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Summer Research Report

Summary:

This report details my work done during the summer of 2008 in Dr. Neal Waxham's lab at the University of Texas Medical School in Houston, TX. My main project was an investigation of the intracellular diffusion of RC3 protein, also known as neurogranin, calmodulin (CaM), a protein involved in the regulation of Ca^{2+} within cells, and CaMKII (calmodulin kinase II), a target protein of calmodulin. Also, I devoted a significant amount of time to constructing an apparatus designed to drive mirrors in a scanning Fluorescence Correlation Spectroscopy (FCS) setup. Our goal is to determine how cellular controls of diffusion, a random process, lead to predictable signaling. Random motion, we find, is not a sufficient force for driving known biochemical pathways.

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1. Important Concepts in the Theory of Diffusion:

The simplest model of diffusion considers only that, in an arbitrary volume, changes in the number of molecules contained in that volume are the result of the diffusion of molecules into and out of the volume across the volume's boundary (Proposition 1). Combined with the proposition that the flux of particles at a given point is proportional to the difference in concentration and directed towards the largest concentration difference (Proposition 2), Fick's Law of Diffusion can be derived.

$P(\vec{x}, t)$ = density of molecules at position \vec{x} and time t .

$J(\vec{x}, t)$ = flux of particles at position \vec{x} and time t .

Proposition 1: $\frac{d}{dt} \int_V P(\vec{x}, t) dV = - \int_{\partial V} J(\vec{x}, t) \cdot \hat{n}(\vec{x}) dS$

Proposition 2: $J(\vec{x}, t) = -D \cdot \nabla P(\vec{x}, t)$

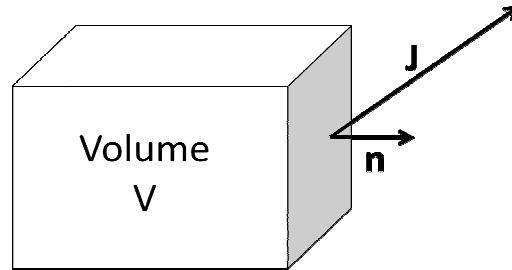
Using the Divergence Theorem:

$$\int_{\partial V} J(\vec{x}, t) \cdot \hat{n}(\vec{x}) dS = \int_V \nabla \cdot J(\vec{x}, t) dV = \int_V \nabla \cdot (-D \nabla P(\vec{x}, t)) dV$$

Because the result holds for arbitrary volumes, V , we have: $\frac{\partial}{\partial t} P(\vec{x}, t) = D \nabla^2 P(\vec{x}, t)$

The last line is the common statement of Fick's Law of Diffusion.

Figure 1 – Volume Element, Flux and Unit Normal Vectors



Throughout the summer, one of the lab's main goals was to determine the actual values of the diffusion constants, D , for various molecules in different parts of live cells. Several physical factors affect this value. Intrinsically, the size of the molecule greatly affects diffusion. Larger molecules inherently diffuse more slowly; a rule of thumb is that the diffusion constant is inversely proportional to the cube root of the molecular weight. Really, this is an estimate which comes from the Einstein formula (a result of statistical physics, not derived here) $D = \frac{kT}{6\pi\eta r}$ and the approximation that the molecule's radius of gyration is proportional to the cube root of the mass (imagine a uniform sphere, for which $M \propto r^3$). T is the temperature, k the Boltzmann constant, r the radius of gyration, and η the viscosity. Below is a table listing estimates of the diffusion constants according to this cube-root rule. Calmodulin kinase II is listed as CaMKII.

Table 1 – Relative Diffusion Constant Estimates According to the Cube-Root Rule

Molecule	Mass* (in Daltons)	Theoretical Diffusion Constant (normalized to GFP)
CaM	42000	0.862
RC3	35000	0.916
CaMKII-alpha	940000	0.306
GFP	26900	1.000

*All of the mass values in the table include the mass of Green Fluorescent Protein (GFP), a dye used to determine the locations of the molecules in the experiments.

Environmental factors also play an important role in determining diffusivity. In cells, proteins interact with other molecules, which may be very large or membrane bound. Proteins held in position via weak or specific binding may not diffuse at all. Also, the "crowdedness" of the environment can change diffusivity, much as shopper motion is limited in a crowded mall. Often, environmental factors determine the diffusion constant much more than the molecular mass.

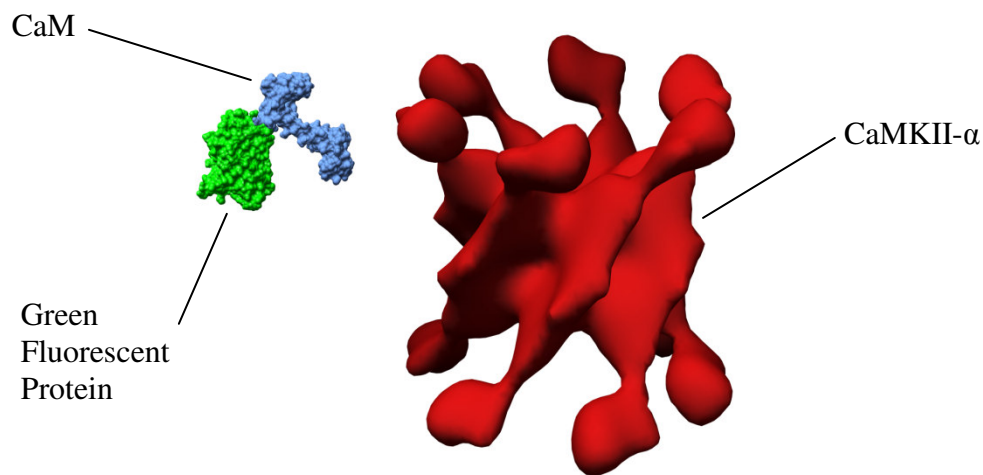
Ultimately, diffusion as random motion in the general sense is the main way which molecules can move around in cells. They aren't self-guided. Despite this lack of direction, biochemical reactions can be discussed as more or less fixed pathways. The loose statistical nature of diffusion must be tightened by regulatory cell structures which focus activity. Forgetting the underlying randomness and multiple interactions would be a mistake, that of overlooking the numerous directing mechanisms.

2. Biological Roles of CaM, CaMKII, and RC3

Calmodulin (CaM):

Calmodulin is a small, dumbbell-shaped, heat-stable protein found in all eukaryotic cells. It has four binding sites for Ca^{2+} ions, and, when activated, serves as a signal protein to alter the activity of various target proteins. Of the four binding sites, two are found at the N-terminus and two at the C-terminus. Calcium binds most slowly to the C-terminus sites, and, in fact, changes at the C-terminus are the rate-limiting steps in many signaling reactions. The activity of CaM as an activator was originally believed to be only a function of calcium concentration levels, but other mechanisms were later found: selective regulation of CaM concentration in areas of the cell where CaM targets are found; regulation of activity via phosphorylation; and controlled regulation of genetic expression of CaM. Activation of CaM by calcium has been shown to lead to changes in plasticity, i.e. long term potentiation and depression, in neurons.

Figure 2 – Protein Structure of Calmodulin (Bound to GFP) and CaMKII- α



Calmodulin Kinase II- α (CaMKII- α):

In general, a kinase is an enzyme which phosphorylates (adds a phosphate group to) another molecule. Calmodulin kinase II, a protein kinase, comes in 4 varieties: alpha, beta, gamma, and delta. In terms of the above-mentioned signaling pathway, CaMKII is a target protein of CaM/ Ca^{2+} . Once CaM has been “activated” by calcium at all four of its binding sites, it can stimulate CaMKII- α . Each CaMKII- α actually consists of twelve subunits, each of which has a binding site for CaM/ Ca^{2+} . However, when any one site has bound to CaM, that site's subunit

becomes active. Via pharmacological tests, CaMKII- α has been linked to long term potentiation and active learning processes associated with the hippocampus.

RC3:

RC3 is a protein which helps to regulate the binding of CaM/Ca²⁺ to CaMKII, especially in pyramidal neurons. The control mechanism lies in RC3's ability to change the affinity of CaM to Ca²⁺ at the C-terminus. In the presence of RC3, the affinity of CaM to Ca²⁺ decreases, which leaves the CaM in an inactive state for a larger percentage of time.

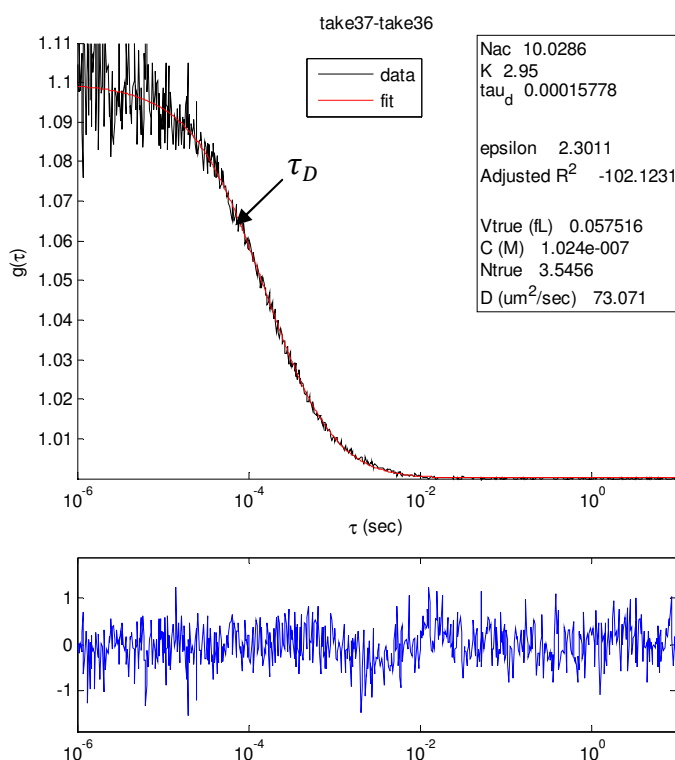
3. Methods Used in the Lab

Two methods produced the majority of the data in this summer's experiments: Fluorescence Correlation Spectroscopy (FCS) and Raster Imaging Correlation Spectroscopy (RICS).

Fluorescence Correlation Spectroscopy (FCS):

FCS implements two-photon laser excitation of a very small volume to stimulate dye molecules to fluoresce. Two-photon excitation methods use two photons of lesser energy to stimulate fluorescence instead of one high-energy photon. Because the energy of the individual photons is reduced, two photons must simultaneously excite the dye molecule; the likelihood of acquiring the requisite energy for excitation is available (with substantial probability) only in a small volume, where the laser is most focused. Constraining fluorescence to this volume prevents excessive photobleaching of dye molecules outside the excitation volume, which would otherwise skew data by causing some species to essentially "disappear" in terms of their fluorescent emissions. The intensity of the emitted fluorescent photons is then recorded by a detector as a function of time (that is, the time of incidence of individual photons upon the detector is recorded), and then the fluorescent intensity at different times is compared, giving a correlation function. Geometric properties of the system, for example, the approximation of the excitation volume as a 3-D Gaussian, combined with Fick's Law of Diffusion, give a correlation function for which $\tau_D = \omega_{xy}/8D$, where τ_D is the time parameter corresponding to the half-maximum value of the correlation curve and ω_{xy} is the Gaussian function's waist (in the lab, about 300 nm).

Figure 3 – Sample Correlation Curve



Correlation function:

$$G(\tau) = \frac{\langle \delta F(t) \delta F(t + \tau) \rangle}{\langle F(t) \rangle^2}$$

$$\delta F(t) = F(t) - \langle F(t) \rangle$$

$F(t)$ is the fluorescent intensity at time t . The curve indicates that as the time parameter, τ , is increased, the correlation between times decreases. Asymptotically, the value of $g(t)$ approaches 1.00, which indicates no correlation between times far enough apart. Below are shown weighted residuals, (differences between model and data).

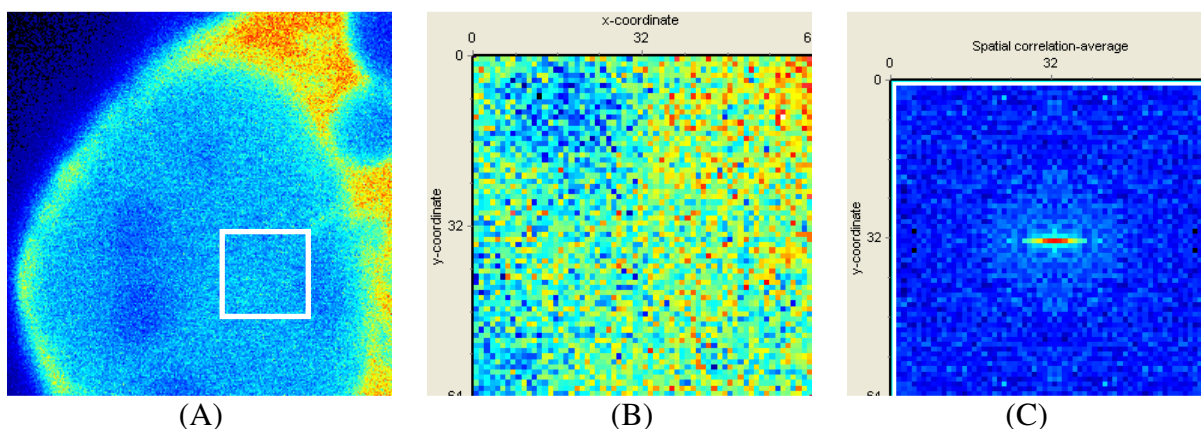
When making measurements, an optical density filter blocks some of the laser intensity and allows a good signal to noise ratio. The fluorescent intensity changes as particles diffuse out of the excitation volume; in order to compute a meaningful correlation curve, these small changes (the signal) must be noticeable compared to the entirety of the intensity observed (the noise). For this reason, extremely low concentrations of dye are preferred (i.e., in Alexa dye calibrations, concentrations of 60-80 nM).

Raster Image Correlation Spectroscopy (RICS):

The second method commonly used was RICS. RICS operates similarly to FCS, but instead of focusing on a single volume, RICS scans a larger region and correlates fluorescent intensity not only in time, but also in space. In the experiments discussed below, the laser scanned a 256 x 256 pixel region in each frame, resting on each pixel for 25.6 μ s. In total, a frame took 3.93 seconds to scan. A hundred frames of the same region were scanned in a total time of 6 min 33 sec. The dwell time must be long enough to collect sufficient intensity data, but not too long, because single-photon excitation is used and photobleaching concerns must be considered. Essentially the fluorescent intensity is autocorrelated in the same way as in FCS, albeit, the formulas are more complicated because spatial and temporal components, including the scanning component, must all be considered. Although the diffusion information can be calculated, in theory, from a single frame, averaging over 100 frames increases the reliability of the result. Slow macroscopic movement of cell parts rather than movement at the particle level has the potential to skew the real molecular diffusion; algorithms which average the intensities over a

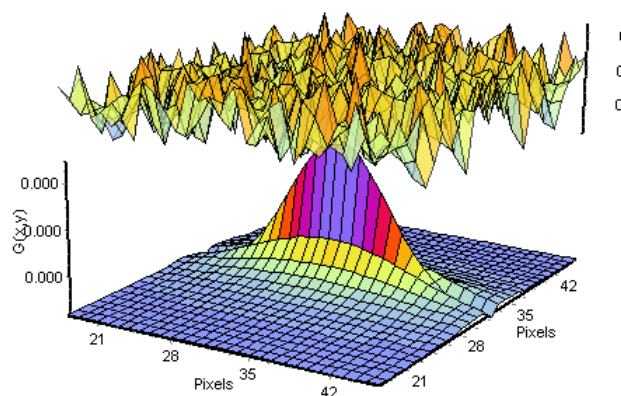
defined number of frames (four in our case) take into account the overall motion of the cell. An example RICS analysis is shown below.

Figure 4 – (A) Raw Data from RICS, (B) Averaged Data, and (C) Contour Graph of the Correlation Function



The original data shows the expression of YFP-RC3 in the nucleus of an HEK cell. The data is averaged over the smaller volume indicated within the nucleus (the box dimensions are 64 x 64 pixels). All three of these pictures were generated by SIMFCS.

Figure 5 – Correlation Function Graphed with Residuals



This correlation function indicates good results. Notice the uniformity of the residuals and the Gaussian shape of the peak. That the peak is almost perfectly flat indicates the presence of only a single species. Two-component models are also possible in RICS. Bad results, often the consequence of aggregation within the region of interest or intense bleaching, have very sharp peaks in the correlation curve and residual graphs. This graph was generated by SIMFCS.

Auxiliary Methods:

All procedures involving cells or cell plates should be conducted in a sterile environment to prevent contamination.

Preparing cell plates with poly-D Lysine:

1. Dilute 10x poly-D lysine to 1x in borate buffer.
2. Filter solution through a 0.2 μm syringe filter and coat the 35 mm plate (approximately 1 mL per plate).
3. Allow the plate to sit for 2-3 hours.
4. Rinse 2-3 times with autoclaved water, letting rest 10 minutes for each wash.
5. Allow the plate to completely dry.

Splitting Cells (a 1/10 split):

1. Start with the main plate of HEK cells; aspirate the growth media from the plate.
2. Rinse off extra media with DPBS (sterile) solution (use enough solution to cover the plate). Gently swirl the DPBS over the plate to wash thoroughly, and then aspirate the DPBS.
3. Add 1.0 mL of trypsin to the plate, and swirl gently.
4. Store the plate in the incubator (37°C with CO₂) for 5 minutes to allow the trypsin to break up the cells.
5. Add 9 mL of DMEM growth media with serum and antibiotic to a new plate and 9 mL of the same media to the trypsin plate. Mix the cells thoroughly in the trypsin media.
6. Add 1 mL of cells from the trypsin plate to the new plate (total volume now 10 mL). Mix the cells in the new solution by shaking gently.
7. Place the cells in the incubator for storage. Cells adhere to the plate and are ready for transfection after about 36 hours.

Transfecting DNA:

1. In 100 μL of DMEM solution mix about 2 μg of DNA.
2. In a separate 100 μL of DMEM, add 8 μL of Lipofectamin2000 and mix well. Wait at most 5 minutes before continuing.
3. Mix the DNA solution with the Lipofectamin solution and let sit for 20 minutes.
4. After the 20 minutes, add 200 μL of DMEM (total volume now 400 μL).
5. Change the media of the cell with 400 μL of DMEM.
6. Add the 400 μL of DNA solution (total volume on plate now 800 μL) and let sit for about 2 hours.
7. After 2 hours, replace the media with 2 mL of DMEM.
8. Cells will begin expressing during the next hour.

Alignment of the Laser for FCS:

1. Turn on the laser cooling system, then the key on the laser to the on position.
2. Open the main shutter and wait for at least an hour for the laser to stabilize.
3. Use the oscilloscope to check that the wavelength emitted is correct (in our experiments, 850 nm). If the wavelength is not correct, adjust using BRF knobs.

4. Mode Lock laser, and then adjust the waveform so that the width of the Gaussian is as desired to keep a 130 fs pulse (dependent on the wavelength, about 8.2 nm for wavelength 850 nm). If the width is not correct, adjust using the BRF (Birefringent) filter and prism.
5. Achieve maximum power output by adjusting the internal mirrors.
6. Now, the laser is operational, the detector must be aligned. Turn out the lights in the room, and set the optical density filter to 2.0 (a relatively high value, to avoid blasting the detector with too much energy).
7. While monitoring the detector reading (using Flex01D, the data collection program), adjust the x and y calibration knobs to move the detector fiber to a position which achieves maximum signal.
8. Place the green signal slide on the sample tray, and observe the green fluorescent spot through the viewfinder, making sure there is water between the objective and the slide. Position the crosshair precisely over the green spot.
9. Adjust the sample tray to the left and right, making sure that the fluorescent spot moves along the same horizontal axis as marked by the crosshair. The spot will also fade in intensity; make sure that the fading is symmetric to the left and right of the crosshair.
10. Repeat in the vertical axis.
11. Use an Alexa dye calibration test to make sure that the apparatus is aligned correctly. This must be done every time the laser is used. Of importance is the total number of counts per molecule achieved (noted in FCSseriesGUI as ϵ), and the structure parameter of the point spread function K.

Calibration of RICS apparatus:

1. Turn on the laser, start the computer, and, using the program LSM 510, set the tube current to 6.1 A, the zoom to 10, the frame size to 256 x 256, and change the objective to the 63x lens. Depending on the exact nature of the experiments, different combinations of filters and dichroic mirrors will be used.
2. Wait 1 hour for the laser to stabilize.
3. Mix Alexa-488 dye solutions of concentrations 10, 5, 2.5, 1.25, and 0.625 μM .
4. In the plastic sample tray, add a drop (100 μL) of the lowest concentration solution and immerse the 63x water immersion lens in the drop.
5. Center the lens on the drop using x- and y-axis tray adjustments.
6. Collect 100 frames, with no delay, of data using the time series function (run time should be 6 min 33 sec).
7. In the RICS analysis subprogram of SIMFCS, autocorrelate the data, and complete a fit using the following parameters:

4. Experiments and Results

Question 1:

What are the diffusion constants of GFP, GFP-CaM, GFP-CaMKII, and GFP-RC3 in cells?

Experiment 1:

The first goal was to learn the actual diffusion constant values in different parts of HEK cells. Nuclear and cytosolic regions were considered. In this set of experiments, we transfected cell plates with each of the three protein/dye combinations and also one plate with GFP dye protein as a control. After waiting for the cells to express, we changed the media from DMEM to a clear calcium media (0.8 mM MgCl₂, 2.0 mM CaCl₂, HEPES+HBSS) and scanned cells on each of the plates using the FCS apparatus (glass-bottomed plates must be used with FCS). The laser wavelength was set to 850 nm, Alexa-546 dye was used to calibrate the apparatus, and the optical density was adjusted to achieve an optimal noise to signal ratio. Data analysis for FCS was completed using the in-house MATLAB application FCSseriesGUI.

RICS was also used to determine these values. The 488 nm wavelength laser was used at tube current 6.1 A. Other parameters include: 256 x 256 pixel scanning region, zoom 10, and a 63x water-immersion lens, dwell time 25.606 μ s. One hundred frames were taken in sequence (3.93 s/frame), over a total time of 6 min 33 sec. Data analysis of RICS experiments was conducted using SIMFCS.

Results of Experiment 1:

Table 2 – Results of FCS and RICS Experiments Determining Diffusion Constants in Cytosolic Cell Regions

Molecule	FCS Diffusion Constant	RICS Diffusion Constant
CaM	2.4 $\mu\text{m}^2/\text{s}$	18.3 $\mu\text{m}^2/\text{s}$
RC3	18.5 $\mu\text{m}^2/\text{s}$	11.9 $\mu\text{m}^2/\text{s}$
CaMKII-alpha	0.9 $\mu\text{m}^2/\text{s}$	1.6 $\mu\text{m}^2/\text{s}$
GFP	15.6 $\mu\text{m}^2/\text{s}$	11.8 $\mu\text{m}^2/\text{s}$

These diffusion constants follow the general pattern expected in some cases, but not in others. It is clear that the diffusion constant for CaMKII is the lowest, as was projected due to size, though the value is lower even than expected. This extra immobility is likely due to CaMKII binding with either itself or with other cellular components. The diffusion constant for CaM was much lower than that for GFP-RC3 in the FCS results only. Possibly this is due to CaM's higher reactivity with cellular proteins, which "weigh it down." The RICS data might not be as reliable because of spatial averaging over the entire region of interest. Even for the same molecule, because diffusion constants are so sensitive to environment, averaging might group unlike individuals from different parts of the cell. Conversely, these results suggest that RC3 is not very interactive with many other cellular proteins.

Question 2:

Does the concentration of calcium ions in the HEK cells affect the diffusion of GFP, GFP-CaM, GFP-CaMKII, or GFP-RC3 in the nucleus and/or the cytoplasm?

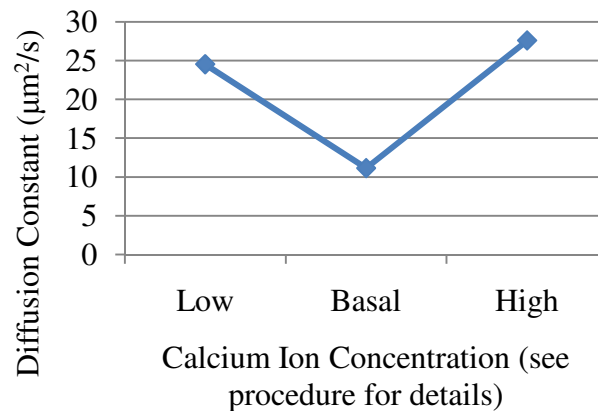
Experiment 2:

This experiment was conducted similarly to the first, except that the Ca^{2+} level in the cells was varied. The natural calcium concentration in a cell is termed the “basal” level. If the cells are bathed in a calcium-rich solution, the addition of ionomycin (a chemical which creates holes in cell membranes) causes a rush of Ca^{2+} into the cytosol, resulting in higher concentrations of calcium. Bathed in calcium-poor solution (10mM EGTA, 0.8 mM MgCl_2 , HEPES+HBSS), the addition of ionomycin drains the cells of their calcium and leaves calcium-poor cells. The diffusion constants of the various proteins were measured in the basal, high, and low cellular concentrations of calcium. The diffusion of GFP provides a control against which the mobility changes of the other proteins can be compared.

Results of Experiment 2:

The diffusion constants we measured for GFP-CaM, GFP-RC3, and GFP-CaMKII do appear to be a function of calcium concentration in the cell, but not in a significantly different way than for GFP alone, which was the control. Thus, we cannot say that any of the observed changes are due to the proteins of interest rather than GFP dye itself. The pattern observed follows the general trend shown below:

Figure 6 – Diffusion Constant of GFP vs. Calcium Concentration



Question 3:

How does the diffusion constant of GFP-CaM alone compare to the diffusion constant of GFP-CaM bound to CaMKII?

Experiment 3:

This experiment was conducted in vitro so that only CaM/CaMKII interactions would be considered. Three trials were conducted. In each trial, a 1x PBS solution was mixed to 40 nM GFP-CaM and 200 mM Ca^{2+} (recall that calcium facilitates the CaM/CaMKII interaction). In this solution, CaMKII concentrations of 80 nM, 40 nM, and 20 nM were added. In each trial, first the diffusion of GFP-CaM was recorded, then the diffusion constant of GFP-CaM + Ca^{2+} , and finally the diffusion constant of CaM+ Ca^{2+} +CaMKII. A two-component model was used to analyze the final mixture because both GFP-CaM and GFP-CaM bound to CaMKII are present in the solution (two species, each with its own diffusion constant). Analysis was done with FCSseriesGUI.

Results of Experiment 3:

The results indicate a diffusion constant for GFP-CaM of $75.0 \mu\text{m}^2/\text{s}$ and a diffusion constant for CaM+CaMKII of $15.9 \mu\text{m}^2/\text{s}$. First, note that these values cannot be reasonably compared to the intracellular diffusion constants because these measurements were taken in free media. As expected according to the mass rule, the diffusion constant for the conglomerate CaM+CaMKII was lower than that for CaM alone, but it was actually much lower than expected by the cube root rule of thumb. Actually, even if all twelve sites on the CaMKII protein were bound to GFP-CaM, the additional mass would not be sufficient to cause a more than fourfold lowering of the motion. However, if CaMKII is binding together via autophosphorylation, then this result is reasonable.

Question 4:

How does the expression of GFP, GFP-CaM, GFP-CaMKII, and GFP-RC3 change with time in different areas of HEK cells?

Experiment 4:

To test expression over time, we transfected HEK cells with each of the target proteins. Using a microscope/camera setup, we recorded images of expression after 4, 7, 24, 28, and 47 hours. Time zero was 12:00 p.m. on July 23, 2008 (useful for comparing images in the appendices with the following table). The cells were kept on growth media during the entire experiment.

Results of Experiment 4:

Table 3 – Observations of Cells Expressing Protein as a Function of Time

Cell Culture	Time				
	4 hours	7 hours	24 hours	28 hours	47 hours
GFP	Expression throughout; higher in nuclei		Empty nuclei seen		More empty nuclei
GFP-CaM	Expression throughout; dimmer in nuclei		Few empty nuclei		More empty nuclei
GFP-CaMKII	No expression in nuclei; formation of aggregates				
GFP-RC3	Expression throughout; slightly brighter in nuclei				Empty nuclei seen

5. Building the Mirror Apparatus:

One goal of the lab is to upgrade the FCS system currently in use to a scanning FCS system capable of single particle tracking in vitro and in vivo. This requires the construction of a device for precisely deflecting the FCS laser to different parts of a sample and recording the data as a function of time. Analysis is done based on the correlation function similar to the RICS method. To build the apparatus, the lab ordered two Micromax 671 Series Dual Axis Drivers (one card to drive the x-axis mirror, and the other card for the y-axis), two 18V DC power supplies, and a mirror/galvanometer setup, pre-calibrated at Cambridge Technology. The setup is currently working, but not yet adapted to the output from the computer.

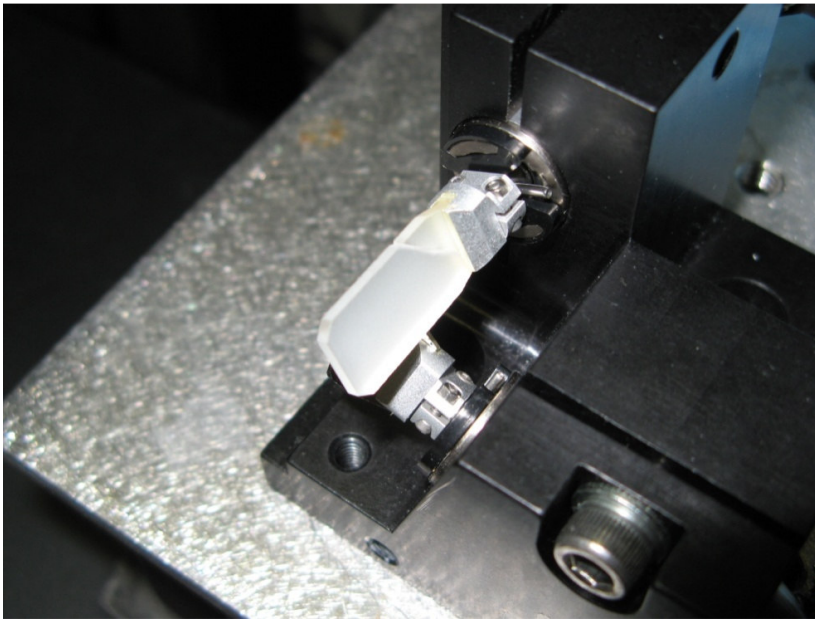


Figure 7 – Oops! We had a small accident with one of the mirrors; fortunately, CamTech was happy to supply us with a replacement for a nominal fee.

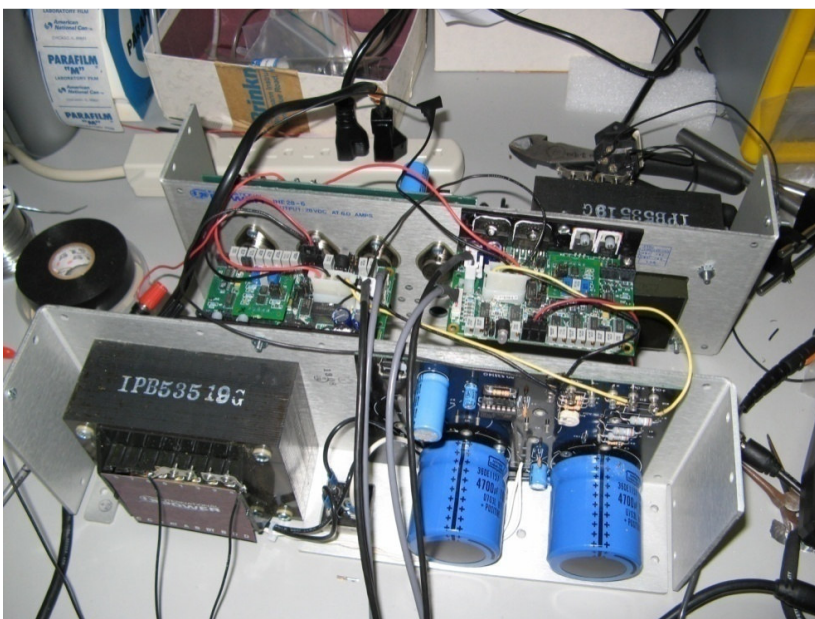


Figure 8 – The finished device. Eventually, housing will be developed and a computer will be connected as the input device.

Appendix A: Time Series Images for HEK Cells Expressing GFP

July 23, 2008:

Image 1 – 16:20

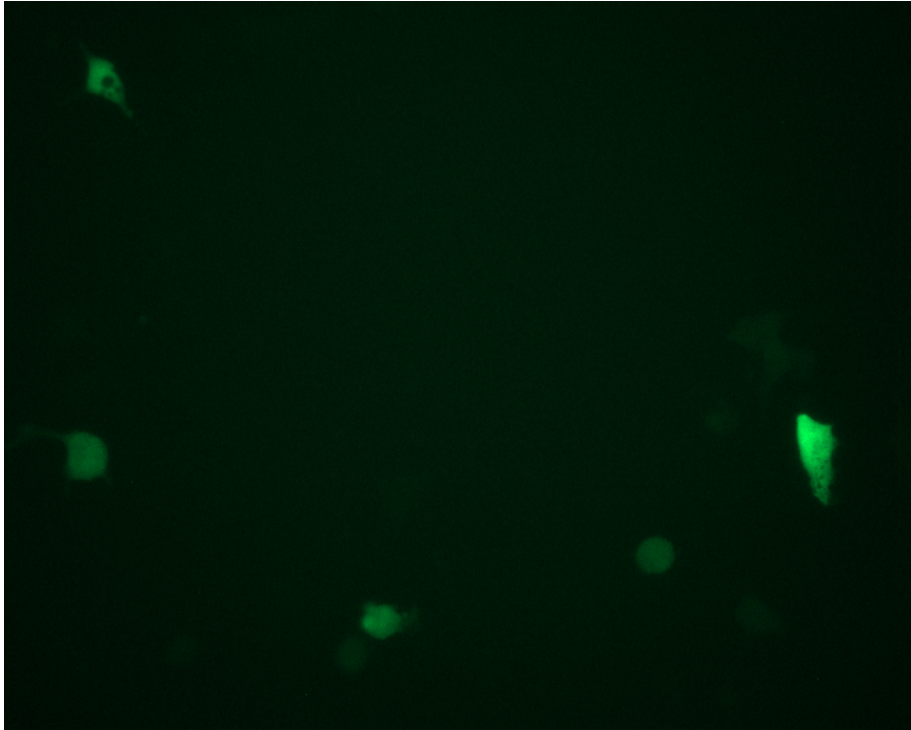


Image 2 – 16:26

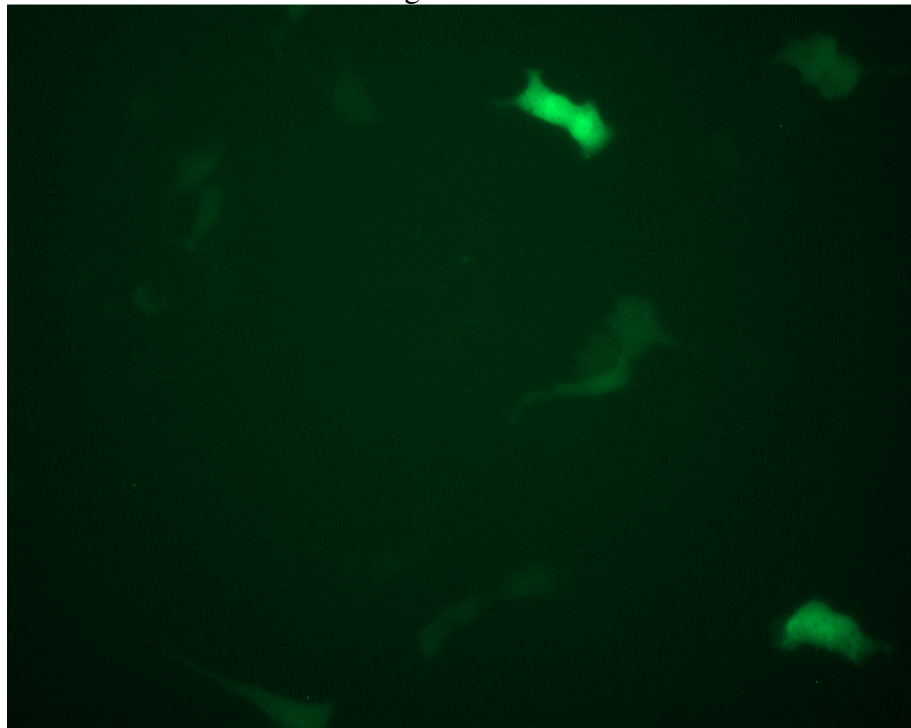


Image 3 – 19:04

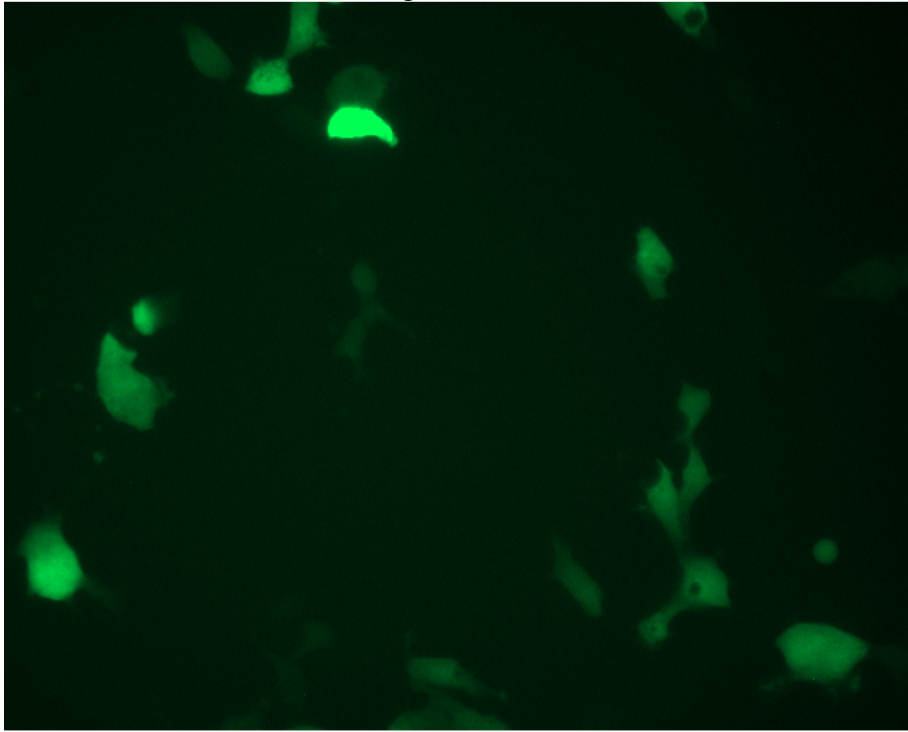
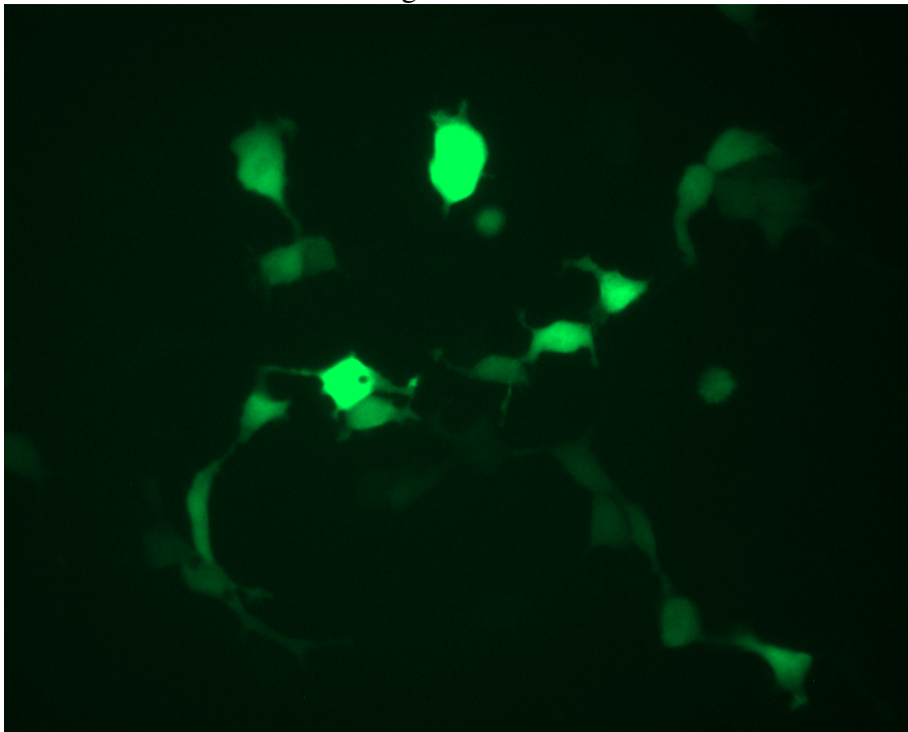


Image 4 – 19:09



July 24, 2008:

Image 1 - 11:58

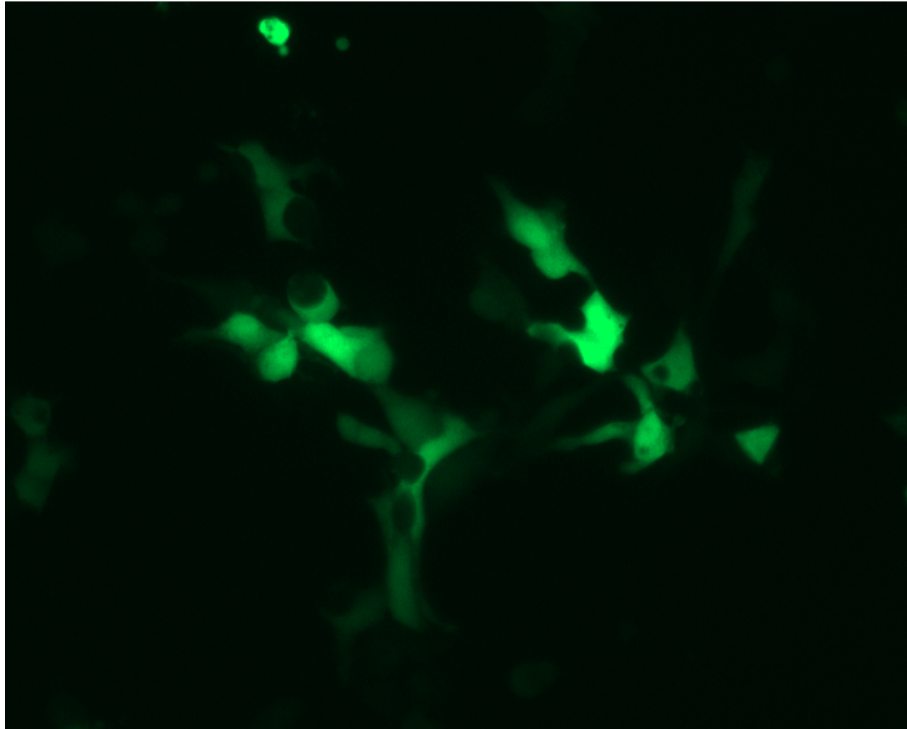


Image 2 - 12:00

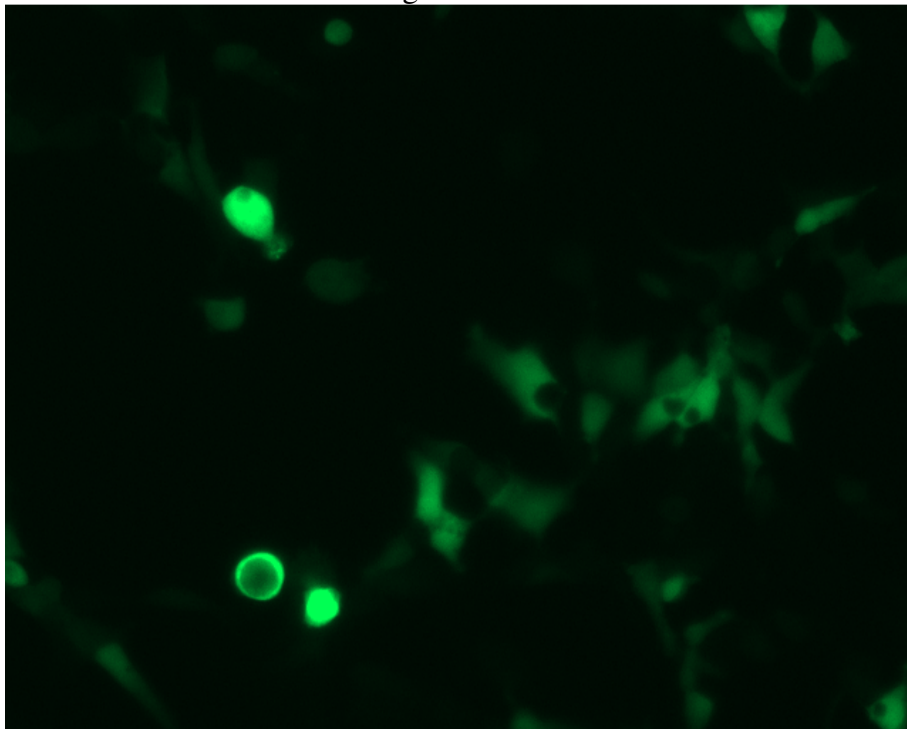


Image 3 – 15:46

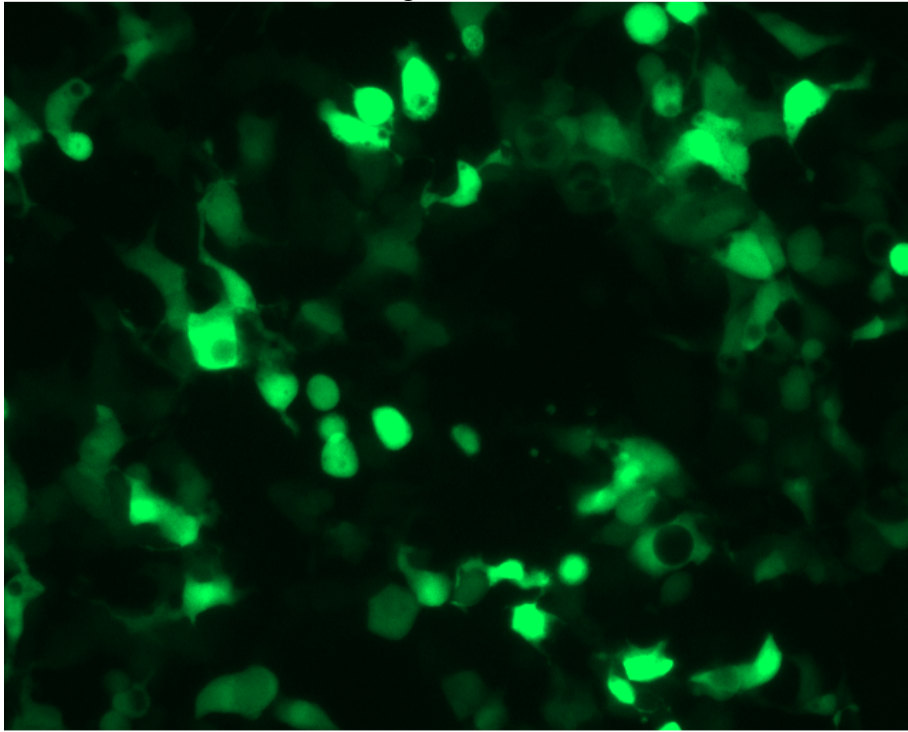
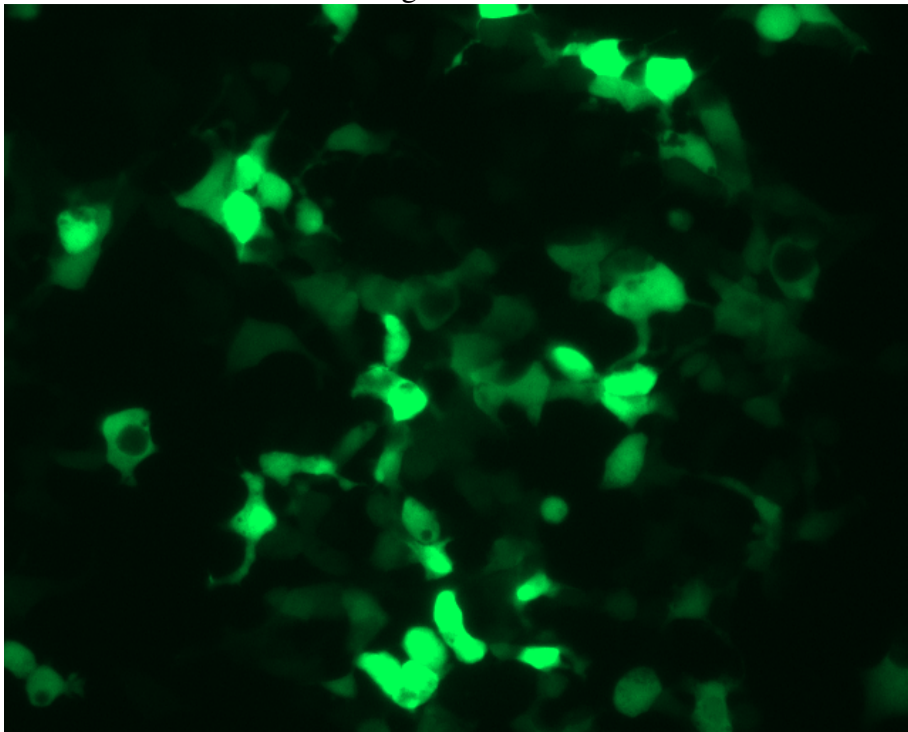


Image 4 – 15:48



July 25, 2008:

Image 1 – 11:04

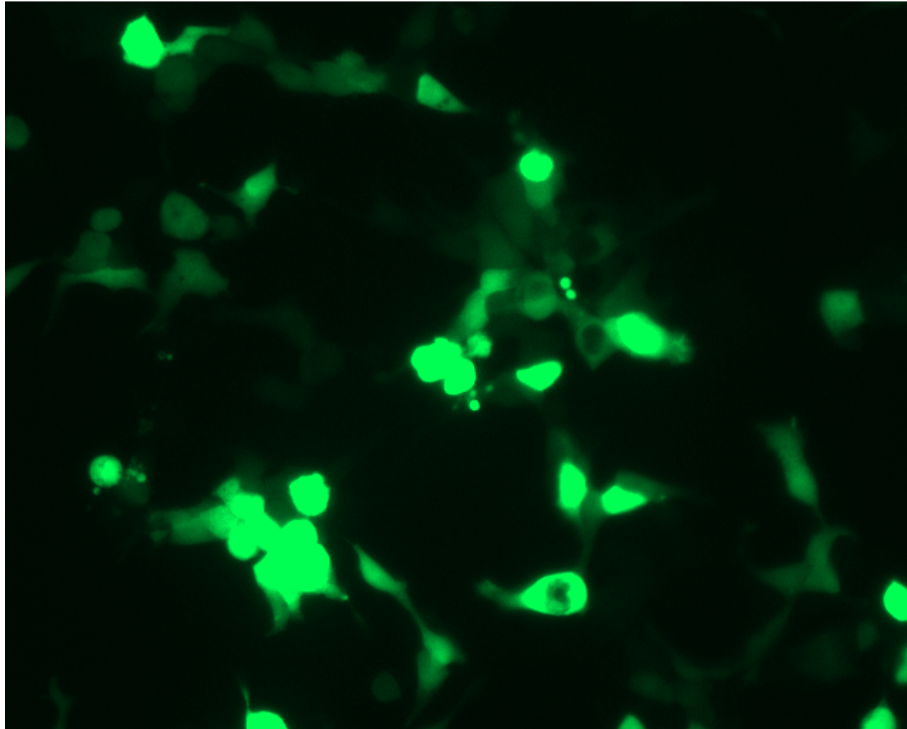
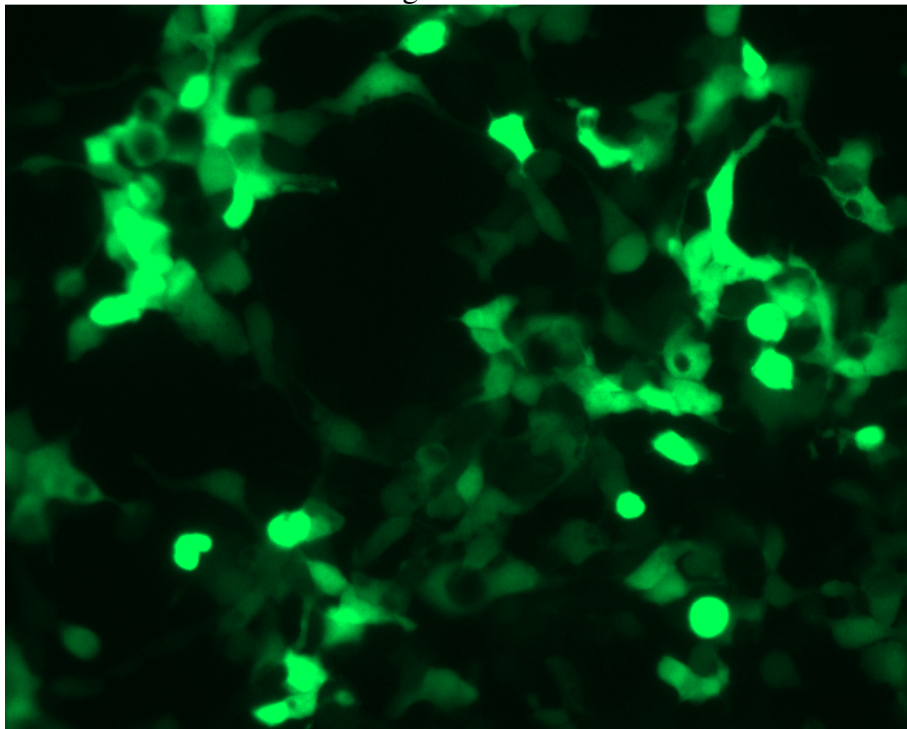


Image 2 – 11:06



Appendix B: Time Series Images for HEK Cells Expressing GFP-CaM

July 23, 2008

Image 1 – 16:35

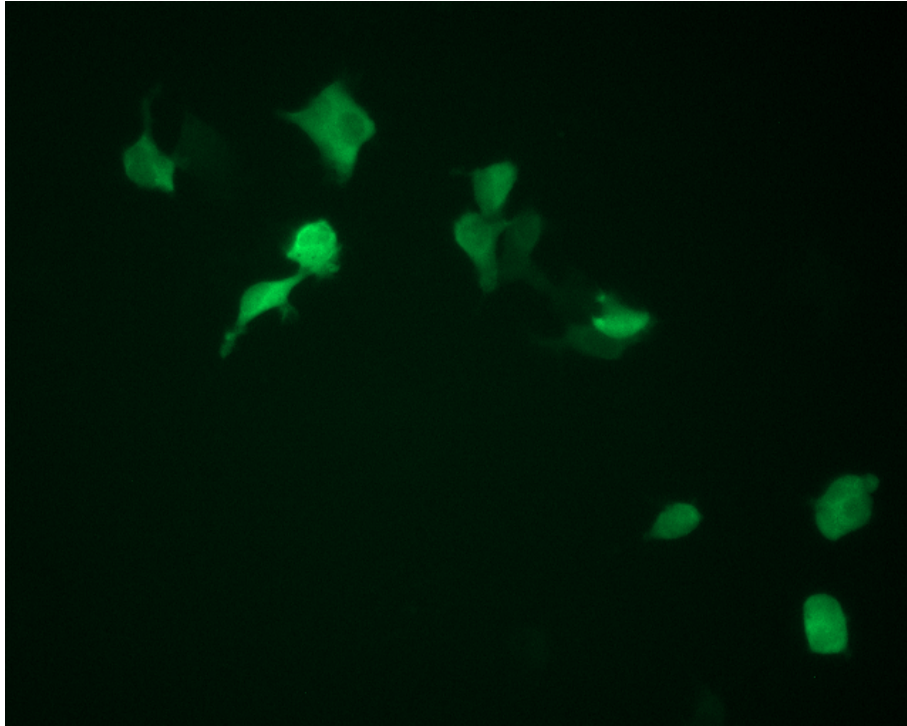


Image 2 – 16:37

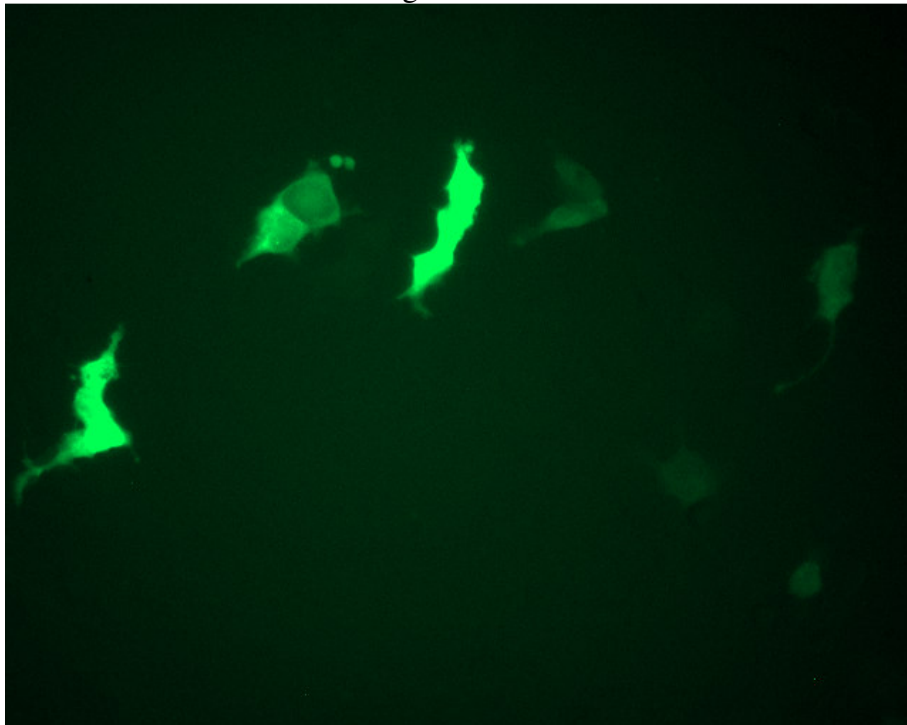


Image 3 – 19:15

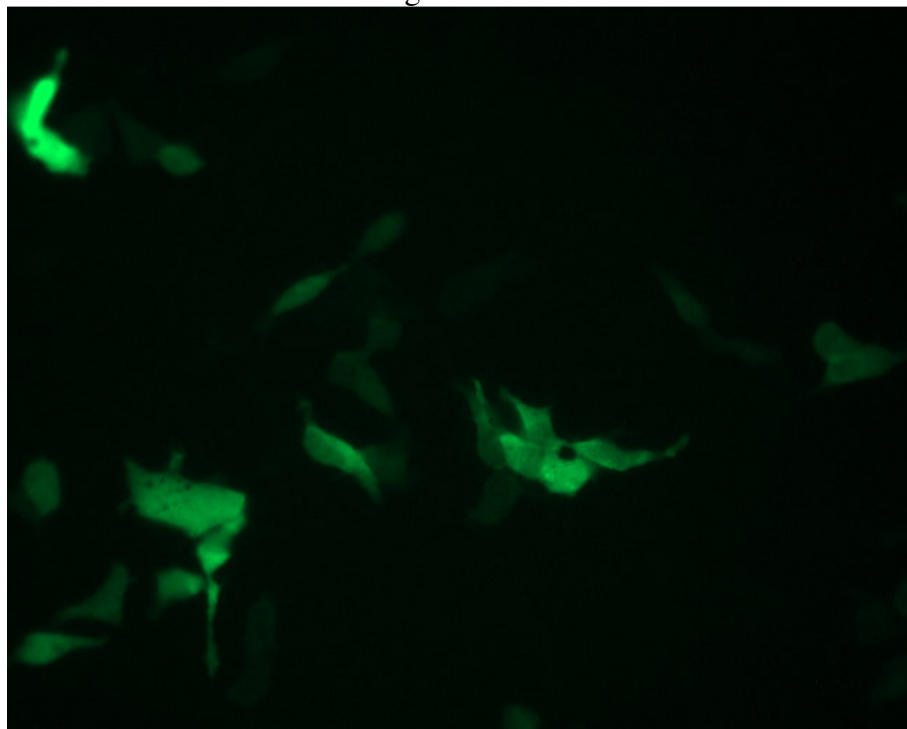
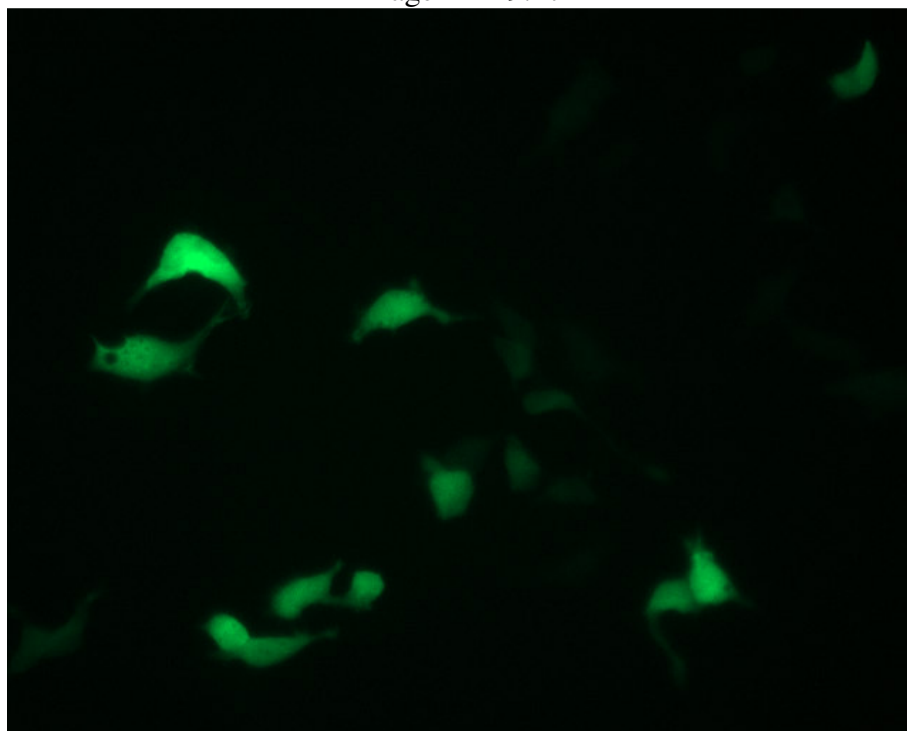


Image 4 – 19:17



July 24, 2008:

Image 1 – 12:06

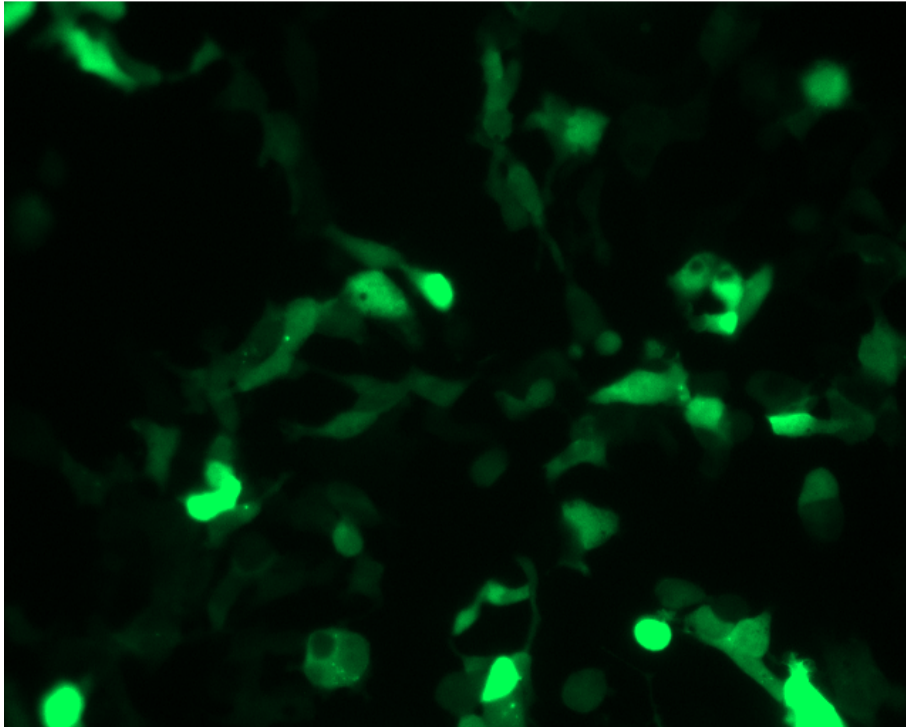


Image 2 – 12:09

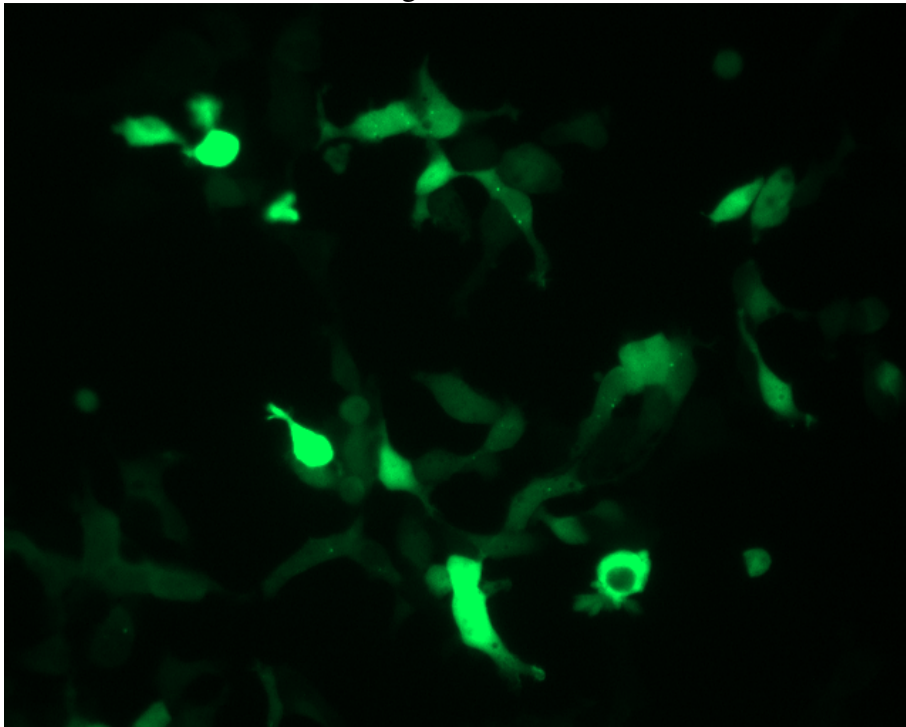


Image 3 – 15:38

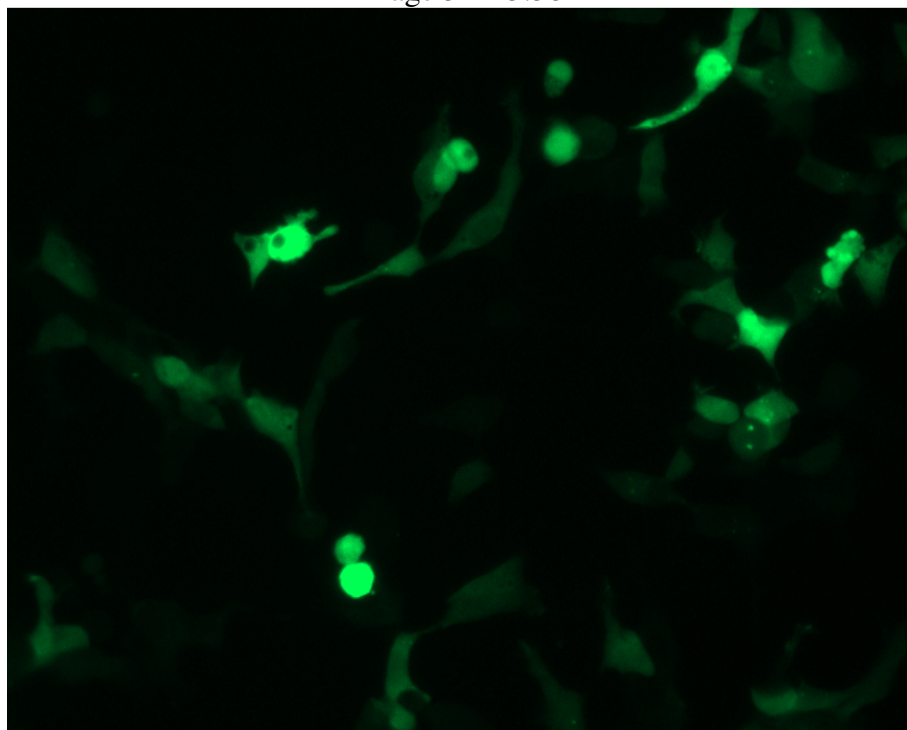
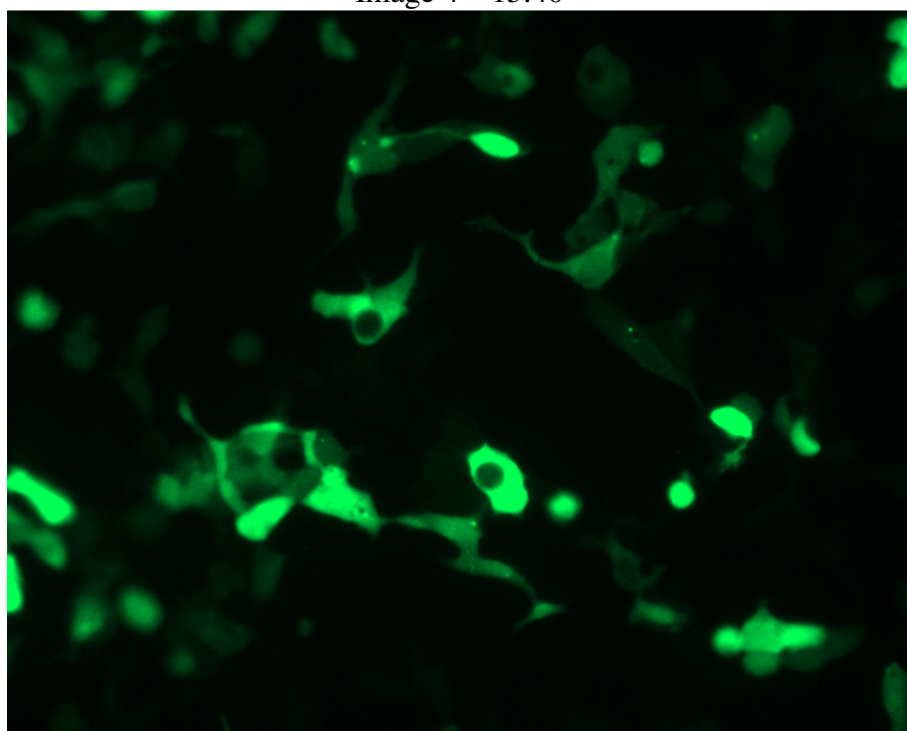


Image 4 – 15:40



July 25, 2008:

Image 1 – 11:09

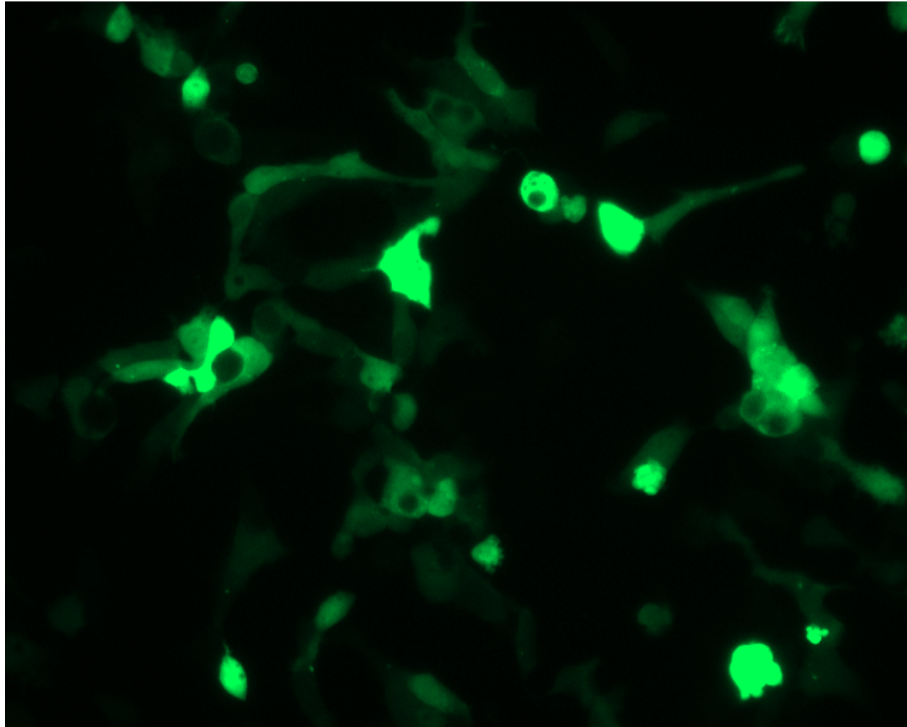
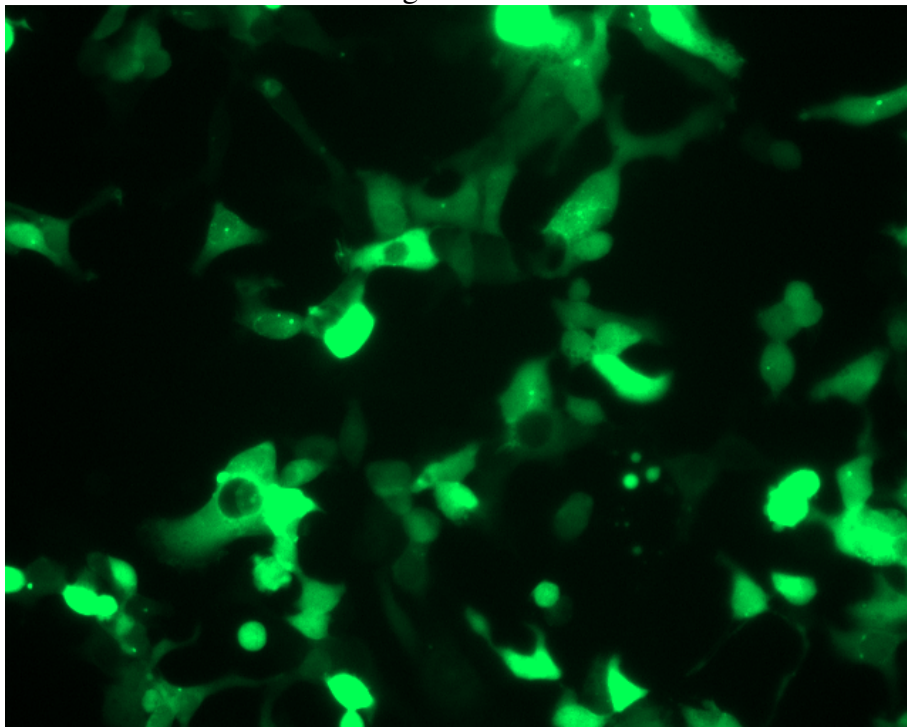


Image 2 – 11:11



6. Appendix C: Time Series Images for HEK Cells Expressing GFP-CaMKII

July 23, 2008:

Image 1 – 16:29

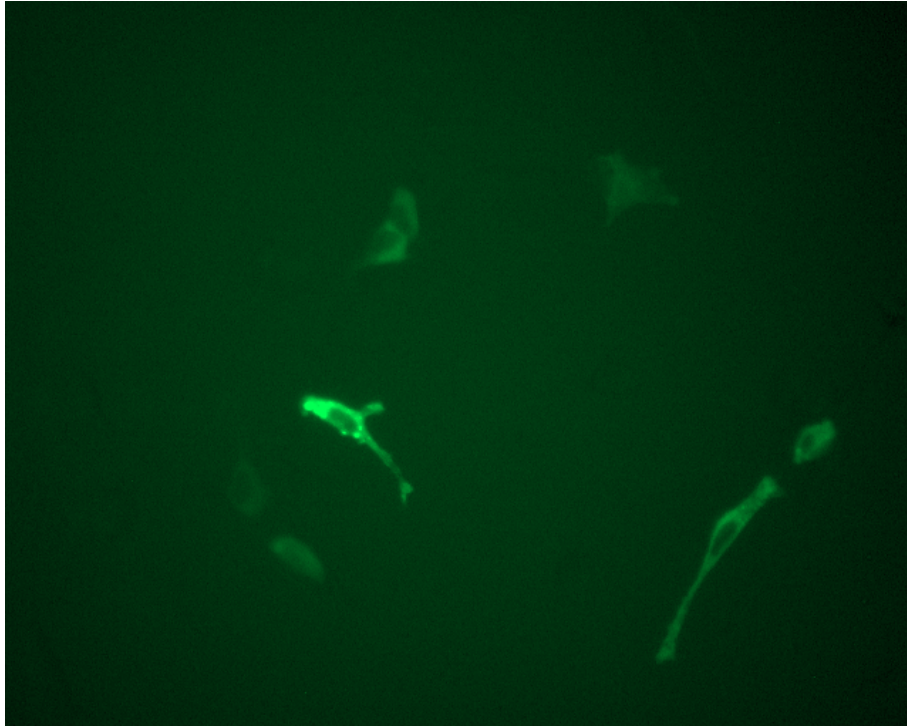


Image 2 – 16:32

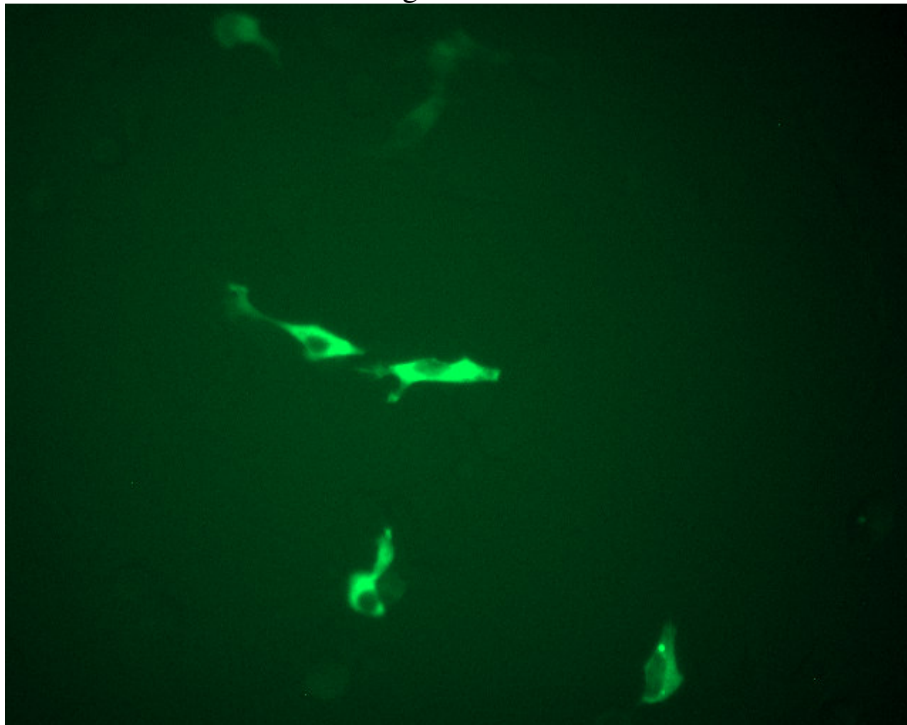


Image 3 – 18:58

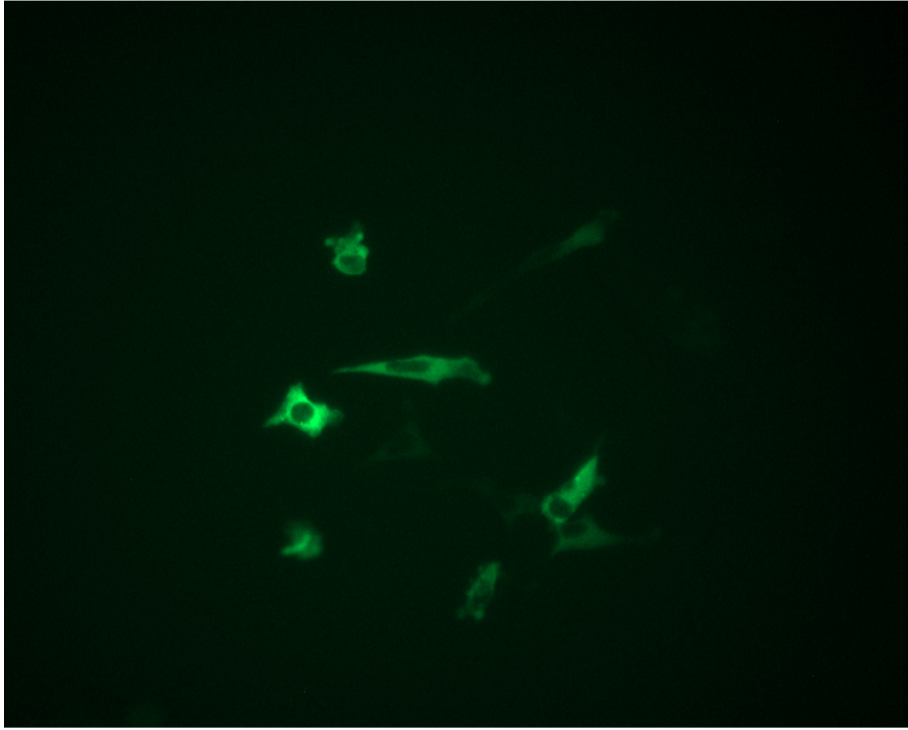
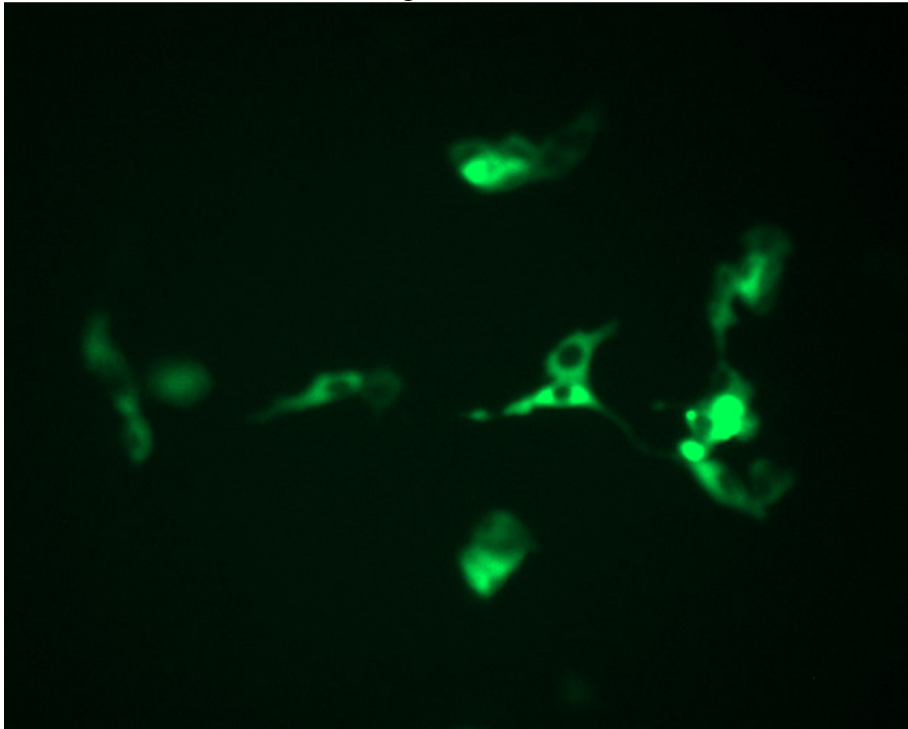


Image 4 – 19:01



July 24, 2008:

Image 1 – 12:03

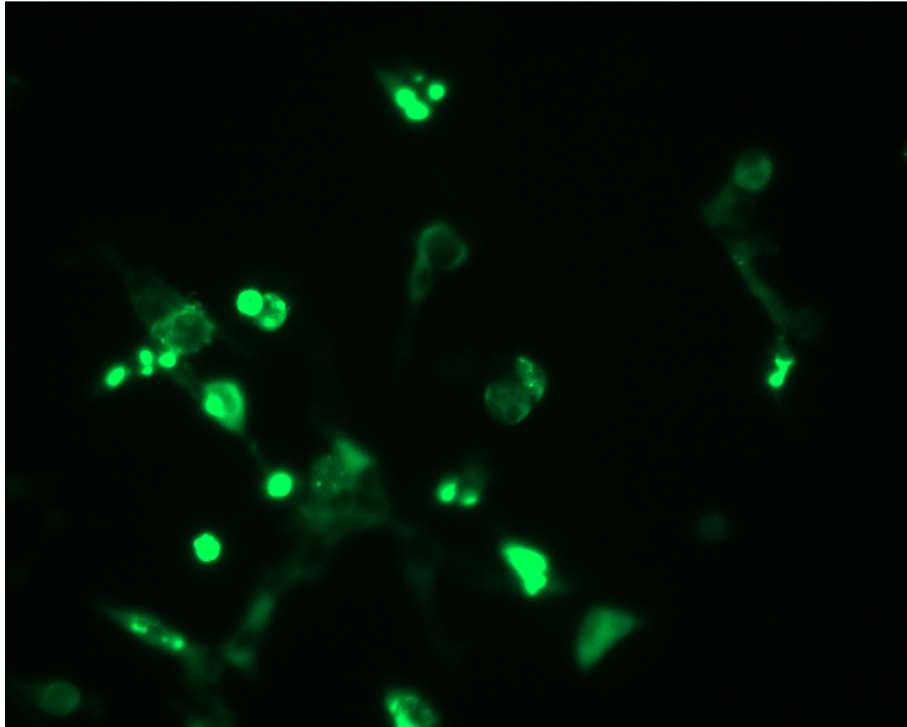


Image 2 – 12:04

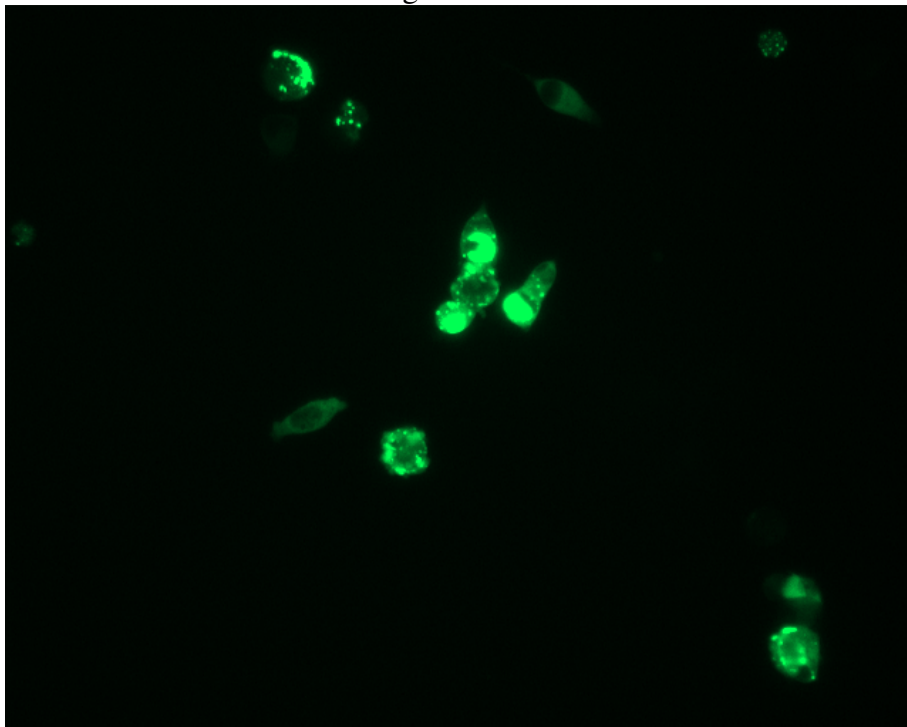


Image 3 – 15:42

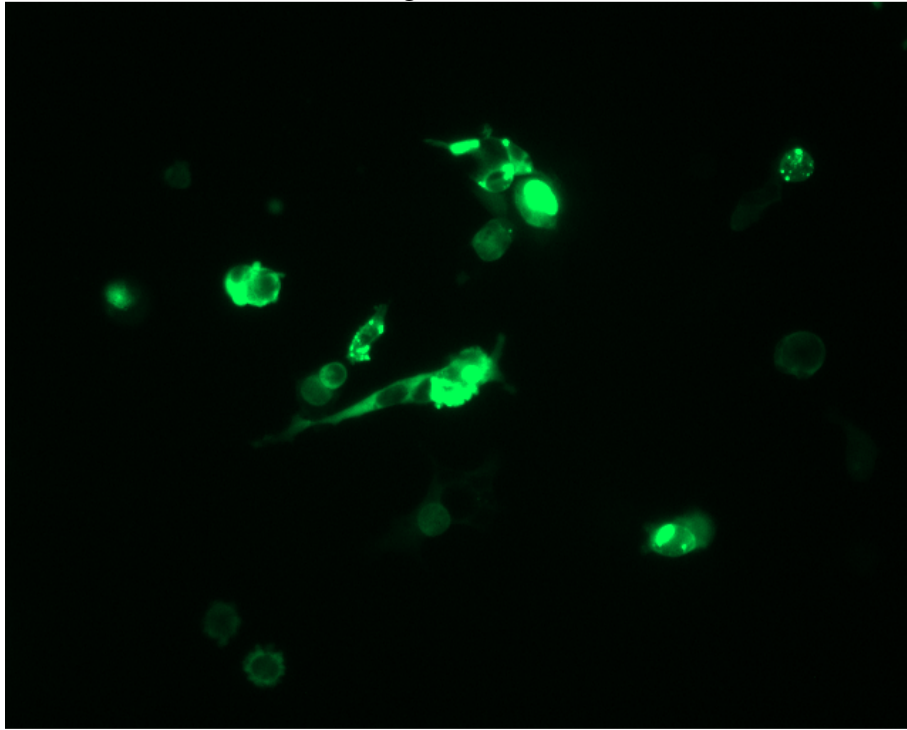


Image 4 – 15:43

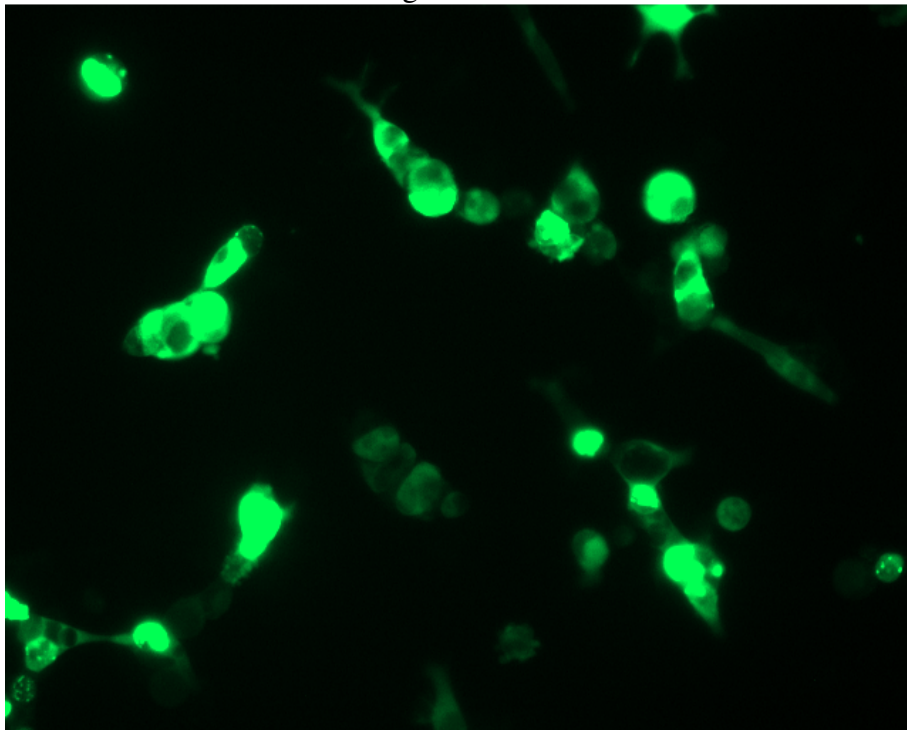
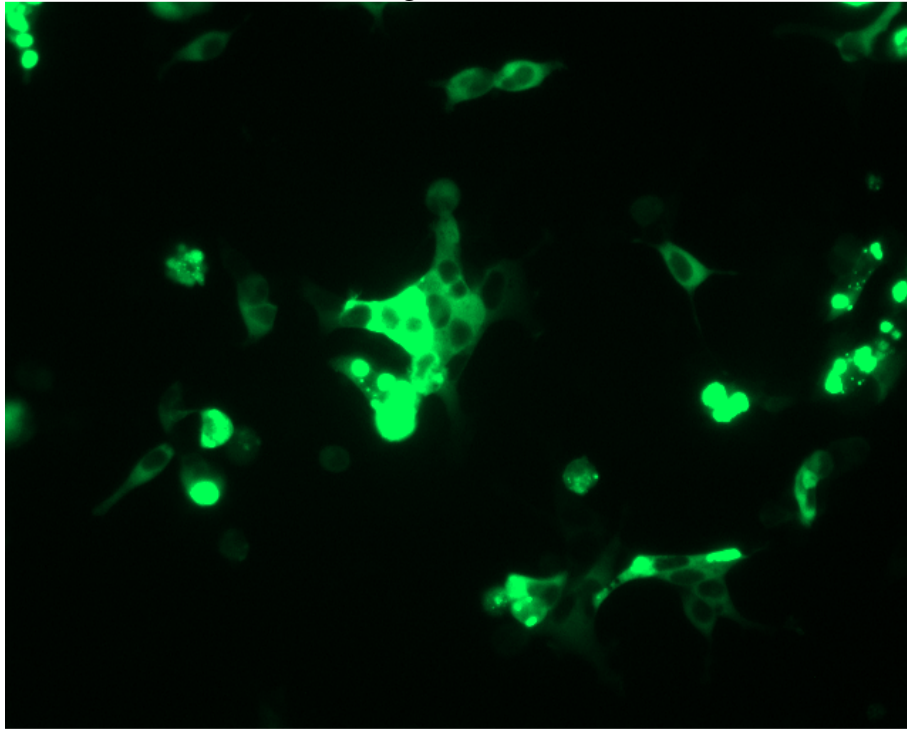


Image 5 – 15:45



July 25, 2008:

Image 1 – 11:19

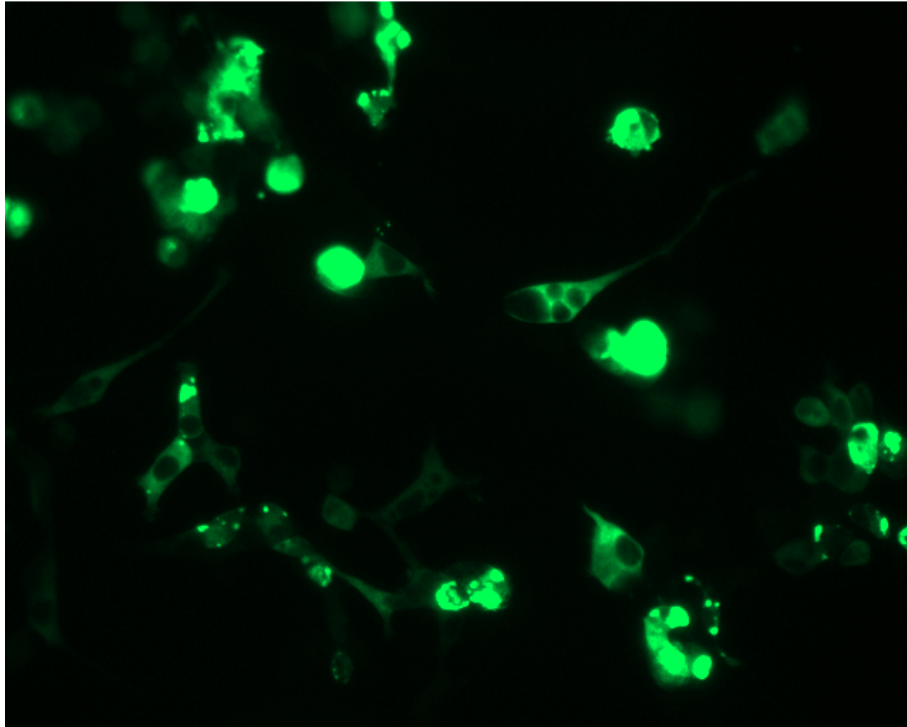
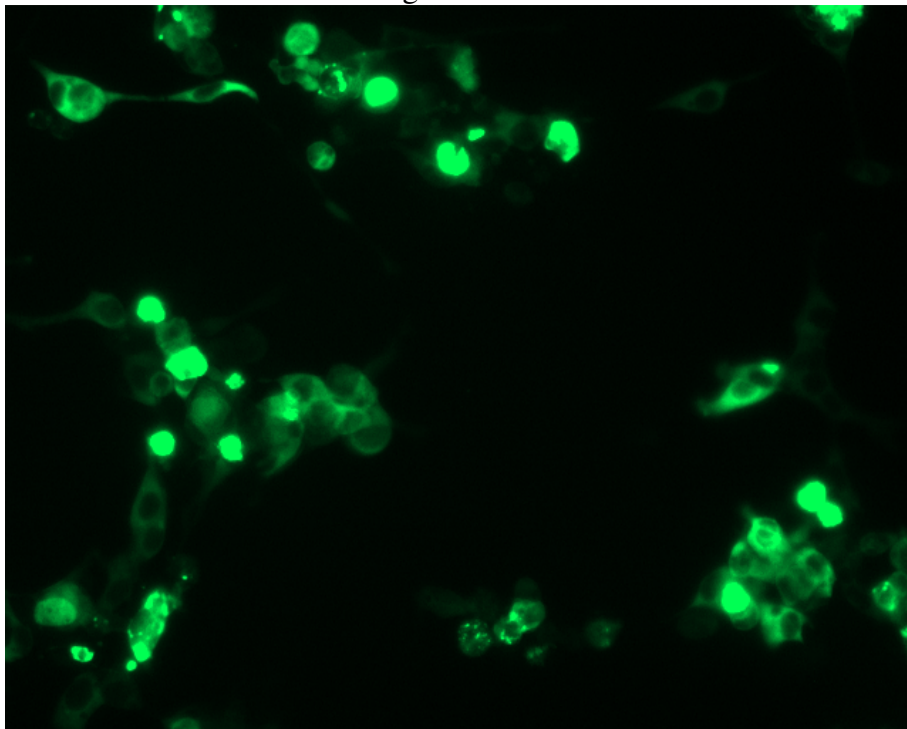


Image 2 – 11:20



Appendix D: Time Series Images for HEK Cells Expressing GFP-RC3

July 23, 2008:

Image 1 – 16:44

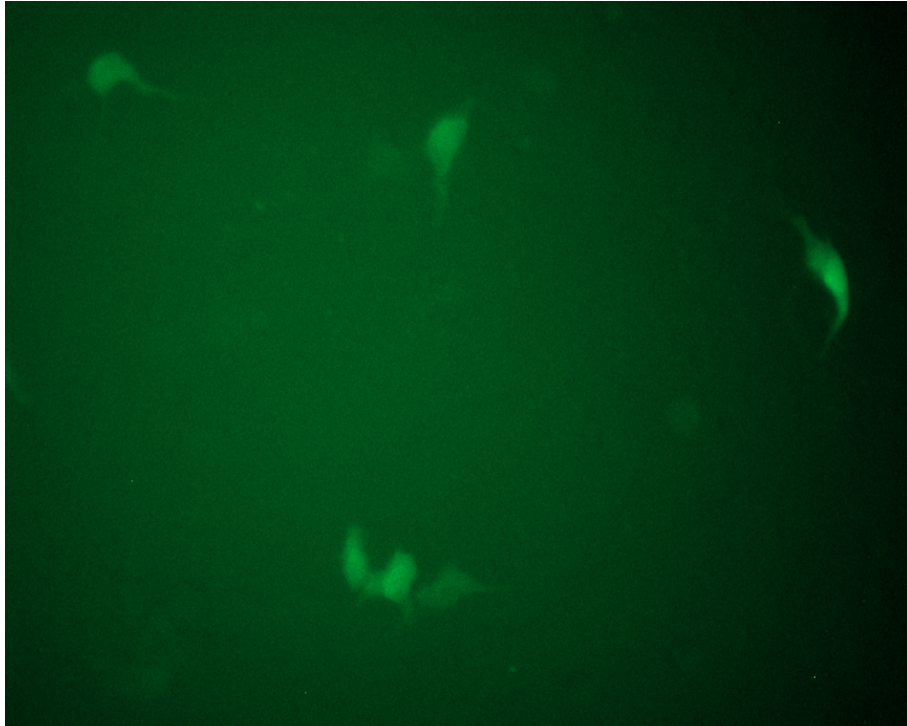


Image 2 – 16:46

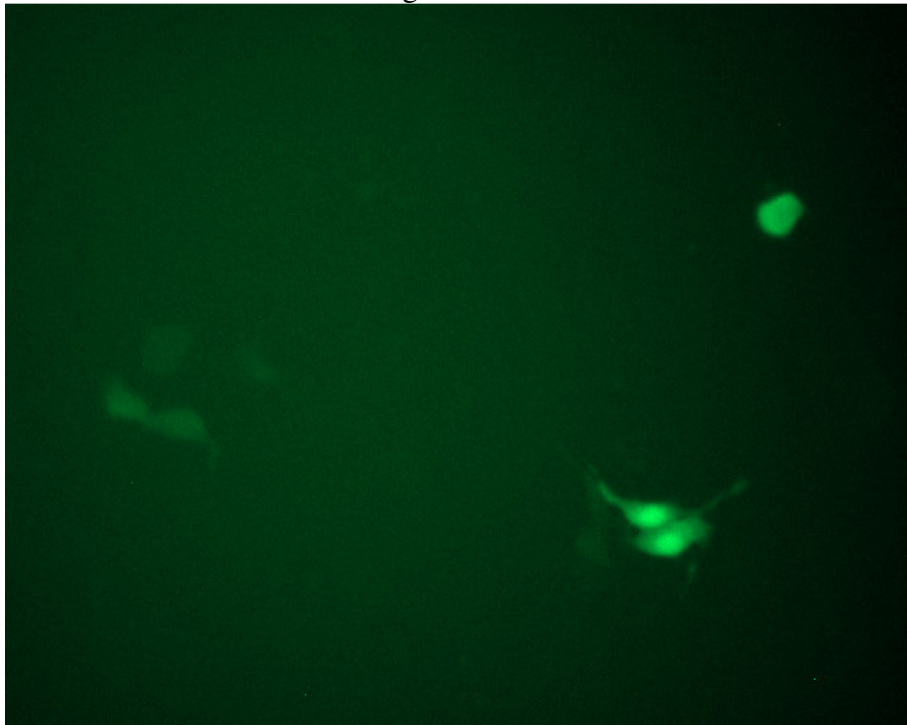


Image 3 – 19:12

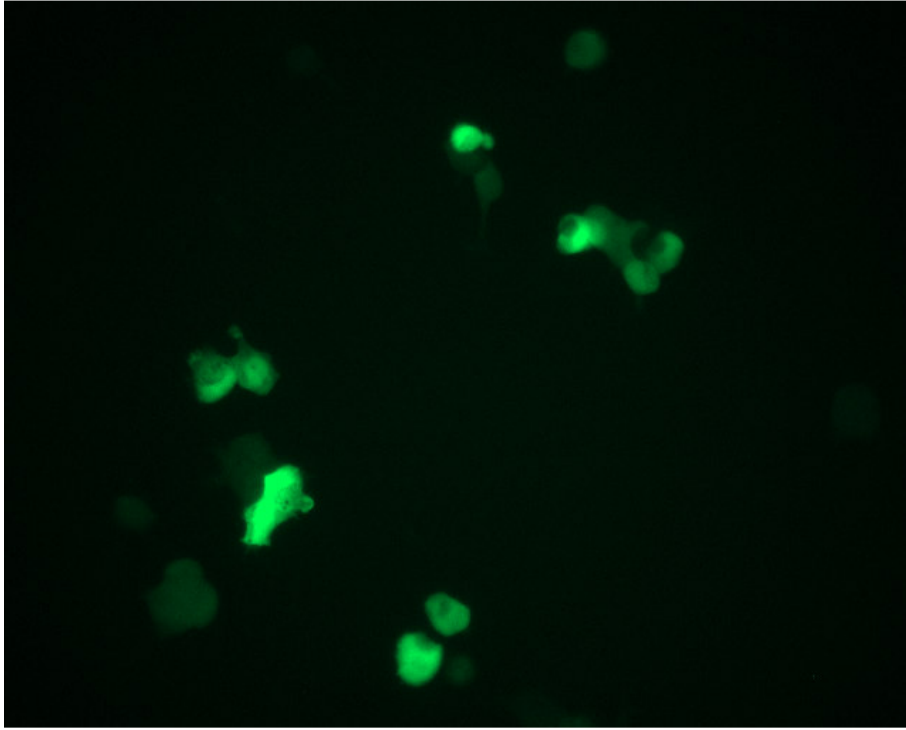
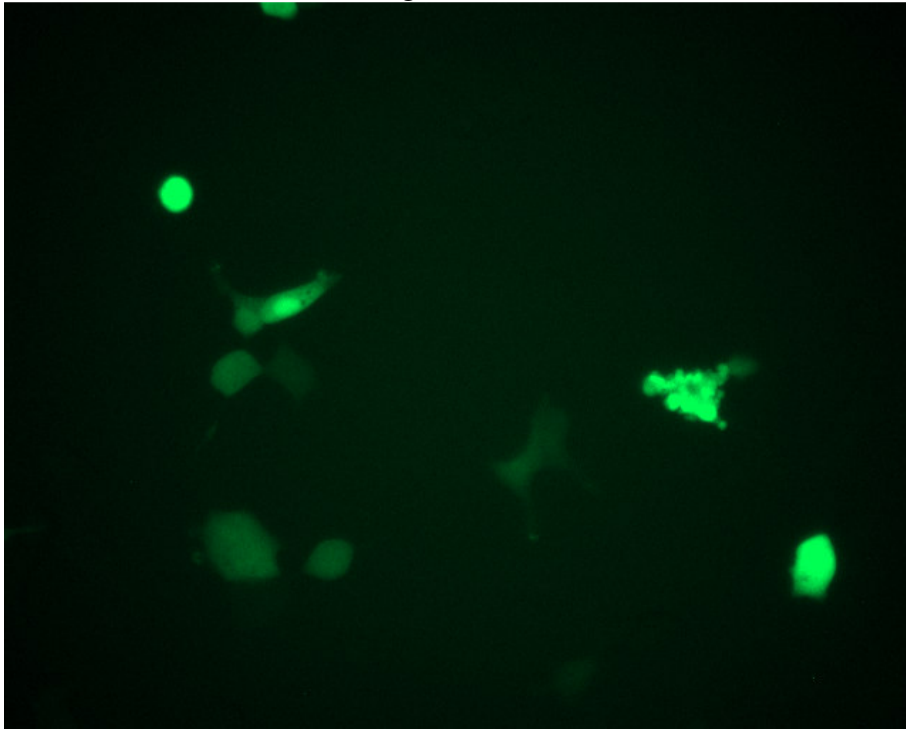


Image 4 – 19:13



July 24, 2008:

Image 1 – 11:55

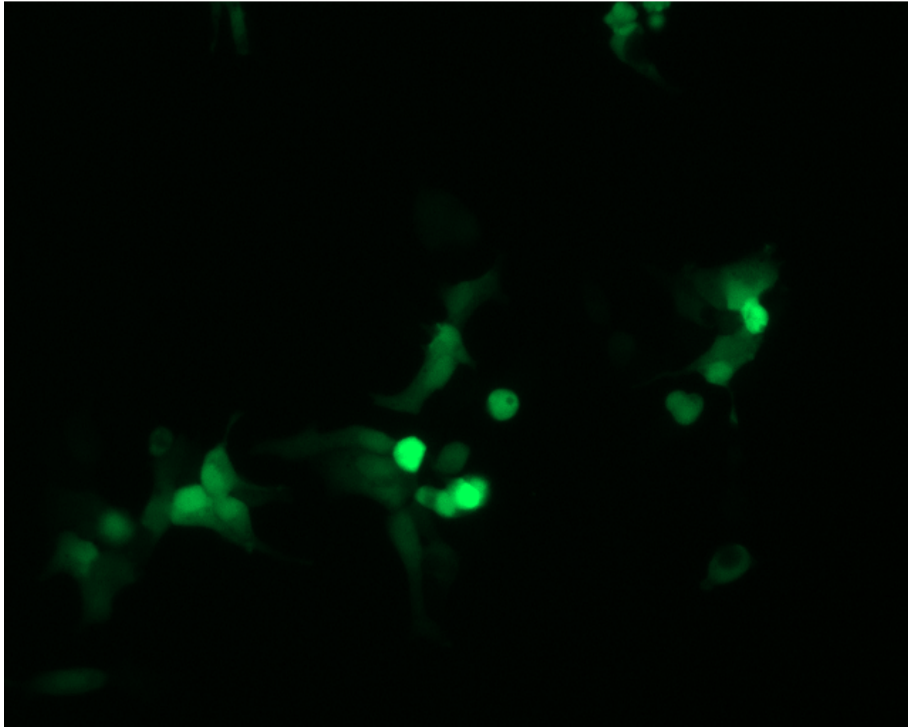


Image 2 – 11:56

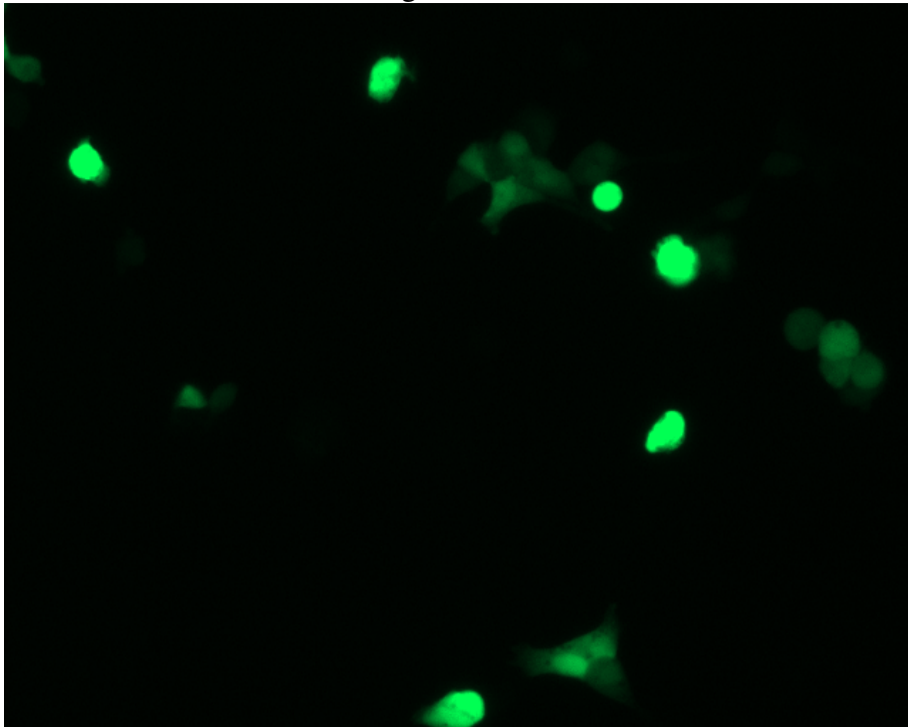


Image 3 – 15:50

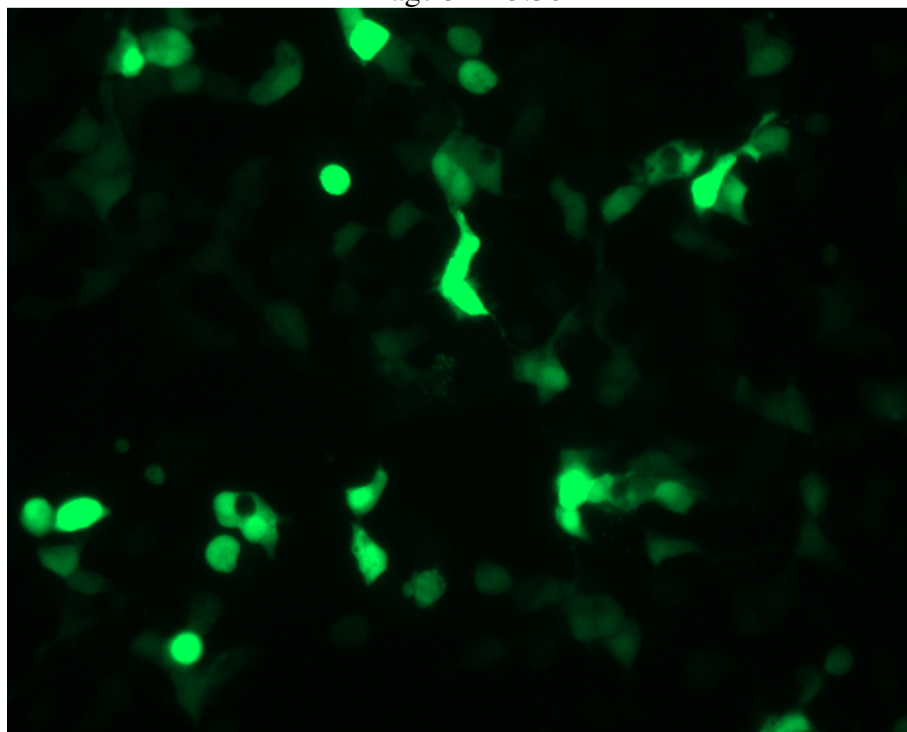


Image 4 – 15:53

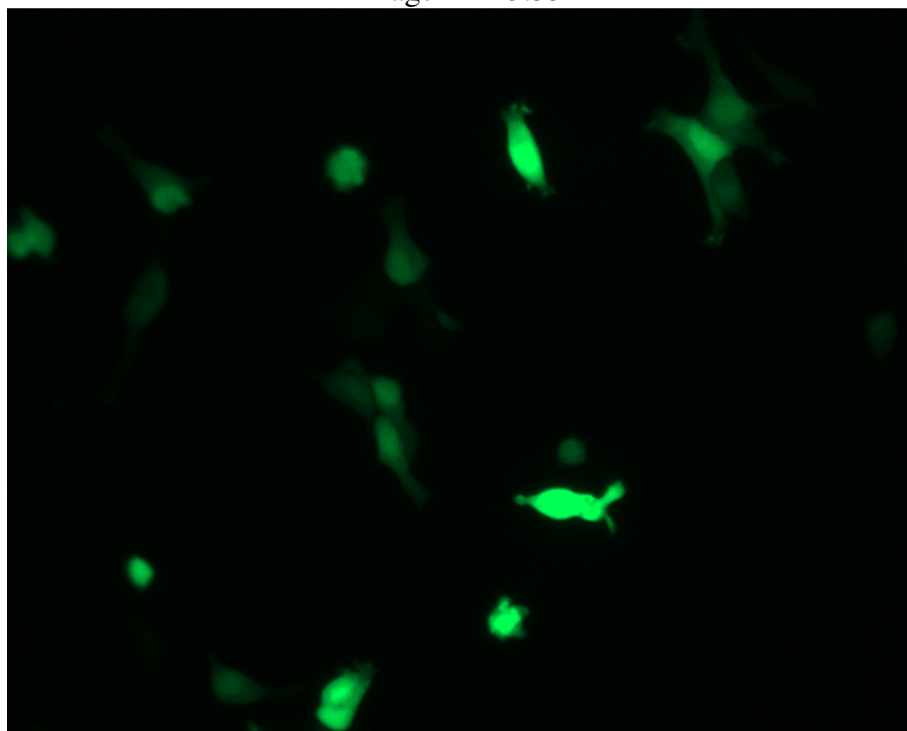


Image 5 – 15:54(a)

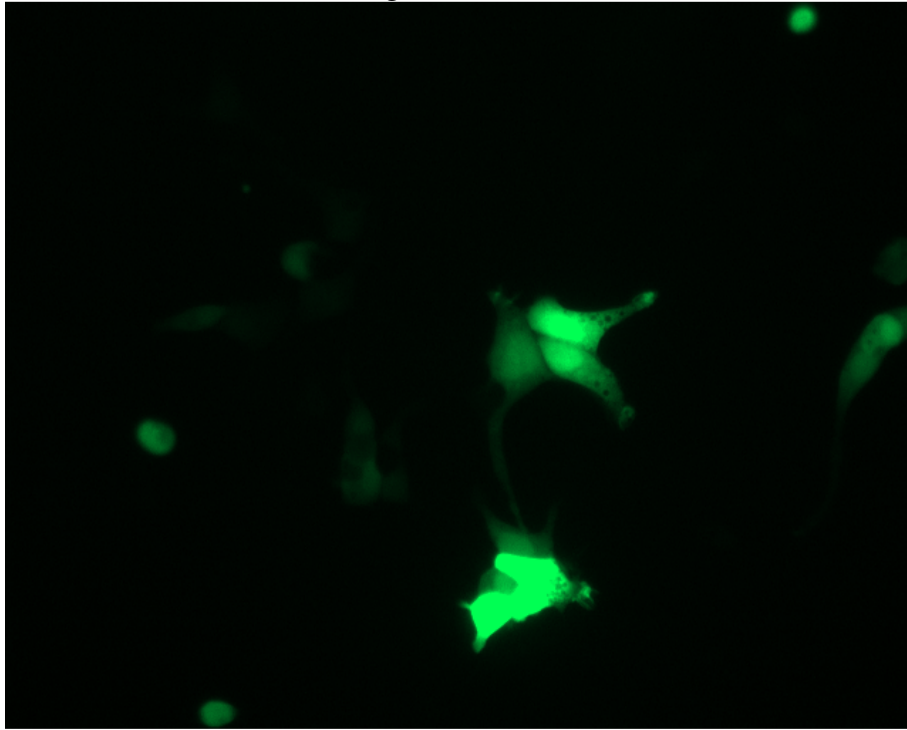
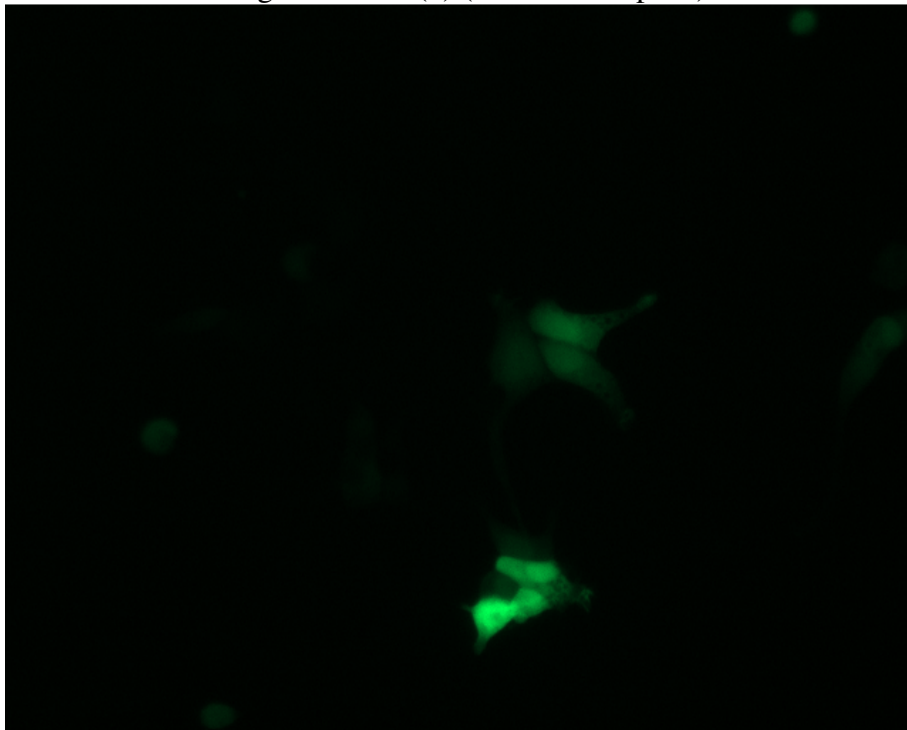


Image 6 – 15:54(b) (low shutter speed)



7. References

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