ANNEXATION OF INTERCHROMOSOMAL SPACE DURING VIRAL INFECTION IN 3D TISSUE MATRIX

A Senior Scholar Thesis

by

ZACHARY AUSTIN CRANNELL

Submitted to the Office of Undergraduate Research
Texas A&M University
A partial fulfillment of the requirements for the designation as

UNDERGRADUATE RESEARCH SCHOLAR

April 2006

Major: Biomedical Engineering

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Approved by:

Research Advisor: Alvin T. Yeh
Associate Dean for Undergraduate Research: Robert C. Webb

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ABSTRACT

Annexation of Interchromosomal Space During Viral Infection

in 3D Tissue Matrix (April 2006)

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The process of viral infection has been shown to be a tightly structured process that exerts precise control over the nucleus. By quantitatively documenting the dynamic nuclear changes in response to nuclear infection of cells suspended in a three-dimensional collagen matrix, an understanding of nuclear reorganization can be elucidated from the tissue. Tissues were cultured *in vitro* in three dimensional collagen matrices which act as scaffolding that mimics the native environment. The cells were then infected with cytomegalo virus (CMV) which was labeled with green fluorescent protein to allow identification of the virus. During viral infection, nonlinear optical microscopy (NLOM) was employed to nondestructively image the nuclear volume over time. Comparing changes in the nuclear volume with control values, we expected to identify several stages of nuclear reorganization that have previously been identified during infection of 2D tissue cultures. Once it has been demonstrated that NLOM can be successfully used to track dynamics of infection, multiple mutant fluorescent proteins can be used to track host, virus and immune response to viral infection.

TABLE OF CONTENTS

Page

LIST OF FIGURES

FIGURE Page

1. INTRODUCTION

The process of viral infection has been shown to be a tightly structured process that exerts precise control over the nucleus[1]. In a study done at the Scripps Research Institute in La Jolla, the genome-wide response to viral infection was quantitatively characterized in an effort to gain a better understanding of nuclear spatial reorganization using Herpes Simplex Virus 1 (HSV-1)[2]. The study was able to quantitatively characterize three clearly defined stages of spatial reorganization of the nucleus in cell cultures. While enlightening, the study was performed on two-dimensional cell cultures that are inherently different than the three-dimensional tissue matrix found within naturally occurring tissues.

Working in conjunction with the original researchers, we proposed to compliment this study by quantitatively documenting the dynamic nuclear changes in response to nuclear infection of cells suspended in a three-dimensional collagen matrix. By studying the nuclear response in three dimensions, a significantly greater understanding of nuclear reorganization can be elucidated from the tissue. As of yet a comprehensive analysis of nuclear infection has yet to be completed on three-dimensional tissue matrices. The information gathered is of a fundamental importance to virologists and immunologists who wish to develop ways of inhibiting viral growth

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This thesis follows the style of the Journal of Biomedical Optics.

2. METHODS

Viral Infection in 2-D

In order to observe viral infection in 2D, cells had to be cultured in a cover slip-bottom Petri dish so they could be imaged using the Nikon Eclipse Telescope confocal fluorescence microscopy system. The virus was labeled with GFP so that when it infected a cell it caused the cell to produce a fluorescent protein that filled the cytosol.

Cells were normally put on Petri dishes when the flasks were split into two flasks. Normal NIH 3T3 murine fibroblasts and GFP tagged NIH 3T3 murine fibroblasts were cultured with a DMEM media supplemented with 10% FBS & 1% Penstrep in T75 tissue culture flasks. As the cells began to become confluent, they were frequently split into two flasks according to standard cell culture procedures. To loosen the cells, 3-5 mL of .25% trypsin was added. The trypsin was poured out after it had coated the bottom of the flask and the flask was returned to the incubator. After the cells were loosened, 15 mL of media were added to the cells and they were mixed thoroughly with the auto-pipetter. A small drop of cell/media mixture was placed in the middle of the Petri dishes and the remaining cell/media mixture was divided between two fresh, sterile flasks and returned to the incubator. Ideally, the cells in the Petri dish would be less than 30-40% confluent. At least two Petri dishes had normal NIH 3T3 cells and at least one Petri dish had GFP tagged 3T3 cells. The GFP tagged dish and one of the normal dishes served as positive and negative controls when fluorescently imaging the viral infection.

The Petri dishes were allowed to culture overnight in the incubator to allow the cells to attach to the bottom of the collagen coated cover slip. After the cells were attached, the media was removed and replaced with a DMEM media that was supplemented with only 2% BCS & 1% Penstrep. Since the serum binds to the virus, a high serum concentration prevents the virus from infecting the cells.

After at least one full day of culturing the Petri dishes in 2% BCS media, the CMV virus was added to one of the Petri dishes with normal cells. The viral concentration that we were given to conduct the experiment with was initially unknown. It was estimated to be roughly .6 million PFU (plaque forming units)/mL. Since we were looking at individual cell response to viral infection, we were not particularly concerned with a specified infection rate. We generally added what we estimated to be far more virus (~1000 μL) than was necessary in order to ensure an extremely high infection rate. All three Petri dishes were imaged at time zero as well as at 24, 48, 72 and 96 hours.

Tissue Engineering

NIH 3T3 murine fibroblasts were cultured with a DMEM media supplemented with 10% FBS & 1% Penstrep in T75 tissue culture flasks to 90% confluence. At this point the cells were suspended in a collagen matrix to create three dimensional tissues.

Tissues were made by emptying the flask of media and introducing 3-5 mL of trypsin into the flask. After the trypsin coated the bottom of the flask, the trypsin was poured

out of the flask. The flask was then returned to the incubator for a period of five minutes. After 5 minutes, the cells should have loosened from the flask and be seen visibly sliding along the bottom as the flask is turned on its side. To collect the cells, 10 mL of media was then introduced into the flask. The cells were gently mixed by pipetting the media/cell mixture up and down several times, being careful to avoid introducing bubbles.

When all the cells were collected, the media was removed and placed in a sealable, sterile test tube. Two 10 microliter samples were then taken from the well-mixed larger sample in order to calculate the cell count using a Bright-line Hausser Scientific hemocytometer. The test tube was then centrifuged at 1000 rpm for 5 minutes.

When the media was done centrifuging, the supernatant was poured off. Media was added to the pellet to bring the cell concentration up to the desired concentration. For making tissues, it is best to have the cells concentrated between 10^6 and 10^7 cells/mL of tissue. Since 0.5 mL of cell/media is used to make roughly 6 mL of tissues, this translated to a concentration of 20 million cells/mL.

The following steps were performed on ice blocks to prevent the collagen from prematurely renaturing. In a sterile mixing cup 0.6 mL of reconstitution gel was combined with 1.2 mL DMEM at five times concentration. Next 3.9 mL of collagen T1 Rat tail was added while continuously stirring the mixture. Before the next step, the

mixture's pH had to be brought up to physiological pH. This was normally accomplished by adding 1-2 drops of sterile NaOH solution. Next the 0.5 mL of cell/media was added to the mixture and stirred well. The mixture was then quickly distributed amongst several wells in a well-plate. For the purposes of our research, we normally put 1 mL of mixture into 6 wells of a 24 well-plate. Smaller or larger tissues can be made by aliquoting the mixture into smaller or larger sterile well-plates. The well-plate was immediately placed in the incubator. This allowed the collagen to renature quickly and suspended the cells uniformly before the cells sunk to the bottom of the tissue.

After roughly five minutes, the collagen was renatured and the cells were properly suspended. The collagen was confirmed to be adequately renatured when the tissue-mixture did not move when the well-plate was tilted. After the tissues were gelled, 1 mL of media was added to each tissue and the tissues were returned to the incubator. The color of the media was generally used as an indicator as to how often the media needed to be changed. Pink and red media indicated a desirable pH for the media. As the cells consumed the nutrients in the media, the media became more acidic and turned to an orange or even a yellow in extreme cases. Normally the media was changed every 1-3 days as the media took on an orange colored tint.

Imaging the Cells in 3D

To image a tissue in 3D, the tissue was removed from the well-plate and placed in a 5 cm Petri dish. The tissue was immersed in PBS solution and imaged using nonlinear optical microscopy (NLOM). NLOM excites tissues and provides images in a plane of view at a specified depth. By sequentially obtaining images at varying depths, a three dimensional reconstruction was created. These reconstructions were in turn used to gain information on the dynamics of viral infection and the nucleus.

Viral Infection in 3D

We were not able to fully develop the protocol for viral infection in 3D as time ran out before we were able to establish a protocol that produced reliable results. The procedure we followed was similar to the process of viral infection in 2D. Tissues were made according the procedure previously described. When we wanted to infect a tissue, we removed a tissue from the well-plate and placed it inside a 5 cm Petri dish. We always used at least two tissues made from NIH 3T3 fibroblasts: one as a control and one for viral infection.

We then cultured the tissues in 2% BCS media for one day and infected with the virus the following day. Since much more media is required to sustain a tissue as opposed to a 2D cell culture, much more virus was added to the tissue so all of the virus would not be neutralized by the serum. Since control data for the NIH 3T3 tissues had already been

obtained, the tissues were not imaged at time zero. The tissue was imaged at 24, 48, 72 and 96 hours after infection.

To find cells that were infected with the virus, the data collector scanned around the tissue until they found a cell that fluoresced. He then zoomed out in the z-plane until they reached the top of the cell. Once he was at the top of the cell, he could sequentially scan down through the cell to obtain image slices that could be reconstructed into 3D stacks.

Image Analysis

After the images were stacked, a head on and a side view of the stacks were used to obtain information about the nuclei. Assuming the nuclear volume could be modeled as ellipsoids, the volume of the nuclei was taken to be $4/3\pi abc$. Figure 1 shows how image stacks can be used to approximate nuclear volume.

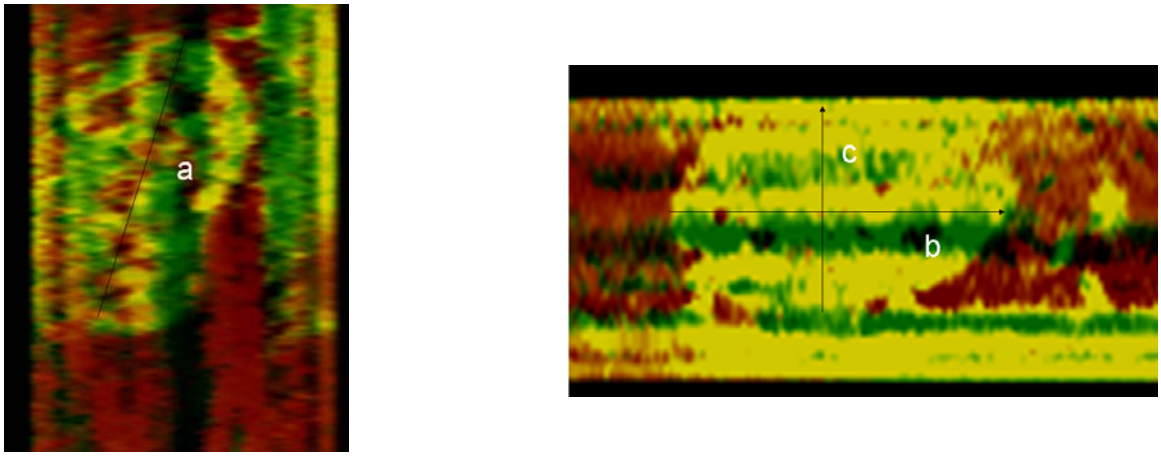

Figure 1: Approximating Nuclear Volume as an Ellipsoid

3. RESULTS

Two Dimensional Results

We were able show definitive viral infection in two dimensions. This was used mainly for confirmation that our viral stock was functional and to test the viral stock we built up. Figure 2 is proof of positive viral infection in two dimensions.

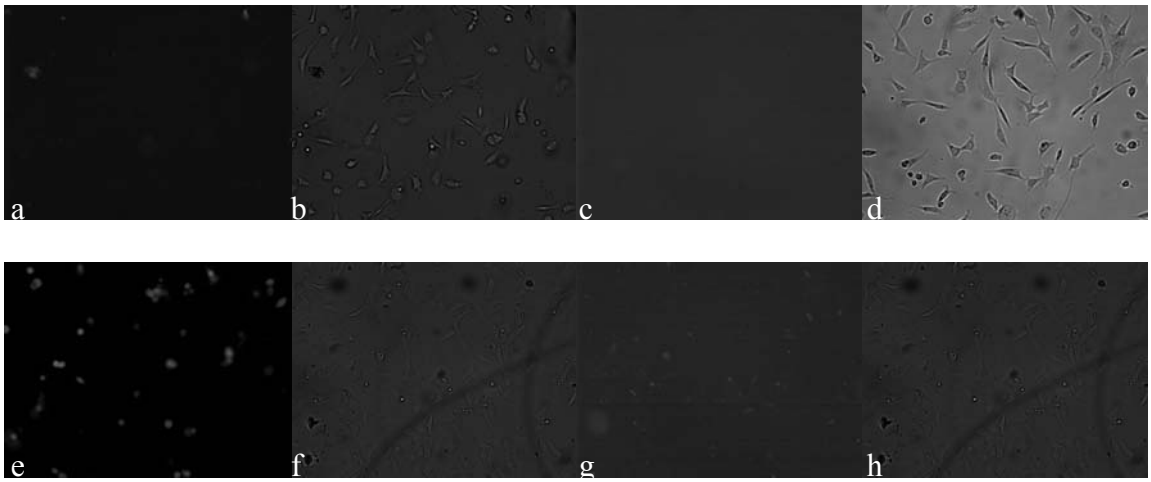

Figure 2: Fluorescence images from day one (a) and day three (e). Note the increase in fluorescence over time indicating viral infection. Images (b) and (f) are light microscopy images of the same cluster of cells. Images (c) and (g) are beginning and end fluorescence images of the control sample. Note the lack of localized fluorescence. Images (d) and (h) are light microscopy images from day one and day three of the control sample.

Three Dimensional Results

The three dimensional protocol failed to yield a confirmed case of viral infection. During the best trial, we showed indications of infection in the two tissues that were infected through fluorescence; however the control also fluoresced when it should not have shown anything. One possible explanation for this phenomenon was cross

contamination from one sample to another. Contamination is unlikely given the strict culture protocol observed and the fact that we were unable to infect any cells in tissue again.

We were able to gather control information about the size of NIH 3T3 nuclei. The average nuclear volume was shown to be 65.6 μm^3 with a sample size of 22.

4. DISCUSSION

Expected Results

This experiment was modeled after a similar experiment that was carried out in two dimensions. According to their calculations on two dimensional cell cultures, they saw an increase in nuclear volume of 57 μm^3 h^{-1} which is roughly what we expected to see in three dimensions. We were looking to confirm their results in three dimensions. Unfortunately, our three dimensional viral infection protocol never yielded worthwhile results.

Two Dimensional Infection

When we infected the two dimensional cell culture we were able to qualitatively identify the process of viral infection. The two dimensional study was used as a means to ensure that our virus was infecting properly. By confirming that the virus was causing an infection, we were able to look at other problems with the system to try to figure out how to make the protocol more successful.

Three Dimensional Infection

Control data was collected on the NIH 3T3 cells that were tagged with GFP proteins. According to the study that we were paralleling, the non-infected cells should have had a nuclear volume of something in the neighborhood of 683 μm^3. Accordingly, that means that our data was off by an order of magnitude. This kind of error is extremely significant and effort should be made in the future to discover why such a large margin

of error was found. If it was a data processing error, the data should be re-examined and re-analyzed. The error could also have been some sort of problem with the cells. In the future, fresh cells should be used from a completely different lineage to eliminate them as the source of the problem.

Beyond control data, little data was collected on 3D tissues due to numerous problems that are discussed further below.

Problems

There were several problems that were encountered along the way that contributed to being unable to collect three dimensional data. At the beginning of the procedure we needed to verify that the virus sample that we were given would infect the cells that we were given. To do this, we attempted to replicate the process of a 2D viral infection on a cover slip bottom Petri dish. The first time this was done, we used the wrong cells. It is important that this procedure is carried out with NIH 3T3 cells because they carry half the genome required for the virus to infect cells and reproduce. Once this problem was identified and rectified, we discovered that it was also necessary to use a much lower serum concentration than is used to culture the cells normally. If a high serum concentration is used, all of the virus is neutralized and can not infect the cells.

Just as the infection protocol was being established on the 2D cell culture, we began to run low on our viral stocks. This brought up the new issue of identifying a method of

building up the viral stocks. Once a protocol was obtained from Scotland for replenishing the viral stocks, there was a moderate degree of difficulty getting the cells to build up the virus and demonstrate proper infection using the new virus we had made. One of the biggest issues we ran into when we tried to build up the viral stocks was that we did not know what the initial viral concentration was. This created a major problem since the amount of PFU used to build up the stocks had to be kept in ratio to the number of cells in the culture. Estimates were made and after some trial and error we were able to carry out the viral replication procedure and demonstrate infection in 2D culture.

After the viral stock were built up and the 2D infection protocol was established, we had little time left in the semester to establish an effective protocol in 3D. The one trial we were able to run seemed to be running well until we compared our infection results to our control results. The control fluoresced as much as the infected samples meaning that we could not attribute the fluorescence in the experimental samples to the virus. This unexpected cell behavior could be the result of a number of causes. We suspect it was because of a mislabeled or mishandled cell lineage from before we obtained the cells. Whatever the reason, in the future, a fresh cell line should be purchased instead of using frozen cells with long lineages and high passage numbers.

5. CONCLUSION

Despite the effort put into this project, the results were disheartening as we were neither able to confirm nor repute the observed behavior shown by Monier[2] in the 2D study. Given more time, I am confident that we would have been able to observe viral infection in three dimensions. Nevertheless, we were able to collect control data which can be used to build off of for future studies.

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