+} signaling cascade that introduces and commits the virus into the cell cycle.

Calcium signaling in response to different concentrations of growth factors was monitored by genetically-encoded sensors in the cytosol and nucleus *in vivo.* Characterization of cells was classified under the following categories: transient, delayed transient, oscillatory, delayed oscillatory, and elevated. These responses yielded an understanding of the effects of growth factors in NIH 3T3 fibroblasts and can serve as a basis of comparison for future experiments with cells infected with the polyomavirus.

Materials and Methods

DNA amplification of sensors

Mammalian expression plasmids containing genetically encoded sensors were transformed into E. coli and purified. Under separate experiments, 1 µl DNA of each respective

sensor was transformed into DH5α E.coli and streaked onto LB/AMP agar plates. The following day, a single colony was extracted and inoculated into 5 ml LB and 7 µl AMP and left to grow at 37°C overnight. The bacterial culture containing the genetically encoded sensor was purified through a midi-prep kit (QIAGEN) to obtain a concentration of approximately 1000 ng/µl for subsequent use in live-cell imaging.

Mammalian cell culture

Experiments were conducted according to the figure below.

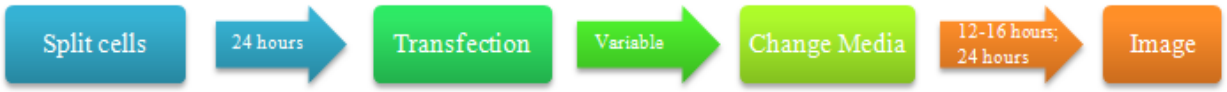

Figure 4. General experimental layout

NIH 3T3 mouse fibroblast cells were obtained from liquid nitrogen storage for usage and plated in sterile cell culture dishes. Cells were maintained at 37ºC at 5% CO_2 in Dulbecco's Modified Eagle's media (DMEM) supplemented with 10% fetal bovine serum and 1% penicillin/streptomycin and discarded after sixteen passages. For general maintenance, cells were seeded to achieve full confluence by day 5 with a replenishment of new media on day 3. To test calcium signaling *in vivo*, cells were split onto poly-l-lysine coated imaging dishes to reach 50-70% confluence by day 2.

Transfection with DNA encoding sensors

Transient transfection of NIH 3T3 cells occurred 24 hours after plating them on 3.5 cm glass bottom imaging dishes. To optimize the transfection efficiency, different chemical transfection agents (TransIT-LT1 transfection kit (Mirus) and a specialized TransIT-3T3 (Mirus) transfection kit) were assessed. Approximately 250 µl of OPTI-MEM and 3 µl of TransIT-LT1 were allowed to mix for 5 minutes before the introduction of 1 µg of either a cytosolic (D3cpv

pc3') or nuclear-targeted (D3cpv nuc pc3) DNA encoding sensor. The mixture was allowed to sit for another 15-20 minutes before its addition to the imaging dishes. Transfection of cells using the specialized kit was performed according to the manufacturer's protocol. After 12-16 hours, or 24 hours the media was changed to a serum-free medium.

Imaging experiments

All cells were imaged between 24-48 hours post-infection. Imaging data were collected with an Axiovert 200M microscope inverted fluorescence microscope (Zeiss) equipped with the following filters:

YFP	Excitation (nm) 495/10 Dichroic Mirror 515 Emission (nm) 535/20 or 542/50 Neutral density #1; 10% light transmission
CFP	Excitation (nm) 430/24 Dichroitic mirror 455 Emission (nm) 470/24 Neutral density #1; 10% light transmission

The microscope was set for viewing with a 40X objective under oil immersion. Cells were removed from incubation and rinsed with HEPES Hanks Buffered Salt Solution (HHBSS, pH 7.4) to fully remove previous media. Cell regions were selected with CFP spot

measurements within the range of 2500-6000 fluorescence counts. Additionally, a background region was placed on a region of the dish that did not contain cells. This region was used for background correction of all the imaging data. Cells were conditioned with 1 ml of HHBSS and a baseline (i.e. resting ratio) was collected for fluorescent cells within view. The ratio signal is defined as the background subtracted fluorescence intensity in the FRET channel divided by the background corrected fluorescence intensity in the CFP channel $(I_{FRET} - I_{background}) / (I_{CFP} - I_{background})$. At approximately 500 seconds, cells were treated with working concentrations of growth factor[3-4] and responses were observed. Images were acquired every 10-15 seconds. Once the ratio returned to baseline level, the dish was washed-out by removing HHBSS and conditioning with Ca^{2+} - free HHBSS for calibration. A minimum ratio (R_{min}) was then collected by chelating calcium ions with a solution of 10 µl ionomycin, 30 µl 0.5M EGTA, and 960 µl Ca^{2+}-free HHBSS. A maximum ratio (R_{max}) followed by introducing high levels of calcium into the cells to observe optimum saturation of the sensor through a solution of 10 µl ionomycin, 10 µl 1 M $CaCl_2$, and 980 µl HHBSS. Each experiment was background corrected and graphs were replicated in KaleidaGraph.

Testing of different growth factors

Two types of growth factors were tested to determine which one was more efficient in studying calcium signaling responses in 3T3 fibroblasts—fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF). Fresh aliquots of each were prepared in reference to working literature values and were used as treatment in live-cell imaging of transfected cells. The working concentration of FGF and PDGF were 10 ng/ml[4] and 20 ng/ml[6] respectively. Each experimental run was conducted independently of all others.

Testing different concentrations of PDGF

Varying concentrations of PDGF ranging from 10-30 ng/ml were tested in cells using either a genetically cytosolic encoded sensor or a nuclear-targeted sensor to better characterize the nature of calcium responses.

Results

Optimization of transfection conditions

In preliminary trials, NIH 3T3 fibroblasts were obtained from liquid nitrogen storage and plated onto sterile cell culture plates. The cells achieved full confluence by day 5 and were ready to be plated on 3.5 cm glass-bottom imaging dishes. However, when imaging runs were conducted, the wash-out treatments with HHBSS and Ca^{2+} HHBSS also washed the fibroblast cells off of the glass bottoms—restricting the ability to perform an imaging experiment. To more firmly attach the cells to the imaging dishes for imaging and treatment purposes, 200 µl of poly-l-lysine were added solely onto the glass portion of the imaging dish and placed in the incubator at 37ºC for approximately 5 minutes and aspirated off to dry. Incubation of the imaging dish with poly-l-lysine for 5 minutes proved to be more effective than coating the dish at room temperature. However, incubation of times longer than 30 minutes was observed to be non-effective. In this case, the cells often experienced cell death within day 1 or day 2. All coated dishes were prepared immediately before plating on imaging dishes. Other possible coatings are collagen and fibronectin.

Once the cells were coated properly with poly-l-lysine, the appropriate amount of cells needed to be plated onto the imaging dishes as to improve transfection. Numerous preliminary trials had a transfection efficiency of about 20%, that is, in a field-view of 10 cells only two cells would be transfected. Cells that were transfected had to meet certain conditions to be used in

imaging experiments: 1) the sensor had to be within a reasonable range of CFP counts (2500-6000) and 2) relatively within the same plane as other cells so the microscope could acquire accurate data. However, many cells were too bright to image, too dim to image, or not within the same plane.

Another complication lays in the future application of the thesis experiment with infection with polyomavirus. Approximately 60-70% of cells can be infected with polyomavirus and to study calcium responses in those cells with genetically-encoded sensors would mean that cells must express the genetically encoded sensor and be infected by polyomavirus—lowering the percentage further. However, both the problems could be slightly alleviated with better transfection conditions for the genetically-encoded sensor. To do so, varying concentrations of NIH 3T3 cells were plated onto 3.5 cm glass bottom imaging dishes and experiments were performed. Approximately 550 µl of cells from a confluent 10 cm maintenance dish was found to be the best volume to plate onto imaging dishes for transfection. Volumes above and below this mark often resulted in cell death or extremely variable CFP counts in a given field of view.

Transfection efficiency was also modified through the use of different transfection chemical agents (TransIT-LT1 transfection kit (Mirus) and a specialized TransIT-3T3 (Mirus) transfection kit). Cells transfected using the TransIT-LT1 kit were performed by allowing a solution of 250 µl OPTI-MEM and 3 µl TransIT-LT1 to mix for 5 minutes before the addition of 1 µg of the genetically encoded sensor DNA before its addition to the cells. Cells transfected using the specialized TransIT-3T3 kit were performed according to manufacturer protocol. The specialized TransIT-3T3 kit did not seem to improve overall plate transfection, but rather individual cell transfection. CFP counts conducted with TransIT-3T3 were found to be oversaturated or undersaturated with wide variability from cell to cell. To increase transfection

efficiency with TransIT-3T3, more time may be needed to adjust approximate volumes of cells plated as well as the volumes of all other reagents such as: the authority reagent, OPTI-MEM, and the TransIT-3T3 reagent. However, the TransIT-LT1 was used for further experiments because the transfection efficiency did not appear to be significantly better when comparing one method to the other and the protocol was more familiar.

The last element in increasing transfection efficiency was timing. Time was extremely variable when determining when to plate cells, transfect cells, change media, and image. It was determined that cells had to have at least 24 hours before transfection to enable time for the cells to attach to the imaging dish as well as be stable enough to be transfected. Transfections were carried out in media containing FBS to aid the cells in supplying all the necessary components for healthy transfection. When transfections were conducted under serum-free media, the cells were able to be transfected, but had a more variable CFP count within a field of view. A serum change from serum with 10% fetal bovine serum to serum-free media at approximately 12 hours was conducted—allowing for 8-16 hours of serum-starvation before imaging experiments. It was found that cells that were starved for a longer period were more synchronized and exhibited Ca^{2+} responses that were more similar to one another than cells that were serum starved for only 8 hours. However, the protocol that was followed for subsequent experiment was to transfect the cells in media with serum and change to serum-free media after 12 hours. Imaging experiments were conducted on cells that were serum-starved for at least 12 hours to obtain both healthy and relatively synchronized cells.

For future experiments with infection with polyomavirus, it may be of interest to perform transfection through electroporation.

Calcium responses upon fibroblast growth factor (FGF) and platelet-derived growth-factor (PDGF) treatment.

To determine which growth factor was the most efficient as a source of stimulus for NIH 3T3 calcium signaling, two different growth factors were tested. NIH 3T3 cells were transfected with the nuclear-localized calcium sensor called D3cpv nuc. Cells were placed into serum-free media for approximately 24 hours before imaging experiments to maximize the growth factor response. The two growth factors tested were fibroblast growth factor (FGF) and platelet-derived growth factor (PDGF). Each growth factor was imaged at working concentrations of either 10 ng/ml for FGF or 20 ng/ml for PDGF. These concentrations were chosen based on literature reports for for FGF[4] and PDGF[6] that appeared to have similar experimental conditions. Figures 5 and 6 show representative responses upon treatment with FGF and PDGF, respectively.

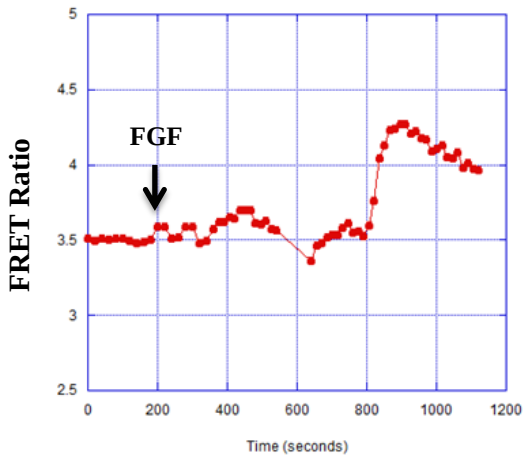

Figure 5. Calcium responses to 10 ng/ml FGF treatment with D3cpv nuc sensor

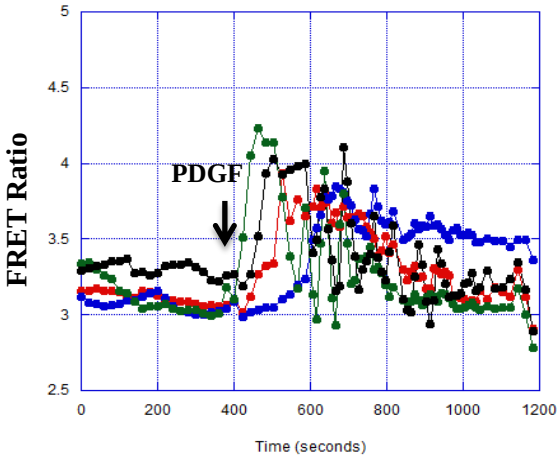

Figure 6. Calcium responses to 20 ng/ml PDGF treatment with D3cpv nuc sensor

The FGF response showed a smaller more delayed response with a ratio change from 3.5 to 4.25, whereas PDGF responses exhibited larger responses upon addition of PDGF with the largest FRET ratio range from 3.0 to 4.0 followed by small oscillations. Only one experiment

was conducted and the result of PDGF as the more effective growth factor is not conclusive. One experiment has high variability and does serve as an appropriate representation. In theory, more experiments should have been conducted with the same concentrations of the different growth factors. However, research conducted by Kuriyama and coworkers analyzed the response of human retinal epithelial cells (RPE) to growth factors under a 24 hour serum-starvation with a Ca^{2+}-binding sensor called fura-2[7]. The table below has been reproduced to display the results of this study.

Growth factor-induced Ca^{2+} transients in RPE cells

Growth factor	*Positive ratio*	*Peak concentration (nM)*
EGF (10 ng/ml)	18/100 (18%)	230 ± 9.7*
FGF (10 ng/ml)	5/102 (5%)	193 ± 4.7
NGF (10 ng/ml)	10/66 (15%)	217 ± 9.3
PDGF (1 ng/ml)	67/97 (70%)	237 ± 4.7
FBS (1%)	52/52 (100%)	774 ± 34.5
Insulin (1 μg/ml)	2/62 (3%)	228 ± 15.8
IGF (100 ng/ml)	0/52 (0%)	—
FN (100 ng/ml)	0/34 (0%)	—

Figure 7. A reproduced table from Kumiyama and coworkers exploring the results of Ca^{2+} transients to growth factor stimulization in RPE cells[7].

The above Figure displays the percentages of positive responses that Kumiyama and coworkers achieved in testing various growth factors in RPE cells. The growth factor with the highest percentage of positive responses as PDGF of 70% whereas FGF had a much smaller percentage of 18%[7]. However, the cells are not the same and the working concentration used in the above experiment of PDGF was different than the one used in this experiment (1 ng/ml versus 20 ng/ml respectively). Nevertheless, the results of Kumiyama's study contributed to the selection of PDGF over FGF in experimental runs.

However, the defining factor in selecting PDGF over FGF was determined by the property of *c-myc*, *c-fos*, *c-jun*, and *JE* as PDGF-inducible genes[13]. Therefore, PDGF was used for all subsequent imaging experiments.

Calcium responses to different doses of PDGF measured by genetically encoded cytosolic fluorescent sensor D3 cpv pc3'

To more thoroughly characterize the effects of PDGF, varying dosage concentrations were tested in NIH 3T3 fibroblasts. This was done to test what concentrations gave rise a calcium response and to examine the reproducibility of those results through the use of a cytosolic sensor and a nuclear sensor. Varying concentrations of PDGF were introduced to NIH 3T3 fibroblasts cells and imaged *in vivo*. Time traces for all the cytosolic experiments are shown in Figures 8a,b,c.

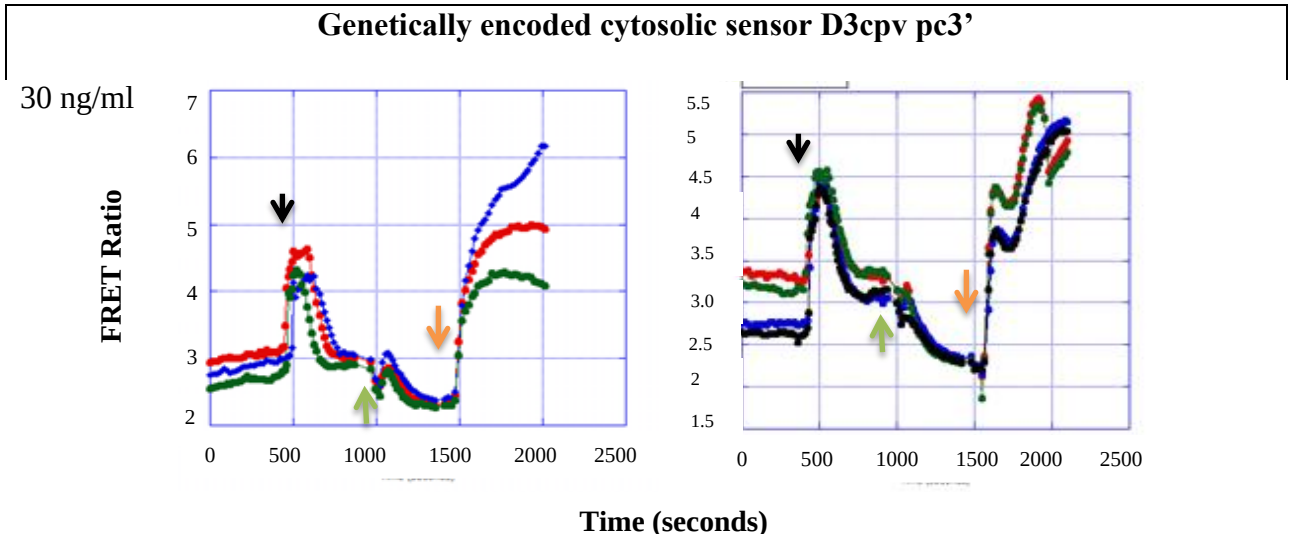

Figure 8a. Time traces for two independent experiments of 30ng/ml PDGF dosage treatment in NIH 3T3 fibroblast cells. Black arrows represent PDGF addition, green arrows represent calcium chelation with ionomycin and EGTA, and orange arrows indicate the addition of $CaCl_2$.

20 ng/ml

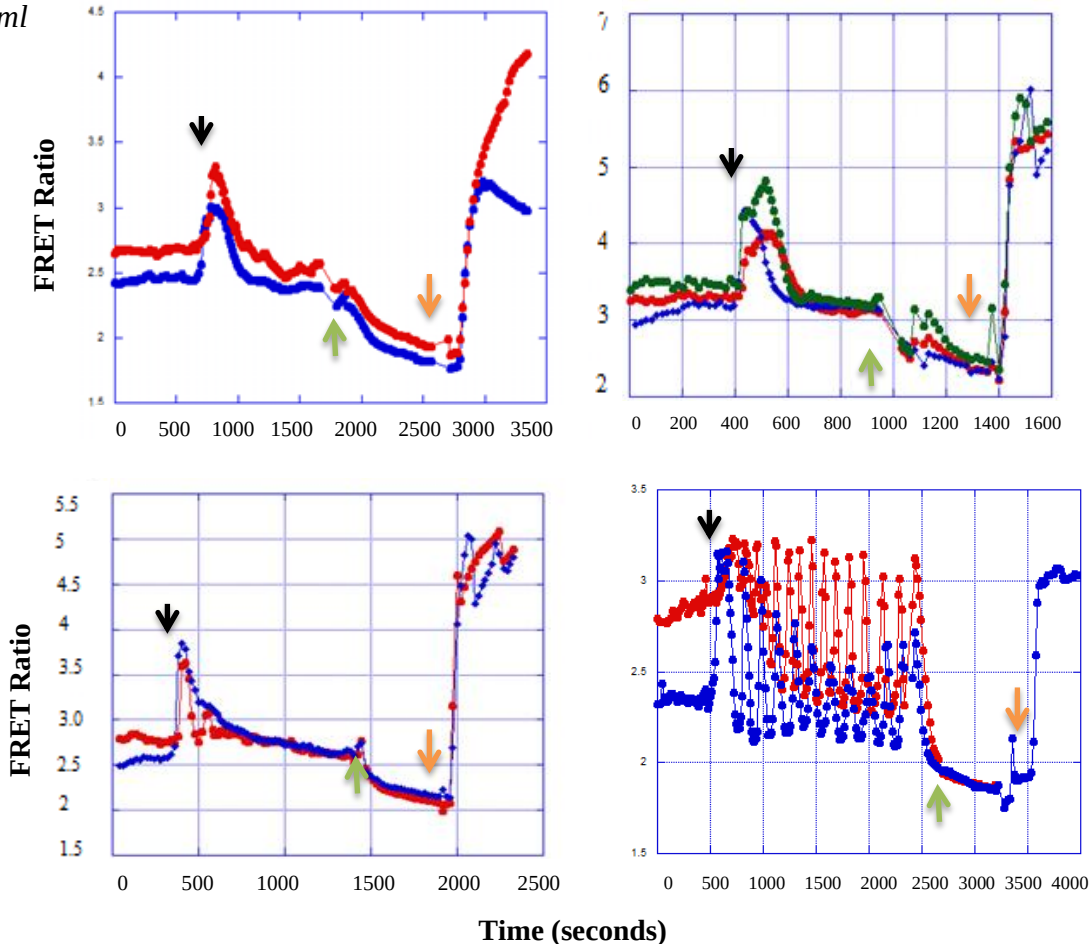

Figure 8b. Time traces for four independent experiments of 20ng/ml PDGF dosage treatment in NIH 3T3 fibroblast cells. Black arrows represent PDGF addition, green arrows represent calcium chelation with ionomycin and EGTA, and orange arrows indicate the addition of $CaCl_2$

10 ng/ml

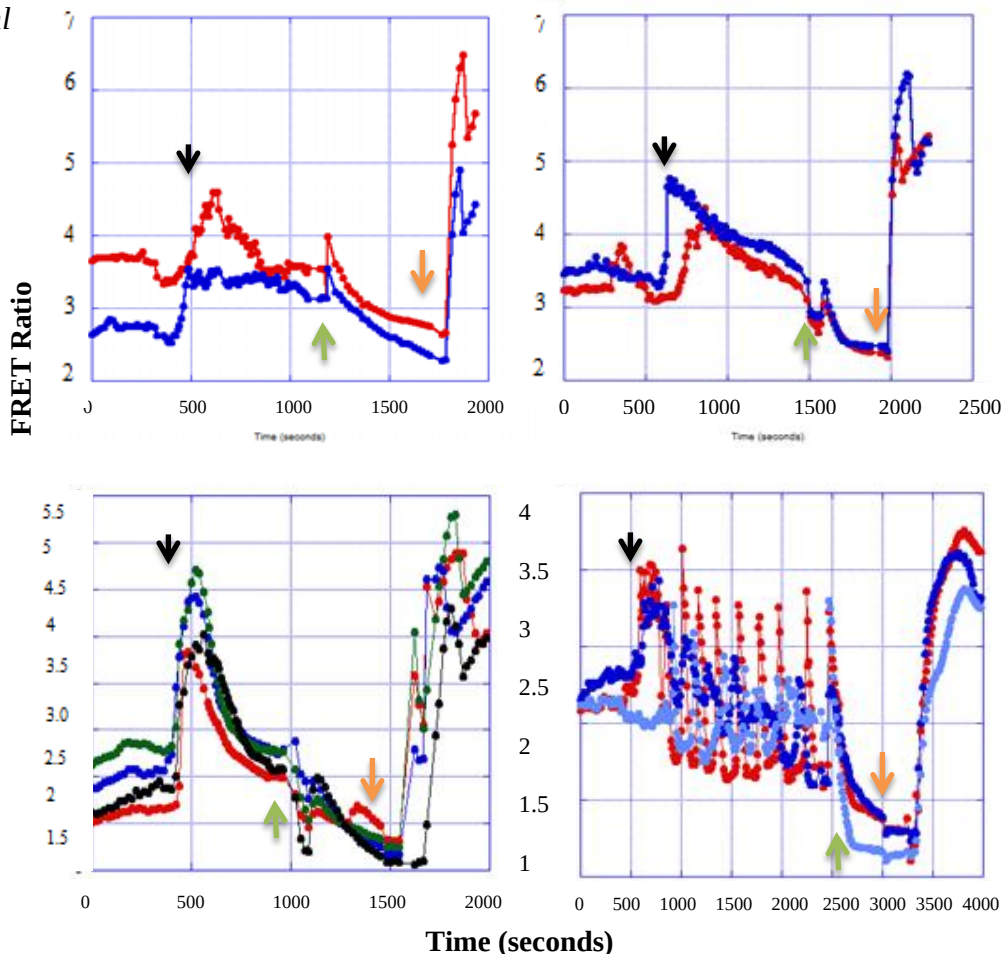

Figure 8c. Time traces for four independent experiments of 10ng/ml PDGF dosage treatment in NIH 3T3 fibroblast cells. Black arrows represent PDGF addition, green arrows represent calcium chelation with ionomycin and EGTA, and orange arrows indicate the addition of $CaCl_2$.

Basal ratios prior to PDGF stimulation varied between 2.0-3.75. Addition of PDGF caused heterogeneous responses. In general, treatment with PDGF caused a rapid rise at the time of addition which is consistent with the elevated levels of calcium upon growth factor stimulation. Heterogeneity was observed in both the magnitude of the responses as well as the nature of the responses (as described below). Following growth factor treatment, cells were treated with an ionophore (ionomycin) along with EGTA (denoted by green arrows) to obtain the minimum ratio (Rmin) followed by ionomycin and Ca^{2+} (denoted by orange arrows) to obtain the maximum

ratio (Rmax) of the sensor in each cell. In future work, the Rmin and Rmax will be used to convert the resting ratio and PDGF-induced ratio change into a specific concentration of Ca^{2+} through the application of mathematical formulas.

The magnitude of the responses was extremely variable ranging from a change in FRET ratio from 0.5-2.5. There appeared to be no connection between the magnitude of the response in comparison to the concentration of the PDGF dosage treatment.

The nature of the responses was categorized under the following groups: transient, delayed transient (characterized by a time-delay between PDGF treatment and onset of the Ca^{2+} response), oscillatory, and delayed oscillatory. Eleven independent experiments were performed and 27 individual cells were monitored. The results are tabulated in the table below.

Nature of response	10 ng/ml	20 ng/ml	30 ng/ml	Total in all experiments
Transient	6/11	7/9	7/7	20/27
Delayed transient	2/11	0/9	0/7	2/27
Oscillatory	2/11	2/9	0/7	4/27
Delayed Oscillatory	1/11	0/9	0/7	1/27
Total in dosage category	**11**	**9**	**7**	**27**

Most responses in the cytosol were characterized as transient responses in which PDGF stimulates a rise in calcium levels and then a decrease back down to basal levels. The variability of the responses was dosage dependent. At 30 ng/ml all responses were found to behave transiently. This response may suggest that the cell's receptors were flooded with PDGF at once and forced to respond, because no oscillations occurred in any trials. However, additional

experiments may be needed to assess if oscillatory character can be observed at 30 ng/ml. At 20 ng/ml, the responses were slightly more variable with 7 out of 9 cells responding transiently with 2 out of the 9 behaving with oscillatory character. The responses at 10 ng/ml PDGF showed the most variability with varying types of results: oscillatory, transient, and low to little response. Of the 4 independent trials and 11 cells, 3 were oscillatory, 8 cells behaved transiently though the delay times were variable from 0-100 seconds. Furthermore, the large variability in the 10 ng/ml suggests that this concentration is most probably at the lower end of the detection limit of the NIH 3T3 fibroblast cells because some trials yielded little to no response whereas others prompted oscillatory character.

Also, the working concentration of PDGF that seems most probable in generating oscillations is around 15 ng/ml. Oscillatory behavior for calcium has been representative of calcium engaging in cell proliferation and activating transcription factors[1]. This concentration is suspected to be ideal because neither 10 ng/ml nor 20 ng/ml dosage responses yield oscillations in all experiments, but rather each displays oscillatory character in solely one individual experiment in each case. These oscillations could have been achieved in solely in one experiment at 20 ng/ml and one at 10 ng/ml because the conditions of the cell were tuned as to use PDGF in a certain manner. This is subject to variation according to the passage of cells, how many cells are on the plate, and transfection conditions.

Calcium responses to different doses of PDGF using a genetically encoded nuclear-targeted fluorescent sensor, D3 cpv nuc.

Resting ratios prior to PDGF stimulation varied between 2.0-4.0—with the bulk of resting ratios in the range of 3.0-4.0. Addition of PDGF produced relatively similar responses. In all trials treatment with PDGF caused an immediate rise at the time of addition which is

consistent with an increase of calcium upon PDGF stimulation. The heterogeneity of the responses was displayed primarily through the magnitude of the responses rather than the nature. Cells were later treated with an ionophore (ionomycin) along with EGTA to obtain the minimum ratio (Rmin) followed by ionomycin and Ca^{2+} to obtain the maximum ratio (Rmax) of the sensor in each cell after the FRET ratios were maintained at a constant level. Along with the cytosolic trials, the Rmin and Rmax will be used to convert the resting ratio and PDGF-induced ratio change into a specific concentration of Ca^{2+} through the application of mathematical formulas in the future.

PDGF dosage treatments in nuclear-targeted sensors were recorded under the same parameters for cytosolic sensors and the following results were attained and are represented in Figures 9a,b,c:

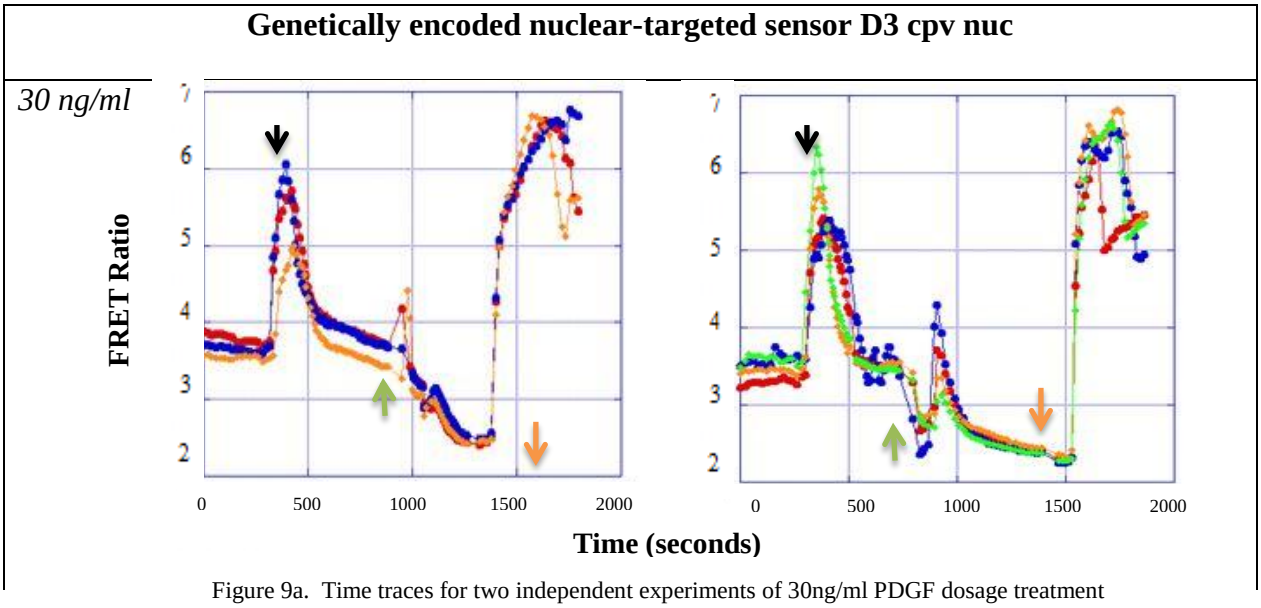

Figure 9a. Time traces for two independent experiments of 30ng/ml PDGF dosage treatment in NIH 3T3 fibroblast cells. Black arrows represent PDGF addition, green arrows represent calcium chelation with ionomycin and EGTA, and orange arrows indicate the addition of $CaCl_2$.

20 ng/ml

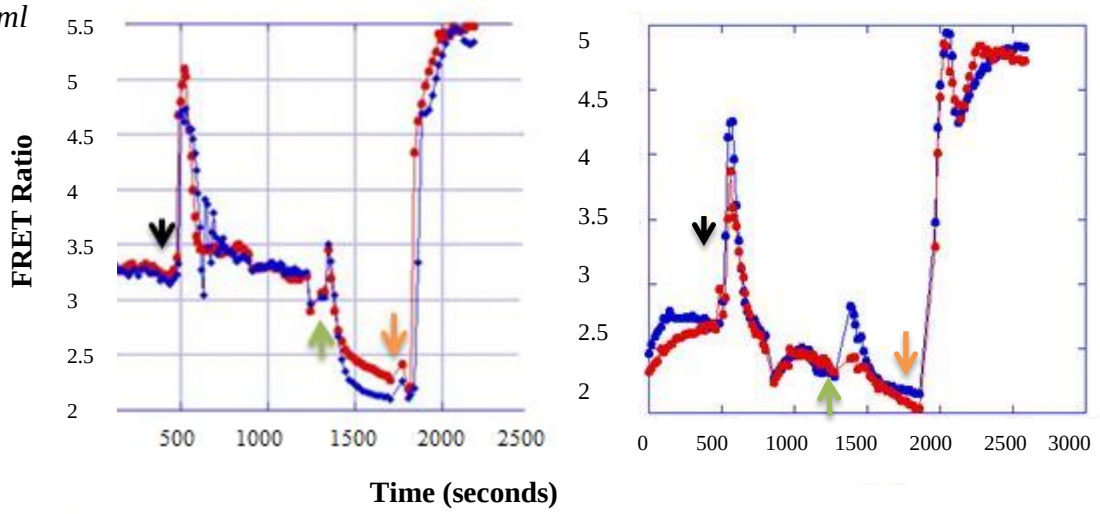

Figure 9b. Time traces for two independent experiments of 20ng/ml PDGF dosage treatment in NIH 3T3 fibroblast cells. Black arrows represent PDGF addition, green arrows represent calcium chelation with ionomycin and EGTA, and orange arrows indicate the addition of $CaCl_2$.

10 ng/ml

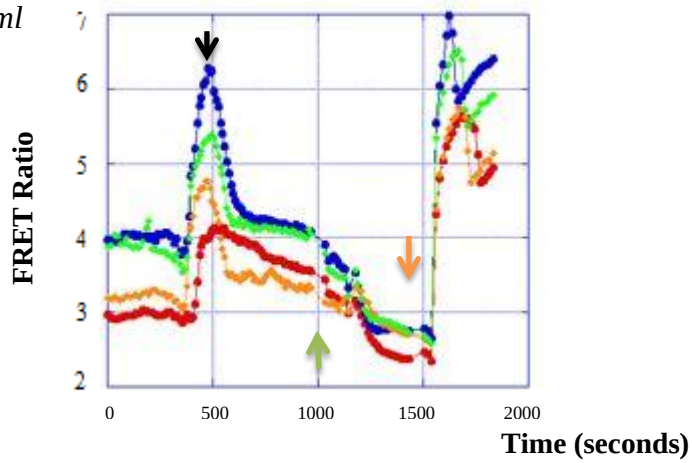

Figure 9c. Time traces for one independent experiment of 10ng/ml PDGF dosage treatment in NIH 3T3 fibroblast cells. Black arrows represent PDGF addition, green arrows represent calcium chelation with ionomycin and EGTA, and orange arrows indicate the addition of $CaCl_2$.

Five independent experiments were performed and 15 cells in total were analyzed. To aid in characterization, a tabulated version of the graphs above are displayed below.

Nature of response	10 ng/ml	20 ng/ml	30 ng/ml	Total in all experiments
Transient	4/4	4/4	7/7	15/15

Delayed transient	0/4	0/4	0/7	0/15
Oscillatory	0/4	0/4	0/7	0/15
Delayed Oscillatory	0/4	0/4	0/7	0/15
Total in dosage category	**4**	**4**	**7**	**15**

The calcium responses to PDGF in the nucleus all behaved transiently under the protocol's conditions. There were no cells that displayed oscillatory character, but some cells did display additional peaks as they returned back to basal levels instead of a smooth decrease. Therefore, at all tested concentrations, PDGF initiates a transient calcium response in the nucleus of all cells. The variability lies in the magnitude of the signal as well as the shape/duration. At 10 ng/ml and 30 ng/ml, the transient peak is a smooth curve, whereas in experiments with 20 ng/ml, the peak is sharp and pointed. However, with only one individual experiment to characterize the nature of nuclear responses to 10 ng/ml PDGF treatment, it is difficult to make conclusions regarding the significance of these differences. More experiments should be conducted to assure the credibility and reproducibility of the results.

Comparison of D3 cpv pc3' and D3cpv nuc

Responses to PDGF dosage in NIH 3T3 fibroblasts were monitored and classified specific to the respective sensors used in the imaging experiments. In the transfection of the sensor into fibroblasts, D3 cpv pc3' generally had more cells within view that were within the acceptable CFP range of 2500-6000 counts, but no more than 9000 counts. However,

transfection of D3 cpv nuc was slightly more difficult, because cells were often extremely saturated with sensor DNA or the distribution of DNA in cells was very variable. This variability made it difficult to find cells within the allowable CFP count range within the same field of view. It is believed that because the nucleus maintains a smaller area than the cytosol, using approximately the same amount of DNA for both may cause excessive sensor expression in the nucleus.

Though the range of resting FRET ratios between the two sensors was relatively similiar (2.0-4.0 for nuclear and 2.0-3.75 for cytosolic), the standard deviation was determined. A t-test was also performed to determine if the resting levels between the two sensors were significant. The average resting ratio for a cell transfected with a cytosolic sensor was calculated to be 2.90 with a standard deviation of 0.40. The average resting ratio for a cell transfected with the nuclear-targeted was calculated to be 3.30 with a standard deviation of 0.45. Therefore, the average values between the two sensors are relatively different, but the standard deviation is approximately the same. However to determine whether the resting ratios are significantly different, a t-testing assuming equal variances was conducted. A p-value of $2.6 * 10^{-5}$ which is much lower than the α level of 0.05, therefore, the resting ratios of the sensors are found to be significantly different from one another. Below is a figure that depicts this significance.

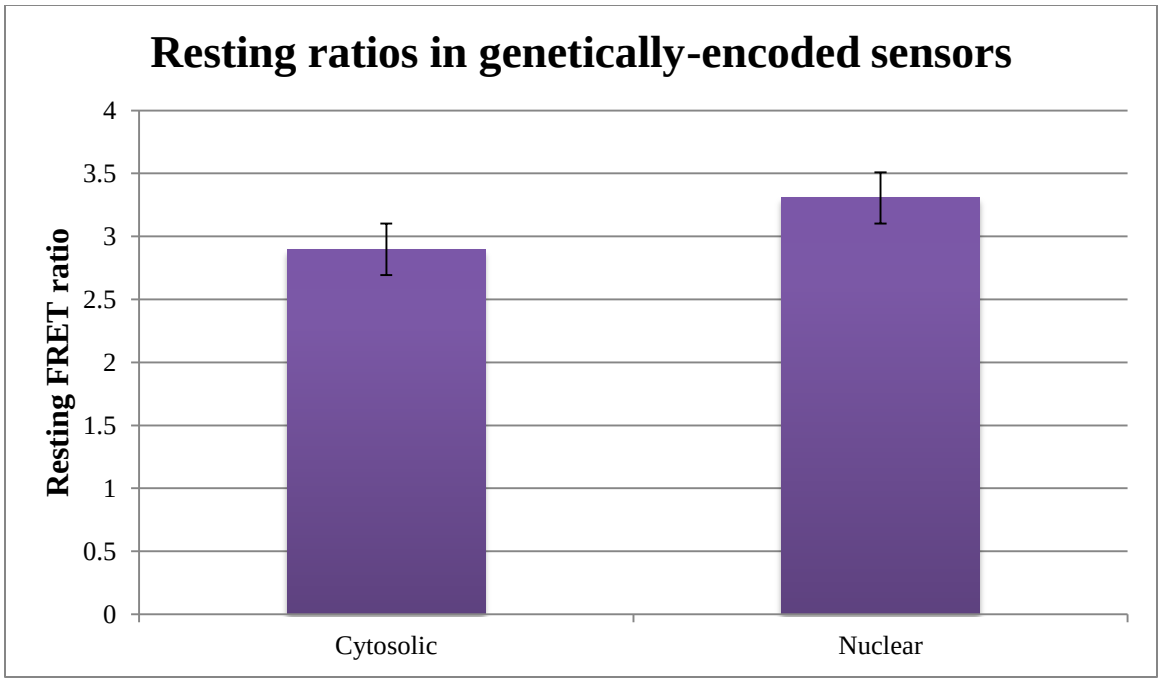

Figure 10. Histogram of the average resting ratios of genetically-encoded sensors

In terms of PDGF treatment, the Ca^{2+} responses were somewhat different between the two types of sensors. Nuclear Ca^{2+} responses were characterized by narrower, sometimes sharper transient peaks, and no Ca^{2+} oscillations were observed. Cytosolic Ca^{2+} responses were much more variable and encompassed the following types of responses: immediate transient, delayed transient, immediate oscillations, and delayed oscillations. However, the bulk of the cytosolic responses were transient, though oscillations occasionally occurred.

For the conduction of future experiments, the cytosolic sensor may be the best sensor to use when observing responses with polyomavirus in cells. In terms of practicality, the cytosolic sensor was much more dependable in terms of transfection and obtaining cells within the usable range of 2000-6000 counts. However, the nuclear-targeted sensor proved to be more difficult with significantly higher resting ratios and oversaturation of cells. Also, because the sensor in the cytosol detected calcium oscillations that may be indicative of transcription factor activation[1], it seems like the most applicable sensor to use for the future purposes of this cell.

Future Directions

Under the experimental protocol utilized for this work, all cells were maintained under serum-free conditions for 11-16 hours or 24 hours. To make sure the responses were not induced solely by serum starvation, experiments with 1% and 5% serum addition should be conducted *in vivo* to assure the veracity of the calcium responses. Once confirmed, the cells may be infected with polymavirus and responses should be monitored within the maximally responsive first hour and compared to the PDGF dosage responses collected. Post-infection responses at the 6 hour mark and the 12 hour mark should be monitored as well so see if the responses mimic dosage responses of PDGF. At the 6 hour mark, early response genes have been found to be undetectable whereas at 12 hours and 24 hours accumulation of early response genes resurfaces. Special attention should be made to quantify any changes in basal levels of calcium in the cytosol and the nucleus since 1 hour post-infection. Because mRNA levels behave in a biphasic pattern in NIH 3T3 fibroblasts, the second accumulation of these early response genes must be analyzed as to determine if the spatio-temporal patterning of calcium signals has changed between that time-frame. By doing so, it may be possible to determine when polyomavirus has been committed to the cell cycle, when it begins to proliferate in cells, and possibly give insight into its method of survival in cells.

If infection of polyomavirus in cells is consistent with growth factor dosage responses, treatment with genestein may be used to knock down tyrosine kinase activated channels to analyze the effect of calcium on the efficiency of polyomavirus to effectively survive and propagate within cells. If the knock down of channels affects the proliferation of polyomavirus, then the results would suggest that calcium is a necessary component to the initiation of polyomavirus into cells. However, if the reverse is true and the knock down of tyrosine kinases

by genestein has no effect on the commitment of polyomavirus into the cell cycle, then the calcium signaling cascade is not suggested to be vital part of the survival of the virus.

Furthermore, the development of a new sensor that would be targeted to both the cytosol and nucleus may be beneficial as well. By using the same sensors used in this experiment (D3cpv pc3' and D3cpv nuc), With this sensor, the ability to spatially differentiate signals nucleus within the same cell is possible, thereby allowing for the analysis between the nature of responses between the two cellular regions. The use of this sensor would also be beneficial for determining where certain types of responses are most likely to occur such as transcription factor activation or gene proliferation. Present research cannot conclude that that calcium signals in the cytosol and nucleus are significantly different. The general consensus in the field is that the cytosol and nucleus should have the same or relatively similar responses to varying treatment. However, the development of this dual sensor would offer more insight into the validity of this generalization by offering the potential to compare responses from both locations side by side. Potential response details that could be analyzed are the nature of the responses, the duration of responses, as well as the initial response time from the addition of treatment. The greatest benefit of this sensor is that discrepancies or similarities between the two sensors could be analyzed more thoroughly because data would be extracted from two spatially different areas within a cell.

All the above steps could also be repeated with the replacement of epithelial growth factor (EGF) as well. Experiments conducted by Garcea and coworkers have identified that PDGF regulates the competence of polyomavirus whereas the progression of the virus is moderated by EGF[13]. Therefore, the polyomavirus may mimic two different growth factors at different stages of viral infection.

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