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Development of a Co-culture System to Mimic the Transfection of HSV-1 from Keratinocytes to Neuronal Cells

A thesis submitted in partial fulfillment

for the requirements for the degree of

Master of Science.

By

David A. Dixon

B.S. University of Dayton, 2011

2014

Wright State University

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WRIGHT STATE UNIVERSITY
Graduate School

May 1, 2014

I HEREBY RECOMMEND THAT THE THESIS PREPARED UNDER MY SUPERVISION BY David Allen Dixon ENTITLED Development of a Co-culture System to Mimic the Transfection of HSV-1 from Keratinocytes to Neuronal Cells.BE ACCEPTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE OF Master of Science.

Nancy J. Bigley, Ph.D.
Thesis Director

Barbara E. Hull, Ph.D.
Director of Microbiology and Immunology Program,
College of Science and Mathematics

Committee on Final Examination

Nancy J. Bigley, Ph.D.
Professor of Microbiology and Immunology

Barbara E. Hull, Ph.D.
Professor of Biological Sciences

Francisco J. Alvarez-Leefmans, M.D., Ph.D.
Professor of Pharmacology and Toxicology

Robert Fyffe, Ph.D.
Vice President for Research and Dean of the Graduate School

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Abstract

Dixon, David Allen. M.S., Microbiology and Immunology Graduate Program, Wright State University, 2014. Development of a Co-culture System to Mimic the Transfection of HSV-1 from Keratinocytes to Neuronal Cells

To more closely mimic the in vivo progression of HSV-1 a novel in vitro method was created. In this study HEL-30 keratinocytes were infected with a 0.1 MOI of HSV-1 and plated unto a membrane with 8µm diameter pores. The membrane insert was then placed into culture wells that contained Neuro-2a cells (N2A). The neurites from the neuronal cells made cell-to-cell contact with the infected HEL-30 cells and the virus was transmitted into the neuronal cells. To determine if infection occurred in the N2A cells the cells were lysed and the lysate incubated with Vero cells to titrate virus plaque forming units. After incubation a virus plaque assay was performed and the results compared with those of uninfected control cells. Methylcellulose was used to protect against the HEL-30 cells lysing and releasing free virus particles into the medium and infecting the N2A cells without cell-to-cell contact. The methylcellulose is viscous enough to stop free virus particles from passage through the pore membrane in the chamber infecting the N2A cells. Therefore, the only way that the N2A cells were infected was if the neurites made direct contact with the infected keratinocytes, which would mimic the “real world” progression of the virus from primary site of infection to neuronal tissue infection. The use of methylcellulose in the co-culture system exerted a profound inhibitory effect on free-floating virus particles. Immunofluorescence

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microscopy was used to confirm cell-to-cell contact between the N2A and HEL-30 cells and to determine morphological differences that occur when the co-culture system is used versus monoculture. This model provides a new system for studying host/virus interactions.

The hypothesis of this study was that a method could be created that mimics the in-vivo transfection of HSV-1 from keratinocytes to neuronal cells using a co-culture system. The neuronal cells would extend the neurite processes and make a connection to the keratinocyte. Then the virus would be transferred into the neuronal cell without diffusing freely into the media environment.

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LIST OF ABBREVIATIONS

HSV-1= Herpes Simplex Virus-1

TAP 1= cellular transporter protein

TAP 2= cellular transporter protein

MHC= major histocompatibility complex

LAT= latency associated transcript

U_S3= HSV-1 antiapoptotic viral protein

gJ= HSV-1 antiapoptotic viral protein

gD= HSV-1 antiapoptotic viral protein

eIF-2α= eukaryotic infection factor 2 α

$\gamma_1 34 \cdot 5$= HSV-1 viral protein

gC= HSV-1 glycoprotein

gB= HSV-1 glycoprotein

mRNA= messenger RNA

N2A= Neuronal 2-a

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NGF= nerve growth factor

HEL-30= keratinocytes

DMEM= Dulbecco's Modified Eagle's Medium

PBS= phosphate buffered saline

M.O.I= multiplicity of infection

FCS= fetal calf serum

IGF-II= insulin growth factor II

IGFBP2= insulin growth factor binding protein 2

IGFPB4= insulin growth factor binding protein 4

TGF-β= transforming growth factor beta

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INTRODUCTION

An in-vitro system mimicking the in-vivo transfection of HSV-1 between keratinocytes and neuronal cells has not yet been well established. A model is needed that keeps the cell populations separate, but allows cell-to-cell contact so the virus can move directly from the keratinocytes to the neuronal cells. This would mimic the "real world" progression of the virus in natural infection. The virus first enters epithelial cells via a break in the skin or through the mucosal membrane, such as the mouth or eye. The virus is then transported via retrograde flow along the axons into the nuclei of neuronal cells where it remains latent (Whitley and Roizman 2001). Cell-to-cell contact is a crucial step for this process to occur (Fig. 1, Fig. 2). In this study, a co-culture system was developed to permit the transmission of HSV-1 from infected keratinocytes directly to neuronal cells. This system involved the passage of virus to neuronal cell neurites protruding through the 8μm pores of a membrane.

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Literature Review

Herpes Simplex Virus 1 and Infection

Herpes simplex virus 1 (HSV-1) is a member of the herpesviridae family. HSV-1 is associated with encephalitis and facial manifestations, such as scaring of the cornea and blindness as well as sores on the lip. In order for initial infection to take place HSV-1 must contact mucosal surfaces or abraded skin to initiate infection. The incubation period for HSV-1 ranges from 2-12 days with an average of 4. The viron is made up of four parts: the electrodense core, which contains the large, double-stranded DNA molecule, a capsid, a layer of proteins that surround the capsid, and the envelope (Roizman et al. 2007; Whitley and Roizman 2001). After infection and the incorporation of the viral genome, the genome transcribes infected cell proteins 0, 4, and 22. These proteins alter the environment inside the cell to allow efficient expression of the viral genes (Roizman et al. 2007).

HSV-1 also has the ability to evade host immune responses. One way in which the virus does this is by encoding a special protein that binds to the transporter proteins transporter associated with antigen processing 1 and 2 (TAP 1 and TAP 2). This binding inhibits the ability of the transporter protein to bind to other viral particles and transporting then into the endoplasmic reticulum. This sequence of events blocks the normal presentation by major histocompatibility protein (MHC)-class 1 to the host immune system (Ward and Roizman 1994). Another host immune response is apoptosis in response to disturbance of cellular machinery by viral proteins. HSV-1 has the ability to block apoptosis. Although it is not fully understood, at least three proteins (U_S3, gJ,

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and gD) are thought to block apoptosis (Ward and Roizman 1994). Finally, HSV-1 can inhibit the function of eukaryotic infection factor 2 α (eIF-2α). In uninfected cells this protein is activated by phosphorylation and once activated it can block all protein synthesis. HSV-1 is able to stop this process by transcribing the HSV-1 protein $\gamma_1 34 \cdot 5$. This protein binds protein phosphatase 1 and directs it to dephosphorylate eIF-2α. This dephosphorylation inactivates the protein thereby allowing the viral genome to be transcribed. Also, cells cannot respond to infection because HSV-1 destroys cellular messenger RNA (mRNA), inhibits DNA transcription, and blocks splicing of mRNA (Ward and Roizman 1994; Whitley and Roizman 2001).

Since HSV-1 becomes latent in and resides in the central nervous system many other manifestations of the disease can result. Viral infection can cause meningitis, myelitis, and radiculitis, among other diseases. HSV-1 has also been isolated from the respiratory tract of adults with adult respiratory distress syndrome and acute onset bronchospasm. Lastly, HSV encephalitis is thought to be the most common cause of sporadic fatal encephalitis (Whitley and Roizman 2001).

HSV-1 infection can be further divided into the initial infection of keratinocytes and latency in the neuronal cells. The mechanism by which the virus enters the cell also depends on the cell type (Nicola et al. 2005).

Keratinocytes and Infection

HSV-1 first infects keratinocytes in the mucous membranes. The virus must first attach and fuse with the plasma membrane of the keratinocytes and this happens in sequential steps. The first step is the binding of the viral glycoproteins gC and gB to

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heparan sulfate molecules on the surface of the keratinocyte (Herold et al.1991; Johnson et al. 1990). The viral particle then fuses with the cellular membrane using the viral glycoprotein gD and the cellular receptor (Johnson et al.1990; Ligas and Johnson 1988). It enters the cell through a variety of mechanisms. Nicola et al. have shown that the virus uses a pH-dependent, endocytic pathway to enter the keratinocytes. Endocytosis, which is sensitive to genistein and wortmannin may be required to bypass the dense layer of actin under either the apical or basolateral plasma membrane of epithelial cells (Nicola et al. 2005).

The basal layer of the epidermis is richly innervated with unmyelinated sensory neurites that form free terminals within the epidermis (Rice et al. 1998; Hilliges et al. 1995; Ulmann et al. 2007). Here the neurites make cell-to-cell contact with the keratinocytes and the virus transfection to the neuronal cells takes place.

Neuronal Cells and Infection

After the primary infection of the keratinocytes, the virus migrates through the axon terminal, which innervates the superficial dermis, into the sensory neuronal cells of the trigeminal ganglia where it efficiently infects them and causes a lifelong infection. In contrast to how the virus first enters keratinocytes, HSV-1 enters the neuronal cells via a pH-independent pathway that is not affected by kinase inhibitors (Nicola et al. 2005). Viral multiplication occurs only in a small percentage of neuronal cells and the viral genome remains latent for the life of the host (Whitley and Roizman 2001). In the neuronal cells the lytic genes of the virus can be turned off and the latent genes can become transcribed (Goins et al. 1999; Stevens 1989). Once latent, the viral genome stays

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in the nucleus as an episome coiled around the nucleosomes (Deshmane and Fraser 1989, Efstathiou et al. 1986; Mellerick and Frase 1987; Rock and Fraser 1985). The promoter of the viral genome stays active during latency and causes latency-associated transcripts (LATs) to become transcribed (Coen et al. 1987; Spivack and Fraser 1987; Stevens et al. 1987). These transcripts cause the lytic viral genome to remain latent. The lytic viral genome can also be re-activated due to stressors such as physical or emotional stress, UV light, fever, or tissue damage (Whitley and Roizman 2001). Once re-activated the virus can produce the previously described symptoms. The virus is transported back to the mucosal keratinocytes via the neurites resulting in recurrent infection.

Co-culture Stimulation via Nerve Growth Factor (NGF)

Upon stimulation, the keratinocyte HEL-30 cell line secrete a wide variety of cytokines. One of these cytokines is nerve growth factor (NGF) (Feliciani et al. 1996). NGF is a neurotropic factor that promotes the proliferation and differentiation of neurons. It also enhances the ability of neuronal cells to produce neurites. Neuronal cells that have been exposed to NGF can also withstand various treatments that would normally cause the cell to go through apoptosis and even provide protection against signs of Alzheimer's disease (Goins et al. 1999; Hefti et al. 1989; Hefti et al. 1986; Korsching et al. 1986; Levi-Montalcini et al. 1975; Levi-Montalcini et al. 1968; Thoenen and Barde 1980).

The in vivo administration of NGF is not very effective. The cytokine's effectiveness is limited by its short half-life and adverse side effects when delivered systemically (Butcher and Wolfe 1989; Scott and Crutcher 1994; Taglialatela et al. 1997). Since stimulated keratinocytes release NGF and therefore can cause neuronal cell

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protection, proliferation, and differentiation without the adverse side-effects associated with systemic delivery, an HSV-1 vector might promote NGF release and protect neuronal cells from various diseases and treatments. This idea has been explored, and shown to work, by inserting an NGF transgene into the genome of HSV-1 while deleting the cytotoxic genes (Goins et al. 1999).

Growth Cone of Neuronal Cells

Neuronal cells produce growth cones and filopodia to direct the outgrowth of neurites in order to seek cellular connections and nutrients. The filopodia detect the surrounding environment and act as sensory extensions of the neurite (Rehder and Kater 1992) (Fig. 3). Filopodia and growth cones are important for this study because after HEL-30 cells become infected they release NGF. The filopodia detect the NGF and direct the growth cone, and by extension the neurite, of the neuronal to the source of the NGF, which is the HEL-30 cell. The growth cone will grow toward the higher concentrations of NGF, which will direct the neurite to the HEL-30 cell, when it establishes a cell-to-cell contact. This in turn will allow virus transfection to take place and the neuronal cell will become infected with HSV-1.

Methylcellulose Properties

To better mimic the in vivo properties of a body, such as the basement membrane, a physical barrier would need to be used that restricts the movement of free virus particles. The substance that is used in this novel method is methylcellulose. Using methylcellulose to conduct a plaque assay is one of the established methods for plaque assays. Methylcellulose inhibits the spread of free-floating virus particles while

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preserving cellular function (Stewart el al. 2001). Since the methylcellulose is made containing the same medium that the cells are cultured in, the cells are able to proliferate and differentiate as normal. The methylcellulose also does not affect adhesion properties of the cells. The only way for virus to spread to other cells is if the plasma membrane of the infected cell is touching the plasma membrane of a non-infected cell. Since the two cell populations are kept separate in this experiment, the only way that the N2A cells can become infected is for the N2A cell's neurites to make contact with the infected HEL-30 cells.

Fetal Calf Serum

Like methylcellulose, fetal calf serum (FCS) is a crucial compound in the proposed method. FCS contains a variety of growth promoting compounds that are required for neuronal and keratinocyte cell growth. The main component of FCS that allows for neuronal cell differentiation and proliferation is albumin (Zheng et al. 2006). Also, a unique component of FCS that other common sera do not have, and which N2A cells require is insulin growth factor-II (IGF-II). This potent mitogen includes the highest number of unique peptides from all of the analyzed growth factors of FCS (Zheng et al. 2006). IGF-II also has binding proteins associated with it that affect cell growth. The main binding proteins are insulin growth factor binding protein 2 and 4 (IGFBP2 and IGFBP4). These proteins increase the half-life of IGF-II, which increases the proliferation effects of IGF-II, and IGFBP2 stimulates cell spreading and proliferation (Zheng et al. 2006).

Several other growth factors are found in FCS that are needed for cell growth. One

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of these factors is transforming growth factor beta (TGF-β). TGF-β is a crucial peptide whose functions include proliferation and differentiation. It also regulates the action of other growth factors by determining either a positive or negative effect of the growth factors (Zheng et al. 2006; Nowak et al. 1999). FCS also contains is glial growth factor (GGF). GGF is one of the isoforms of neuregulin 1 that induce the growth and differentiation of epithelial and neuronal cells through interaction with transmembrane receptors of tyrosine kinase (Zheng et al. 2006; Burden and Yarden 1997). These growth factors are not all inclusive, but they are the main ones that neuronal cells and keratinocytes need that separate FCS from other common cell culture sera (Zheng et al. 2006.

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Materials and Methods

Cell Lines

The Neuronal 2-a (N2A) (ATCC; CCL- 131) cell line was obtained from murine neuroblastoma cells and is adherent. The average length of the cell ranges from 25µm when the cells are first plated and dividing, to 100 µm when the cell is differentiating and producing neurites (Letourneau et al. 1986). N2A cells were cultured in 60mm x 20mm cell culture treated petri dishes (Corning) and incubated in a humidified atmosphere with 5% CO_2 at 37° C. Cells were cultured and maintained with Dulbecco's Modified Eagle's Medium (DMEM) (HyClone) supplemented with 10% or 5% fetal calf serum (FCS) (Fisher brand) and 1% penicillin-streptomycin (10000U/mL Penicillin and 10000U/mL Streptomycin). The HEL-30 cell line was derived from murine epidermal cells of C3H mice. It was also cultured in 60mm x 20mm cell culture treated petri dishes and incubated in a humidified atmosphere with 5% CO_2 at 37° C. Cells were cultured and maintained with Dulbecco's Modified Eagle's Medium (DMEM) (HyClone) supplemented with 10% fetal calf serum (FCS) (Fisher brand) and 1% penicillin-streptomycin (10000U/mL Penicillin and 10000U/mL Streptomycin). The Vero cell line (ATCC CCL-81) was derived from the kidney cells of the grivet monkey and is used in this study for plaque assays. It was also cultured in 60mm x 20mm cell culture treated petri dishes and incubated in a humidified atmosphere with 5% CO_2 at 37° C. Cells were cultured and maintained with Dulbecco's Modified Eagle's Medium (DMEM) (HyClone) supplemented with 10% or 5% fetal calf serum (FCS) (Fisher brand) and 1% penicillin-streptomycin (10000U/mL Penicillin and 10000U/mL Streptomycin). Cells were sub-cultured every two days at a split ratio of 1:6 throughout the course of this study. To

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release the adherent cells from the plate the cells were first washed with 1X PBS and then the plates were incubated with 1mL of a 0.25% Trypsin-EDTA solution (Gibco-Life Technologies) for 5 minutes. The trypsin was then neutralized with 1 mL of medium and the cells were spun down and re-plated.

Virus Culture and Quantification

Stock sample of Herpes Simplex Virus-1 strain Syn 17+ initially obtained through Dr. Nancy Sawtell, Children's Hospital Medical Center Cincinnati, OH. The virus was propagated on 100% confluent monolayers of Vero cells. When cytopathic effect, damage to the cells during viral invasion (plaques), became evident, around day 5 post infection, the cells were spun down and the supernatant was aliquoted into 1mL micro centrifuge tubes and stored at -80°C. A dilution of 0.1 multiplicity of infection (MOI) was calculated by infecting Vero cell monolayers with different dilutions of virus and counting the plaques. The dilution that resulted in a MOI of 0.1 was used for this study.

Cell Viability

After co-culturing for 4 days, leading to around 80% confluency, with or without virus, the N2A cells were detached from the plate using trypsin. The cells were then stained with Trypan Blue and placed upon a hemocytometer.

Immunofluorescent Staining

Representative fields were selected by choosing a field that had on average the same number of cells in it for each image. N2A and HEL-30 cells were co-cultured to approximately 70% confluency. The medium was then removed and the cells were

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stained. A neurite outgrowth staining kit was used to prepare the stains (Molecular Probes-Life Technologies). The 1X working stain solution was prepared by placing 10µL of the cell viability indicator and cell membrane stain into 10mL of 1X phosphate buffered saline (PBS). An application volume of 1.5mL of staining solution per well was then used. The cells were then incubated for 20 minutes at 37°C. After 20 minutes, the solution was aspirated and the cells were washed with 1X PBS. Then 1.5mL of a background suppression dye was applied. The dye was diluted100-fold by adding 100µL of the dye to 10mL of 1X PBS. Once the dye was applied, the cells were visualized on an inverted fluorescent microscope. In order to detect the cell viability stain, an excitation of 495nm was used to produce a green fluorescence. The viability indicator dye is converted by the intracellular esterase activity of living cells to emit green fluorescence primarily in the neural cell bodies. To read the cell membrane stain a 555nm wavelength was used to produce a red fluorescence. Since the thickness of the culture dish is thicker than the ideal glass coverslip, this resulted in some degradation of the resulting images taken on the inverted fluorescence microscope to not be at optimal sharpness. The company does not reference the exact components of the cell viability and neurite outgrowth stains.

Plaque Assay

After infection, the inserts containing the HEL-30 cells were removed and discarded. The medium left in the culture plates was aspirated. The N2A cells were then washed in 1x PBS and removed following the method previously described. After the cells were spun down at 1500rpm for 5 minutes the supernatant was aspirated and the cell pellet was re-suspended. The cell-containing solution was then placed into a -80°C. The cells were put through a freeze-thaw cycle 4 times in order to lyse the cells and free the

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virus. After the fourth cycle, the cells were spun down. The resulting supernatant was then used to infect Vero cells grown to 100% confluency for 2 hours. After 2 hours of absorption, the medium was aspirated and 2 ml of Opti-MEM GlutaMAX (Life Technologies Inc.) containing 2% Methylcellulose (Acros Organics) and 0.5% fetal bovine serum was laid over the Vero cells. The cultures were incubated at 37 °C until plaques began to form, usually around day 5 or 6, and then counted for plaque formation after fixation with 4% paraformaldehyde and staining with 1% crystal violet solution (Sigma).

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Results

Percent Serum Affects Neurite Growth

In determining the best concentration of FCS to use in culture medium to culture the N2A cells, the standard 10% concentration that other studies have used and a 5% concentration were used. When the N2A cells were cultured alone in the 10% FCS medium they produced small, multidirectional neurite processes. When the cells were grown in a medium containing 5% FCS the cells produced larger and fewer neurites with an average length of 75-100 µm (Fig. 4). The ends of the neurites also showed signs of growth cones. The cells grown in the 5% FCS media also produced growth cones. In vivo the cells produce growth cones. So, these cells more closely resemble cells in the human body versus cells grown in the 10% FCS media. Both serum concentrations resulted in N2A cell growth and differentiation although the cells grown in the 10% concentration came to 70% confluency 1-2 days faster than the cells grown in the 5% concentration. Since the neuronal cells grown in the 5 % FCS medium grew longer neurites than the cells grown in the 10% FCS media, in the co-culture experiments the cells were only grown in the 5% FCS media.

Protocol Set-up

The purpose of the study was to be able to keep the two cell populations separate so that each population could be studied and imaged individually. This is why a porous membrane that fits into the culture dish was used (Fig. 5). With the pore diameter being 8µm and the average width of a neurite being 1.1µm there is adequate space for the neurite to grow through the pore without being impeded (Letourneau et al. 1986). N2A

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cells were first seeded onto the bottom of a 6-well culture plate and grown to a confluency of 20% in medium containing either 5 or 10% FCS. After the HEL-30 cells came to the desired level of confluency they were plated on top of the porous membrane insert and placed into each well. This membrane kept the two populations separate, while the pores allowed the N2A neurites to make cell-to-cell contact with the HEL-30 cells. To determine if the two cell populations made cell-to-cell contact and allow the transfection of virus, after the medium had been aspirated a layer of methylcellulose was placed on top of the N2A cells at 40% confluency. The membrane was placed on top of the methylcellulose-coated N2A cells. The HEL-30 cells, which were previously infected with a 0.1 MOI of HSV-1 in a different dish, were then plated on top of the membrane along with 10% FCS medium. Thus, the methylcellulose and the porous membrane separated the two cell populations. Methylcellulose was used to block diffusion of free virus particles to the neuronal cells. The only way that the virus could infect the neuronal cells is for the virus to exit the keratinocytes through exocytosis and then taken into the neuronal cells through endocytosis. The pores would also allow cytokines produced by the two cell populations to flow back and forth throughout the medium.

Co-culture Morphological Changes

When the N2A cells were co-cultured with the 0.1 MOI HSV-1 infected HEL-30 cells, the neuronal cells underwent morphological changes (Fig. 6). The neuronal cells produced fewer and longer neurites. The neuronal cells produce nodules on their neurites. These nodules are presumably neurite processes coming from the main neurite and protruding through the pores in the membrane above.

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Upon co-culture with 0.1 MOI HSV-1 infected HEL-30, the morphology of the N2A cells changes when compared to control N2A cells. The N2A cells co-cultured with the infected HEL-30 cells become rounded and the neurites become rounded as well. The N2A cells also produce more neurites than the control cells (Fig. 7). The N2A cells co-cultured with the infected HEL-30 cells differentiated at a faster rate as well and reach 70% confluency 2 days before the control cells did.

Plaque Assay

To determine if this co-culture protocol allowed for the transfection of HSV-1 from infected keratinocytes to neuronal cells without the diffusion of free virus particles into the media various plaque assays were performed. N2A cells grown in 5% FCS medium alone were used as a negative control, N2A cells grown in 5% FCS medium infected with 0.1 MOI HSV-1 were used as a negative control. Then the N2A cells were co-cultured with the HEL-30 cells as previously described. The HEL-30 cells were infected with 0.1 MOI of virus. Two set-ups were used to determine the efficacy of the protocol with methylcellulose. In the first set-up the medium that the N2A cells were growing in was replaced with methylcellulose once the porous insert containing the infected HEL-30 cells was placed into the culture dish. In the other group only medium was used and not methylcellulose. When the plaque assay was conducted using the lysate from the group in which methylcellulose was used and compared to the group in which no methylcellulose was used, the N2A cells that were grown with methylcellulose had fewer plaques comparable to the initial infection of 0.1 MOI (Fig. 8). The group that was cultured with methylcellulose contained plaques equivalent to the initial infection rate of

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0.1 MOI, while the group that was not cultured with methylcellulose had plaques too numerous to count.

Immunofluorescence

Immunofluorescence imaging was used to show cell-to-cell contact points and cell viability (Fig. 9). All cell populations were plated at the same density and a striking difference can be seen between the N2A cells grown alone or co-cultured with HEL-30 cells (Fig. 10). Co-culturing causes the cells to differentiate at a faster rate and produce more neurites (Fig. 9,10). Immunofluorescence microscopy also revealed neurites of the N2A cells growing up through the pores and making cell-to-cell contact with the HEL-30 cells when imaged from the HEL-30 level of the membrane (Fig. 11).

Images from the same area of the culture dish, at the N2A level and then the HEL-30 level (membrane level), were also taken to permit overlaying (Fig. 12). The overlay demonstrates how the neurites growing through the pores block the differentiation of HEL-30 cells in the area that is occupied by the neurites. The neurites then make cell-to-cell contact with the HEL-30 cells and virus transfection can take place along the neurites. The image reveals small dot-like objects, which appear to be neurites sticking up through the pores in close proximity and sometimes next to the cell membrane of the HEL-30 cells.

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Discussion

In this study a novel in vitro protocol to mimic the in vivo transfection of HSV-1 from keratinocytes to neuronal cells was established. First, N2A cells were cultured to 20% confluency. The old medium was aspirated and the cells were washed with PBS. Methylcellulose was then placed into the culture dishes on top of the N2A cells. A porous membrane with 8µm diameter pores was then placed into the culture dishes. HEL-30 cells were infected at a 0.1 MOI of HSV-1 and plated onto each membrane insert with 10% FCS medium. The culture plate was then incubated for 48 hours at 5% CO_2. After 48 hours, the insert and methylcellulose were removed and the N2A cells were taken off of the plate. The resulting pellet was re-suspended in 10% FCS medium and placed into a freezer. The cells were lysed using freeze-thaw cycles. After three cycles, the cells were pelleted again and the lysate used to infect Vero cells for plaque assays.

Throughout the course of this study, the percentage of FCS in the medium affected neurite outgrowth and N2A cell differentiation. A possible explanation of this phenomenon was studied by Zheng et al. 2005, whose work using embryonic stem cells showed that lowering the concentration of FCS in stem cells leads to arrest of the cells in the G0/G1 phase. Increasing the concentration leads to normal cell differentiation throughout all phases of the cell cycle (Zheng et al. 2005). Since the current study used a lower concentration of FCS (5%), the cells were not completely starved of serum, but the lower FCS concentration may have slowed cell differentiation and proliferation. This slower growth might be the reason why we saw the N2A cells grown in the 5% FCS medium produce fewer, yet longer neurite processes. Since the cells were in the G0/G1 phase longer, they could produce longer neurites rather than going through the cell phases

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at the normal rate. Future studies could explore on how to arrest the cell cycle of the N2A cells once the neurites are at a desired length without causing the cells to go through apoptosis. This could be applied by creating a more controllable co-culture protocol by arresting the cell cycle and using only one cell-to-cell contact point between the neuronal cells and keratinocytes.

This proposed novel method for studying the transfection of keratinocytes and neuronal cells with HSV-1 is a suitable in vitro model that can be applied to the in vivo cycle of HSV-1. It has already been shown that the progression of HSV-1 infection in vivo is that first the virus infects the keratinocytes and then proceeds to infect neuronal cells by being taken into the cell through the neurites (Rice et al. 1998; Hilliges et al. 1995; Ulmann et al. 2007). The virus becomes latent in the neuronal cells and can then be reactivated to travel back through the neurites to re-infect the keratinocytes, leading to symptoms. The proposed in vitro method in this paper mimics the in vivo progression of the virus; the keratinocytes are first infected with HSV-1 and plated in a co-culture system so in order for the neuronal cells to become infected a cell-to-cell contact must take place by the neurites making contact with the infected keratinocytes. It is only then that the virus can infect the neuronal cells.

Because I did not have access to a confocal fluorescent microscope, this experimental procedure should be repeated using a fluorescent confocal microscope. It would produce images where you could follow the neurite growth through the pores and making contact with the HEL-30 cells. This current research paper was only able to take images at two levels and then artificially overlay them to try and show the growth of the neurite. Confocal images would more clearly show the growth of the neurite through the

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pores and cell-to-cell contact. The purpose of this experiment would be to better show the contact points between the neurites and the keratinocytes.

A future study could look at NGF more in-depth using multiple methods, since infected keratinocytes produce NGF, which acts as a chemotactic and causes neuronal cells to differentiate. First, the method outlined by this thesis could be used as a set up and then NGF could be blocked by using a blocking antibody. Then one could see if the N2A cells would still differentiate at the same rate and if the neurites would still make contact with the infected HEL-30 cells. Instead of using infected HEL-30 cells, one could place pure NGF at a concentration similar to the concentration produced and released by infected HEL-30 cells in the top chamber along with 5% FCS medium and see if the N2A cells still produce neurites that protrude through the pores. The results obtained by using an electron microscope to verify contact points and viral transfer could be used to determine if the neurites only make cell-to-cell contact by coincidence, or if it truly is a result of the infection causing the HEL-30 cells to release NGF and thereby causing chemotaxis of the neurites to make contact the HEL-30 cell.

It has been shown that nectin-1 (synonymous with herpes virus entry protein C) is a critical entry receptor found on neuronal cells to which HSV-1 attaches and gains entry into the cell (Akhtar, Tiwari, Oh, et al. 2008). Future research could be done to further prove the effectiveness of this novel co-culture method by focusing on the nectin-1 receptor on the neuronal cells. An experiment could be set up using the co-culture method with the addition of a nectcin-1 antibody to block the binding of HSV-1 on nectin-1. This should protect the neuronal cells from infection. Images could be taken to show that the neurites are still making cell-to-cell contact with the infected HEL-30 cells;

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virus transfection should not take place since the antibody is blocking the nectin-1 receptor.

Another possible application of this protocol is to infect the neuronal cells and keep the keratinocytes uninfected. The cells could be cultured in the same positions as described in this study. This application could demonstrate the migration of HSV-1 back to the keratinocytes upon reactivation. Then with these two protocols further experimentation to protect either the neuronal cells or keratinocytes could be undertaken by 1) making the virus stay latent in the neuronal cells by using treatments such as acyclovir, or 2) protecting the neuronal cells from virus transfection. The in vivo applications of these two experiments would be to create a better drug that keeps the virus in latency in the neuronal cells so that it cannot become reactivated. One way to keep the virus in latency and therefore keep the virus from reactivating in neuronal cells is through the use of acyclovir (Sawtell et al. 2001).

Previous studies have only focused on using acyclovir on neuronal cells to keep the virus latent (Smith et al. 2001; Margolis et al. 2007; and Roehm et al. 2011). However, the effects of the drug on other cell populations such as keratinocytes have not been studied. A novel study could use the protocol established in this thesis with infected neuronal cells and acyclovir treatment. The researchers could then determine the different cytokines produced by aspirating the cell culture media and analyzing it. They could then see if the treatment has any effect on the non-infected keratinocytes, such as cytotoxicity by comparing the cell viability of keratinocytes grown in the presence or absence of acyclovir. Another in vivo application would be to develop a treatment that would prevent the keratinocytes from becoming infected in the primary stage of infection such

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as a type of injection that contains a molecule that binds to and blocks the different receptors that HSV-1 binds to.

This established protocol could be used for any future research that requires cell-to-cell contact through processes, while keeping the two cell populations separate. It could also be used to show how cytokines produced by one type of cell can influence the differentiation and proliferation of another type of cell. Future research could focus on the cytokines produced by the infected keratinocytes and how these cytokines affected the neurite and cellular growth that has been seen in this study.

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Summary

The purpose of this study was to develop a novel co-culture protocol to culture neuronal cells and HSV-1 infected keratinocytes while keeping the two populations physically separate. This protocol mimics the in vivo progression of HSV-1 from the primary infection of keratinocytes to transfection of the neuronal cells. The results of this study suggest that this protocol permits studying the transfection of HSV-1 from keratinocytes to neuronal cells. This protocol may also be helpful in studying virus transfection between two other cell types. This protocol is simple to establish and to make morphological observations, and the materials are easily obtainable. When using the membrane inserts in the culture dish with methylcellulose covering the N2A cells, the protocol allows the N2A neurites to make contact with the infected HEL-30 cells and thereby allowing viral transfection into the N2A cells. The methylcellulose inhibits N2A infection by free-floating virus particles that have been released from lysed HEL-30 cells. Therefore, the only way that N2A cells can become infected with HSV-1 is through cell-to-cell contact made by the N2A neurites attaching to the HEL-30 cells.

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Figures

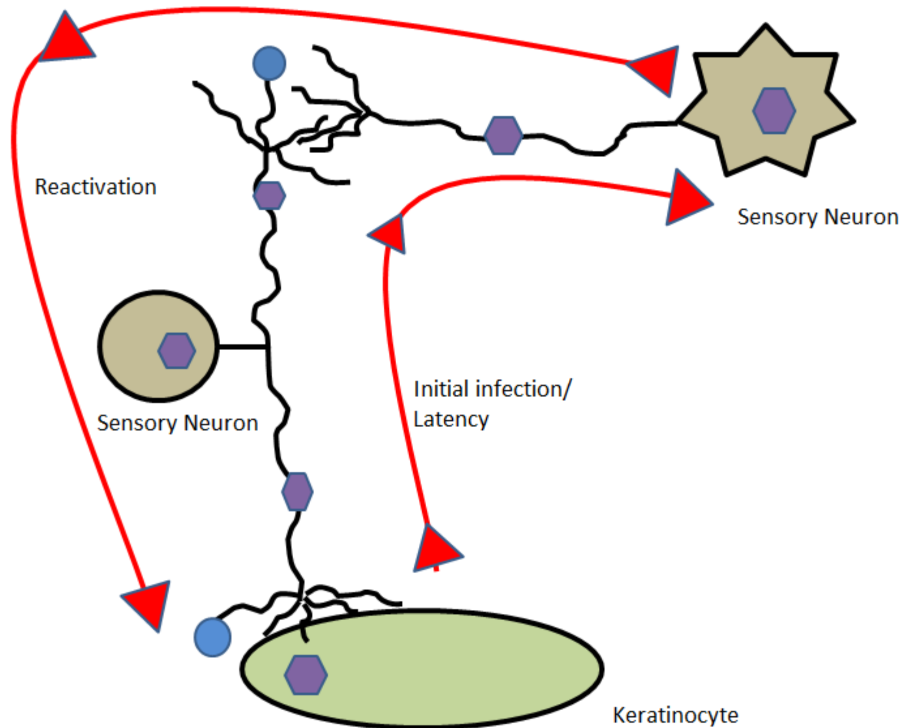

Figure 1. Model of HSV-1 life cycle based upon Lachmann (2003) and redrawn to better show HSV-1 lifecycle. Hexagons represent HSV-1 without the viral envelope in the cell and circles represent HSV-1 with viral envelope when it is transported of the cell via exocytosis before the next cell takes it up via endocytosis. This figure depicts the primary infection of HSV-1 in epithelial/keratinocyte cells leading to latency in neuronal cells. Subsequently, it shows the pathway of HSV-1 upon reactivation in neuronal cells to infection of the epithelial/keratinocyte.

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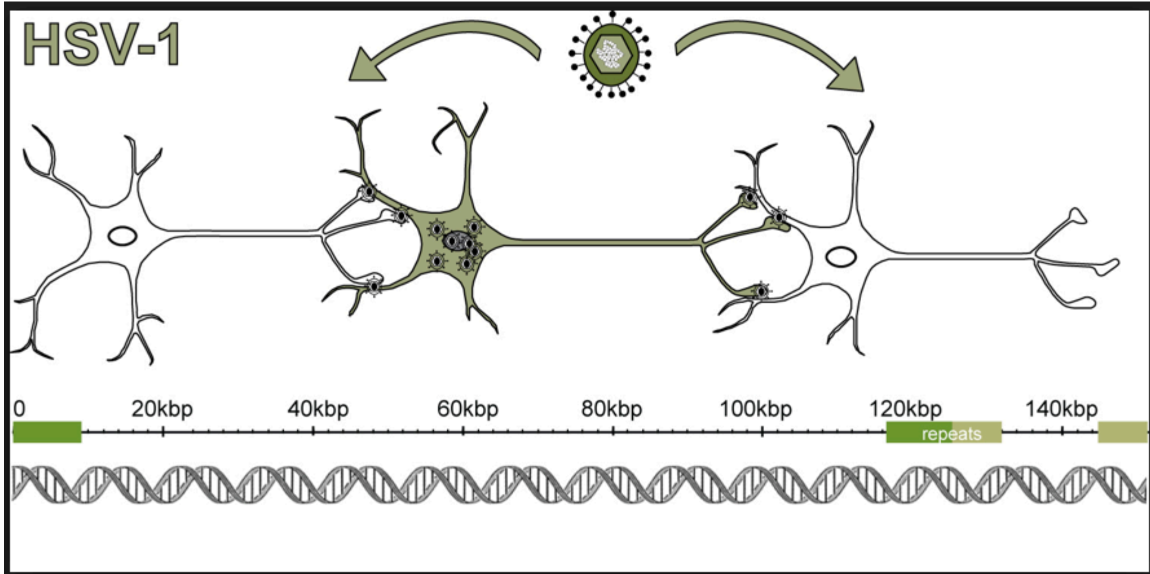

Figure 2 Transmission of HSV-1 along axons in neuronal cells. Image shows the progression of HSV-1 to become latent in neuronal cells or the reactivation of the virus and the migration into keratinocytes. Adapted from Szpara et al. (2010).

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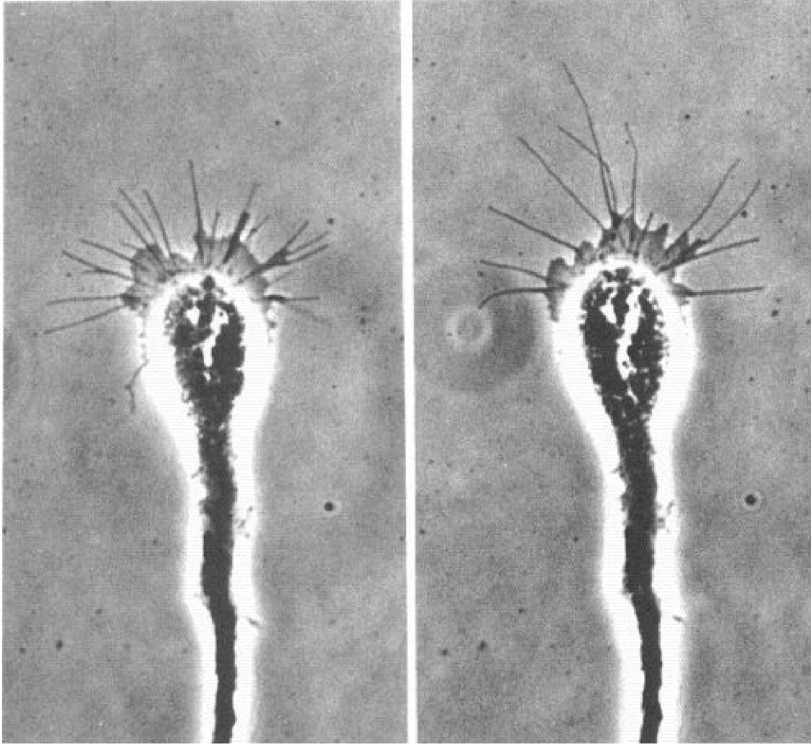

Figure 3 Neuronal growth cone and filopodia. Image of the growth cone on a neuronal cell and the filopodia extending from the growth cone. The filopodia detect the surrounding environment for chemoattractants and nutrients and direct the growth of the growth cone and neurite to the highest concentration of nutrients and chemoattractants. Adapted from Rehder and Kater (1992).

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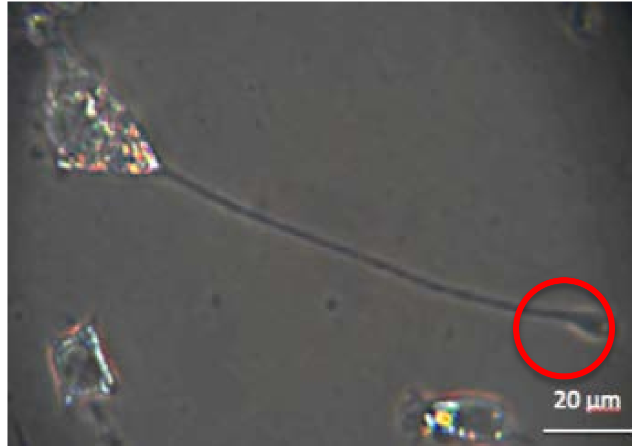

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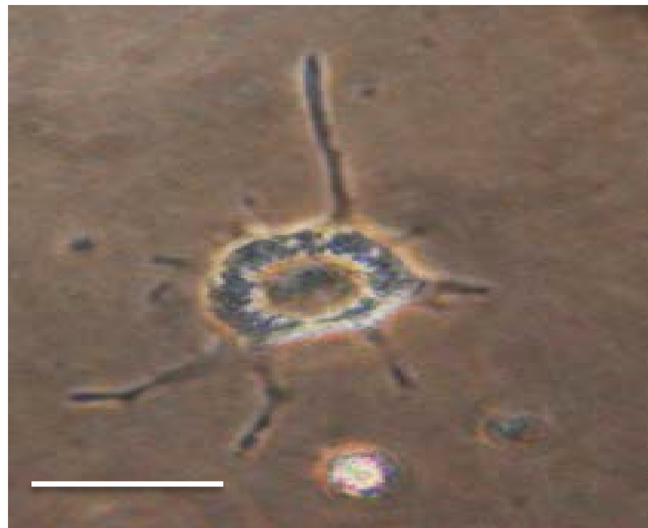

Figure 4. Morphological differences in neuroblastoma cell neurites between using 5% or 10% serum. Image taken at objective magnification 40x. Cells seeded at same time and image taken at day 4 post seeding. A) Using medium that contains 5% FCS causes longer and unidirectional neurite outgrowth. Red circle outlines growth cone. B) Using medim that contains 10% FCS results in smaller multidirectional neurite outgrowth. No growth cones form in the cells grown in the 10% serum. Scale bars represent 20 µm.

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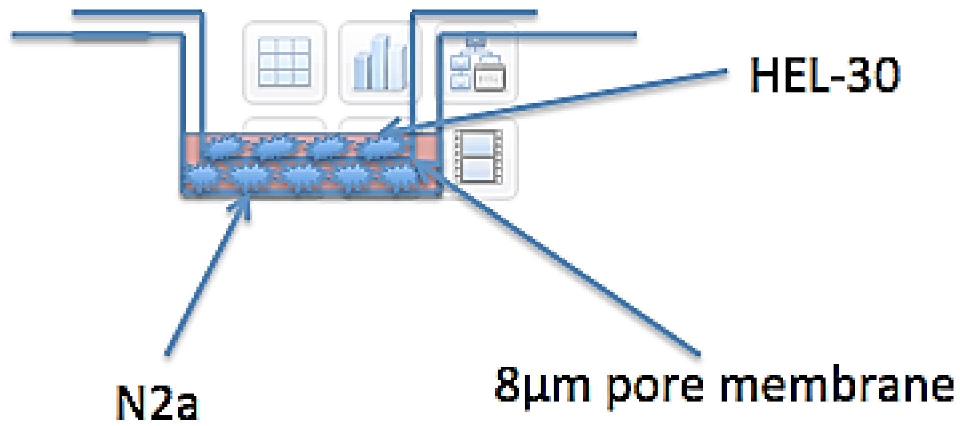

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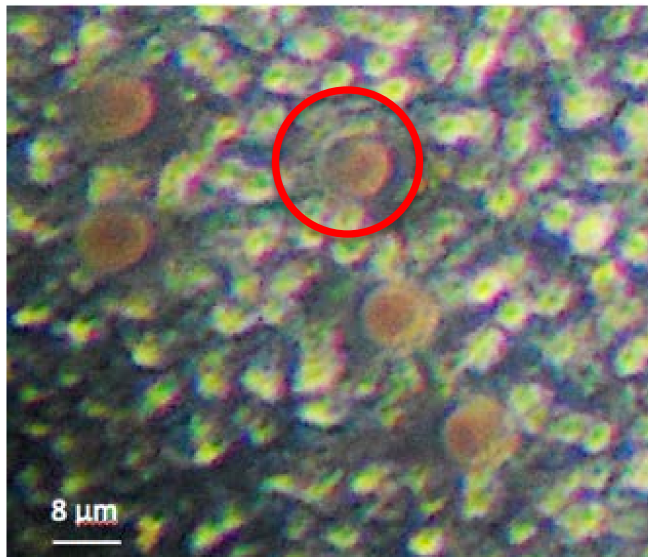

Figure 5. Co-Culture set-up. A) A schematic of how the membrane insert fits inside the culture dish containing the N2A cells. B) Red circle outlines pore in the membrane. All closed in circles are pores.

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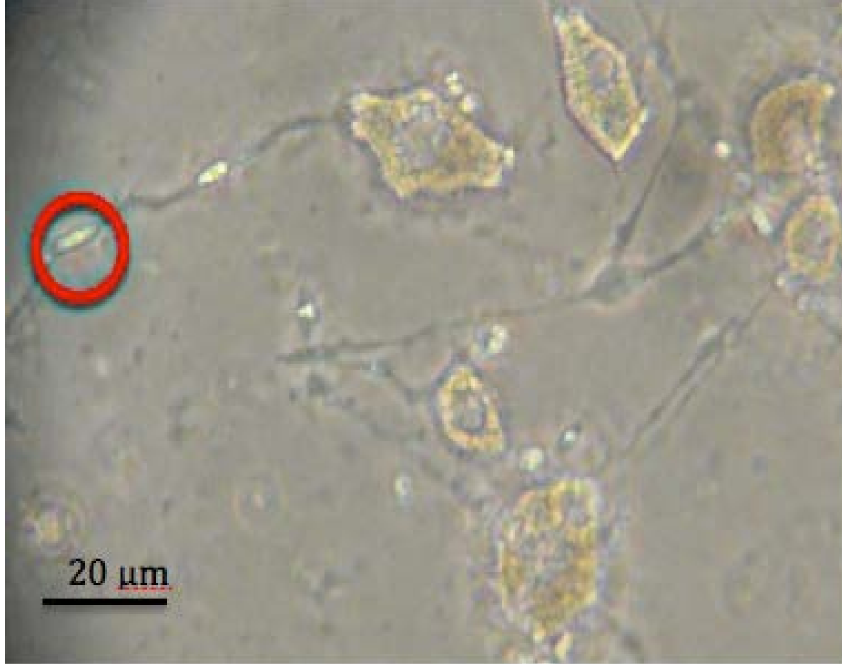

Figure 6. Neurites protruding through the pore membrane. Image taken at day 4 after seeding. Circle outlines a nodule that can be seen in the N2A cells co-cultured with infected HEL-30 cells. The nodules result from the release of NGF by the infected HEL-30 cells. NGF acts as a chemoattractant to the neurites.

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A. B.

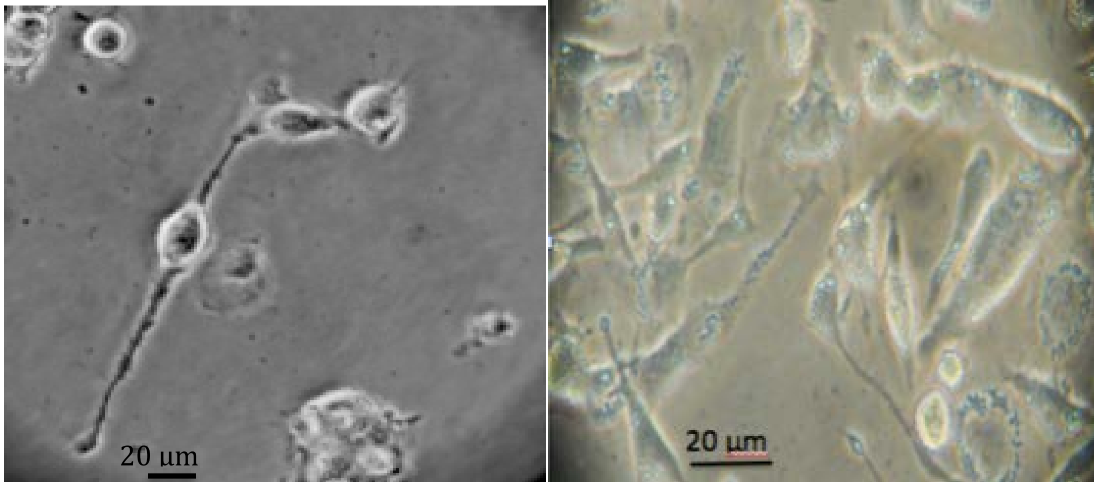

Figure 7. Comparison of morphology of infected and non-infected cells. Morphological changes of infected cells grown in 5% serum 48 hours post infection. A) Image taken at day 4 post seeding of N2A cell grown in 5% FCS without the addition of infected HEL-30 cells. B) Image taken of infected N2A cells. Infected cells become rounded with shorter neurites. Difference in color of the background reflects the color of the media.

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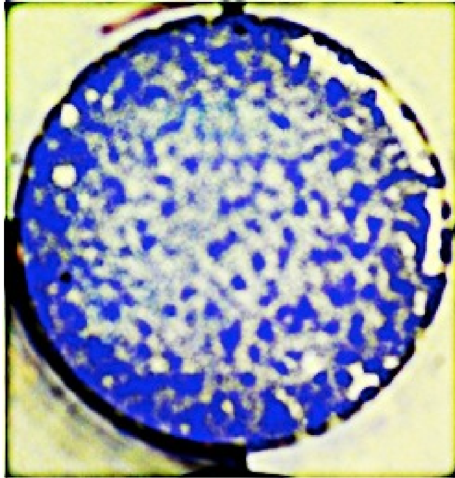
A. B.



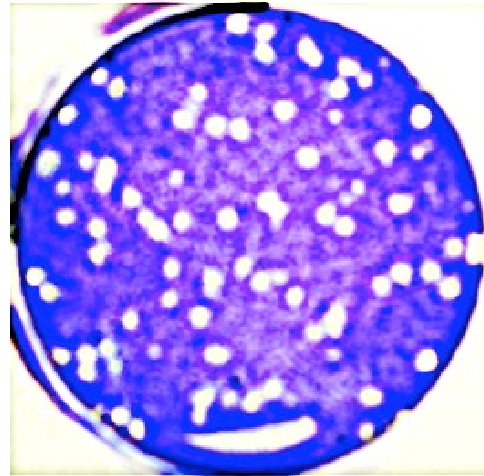


Figure 8. HSV-1 plaque assay. Plaque assay of infected N2A cell lysate grown in 5% serum using the previously described method with or without methylcellulose. A) Co-culture method without the use of methylcellulose. Without methylcellulose in the system free-floating virus particles was able to infect the neuronal cells. B) Co-Culture method with the use of methylcellulose. The use of methylcellulose resulted in fewer virus plaques, indicating a requirement for cell-to-cell contact for virus transfection between the neuronal cells and keratinocytes.

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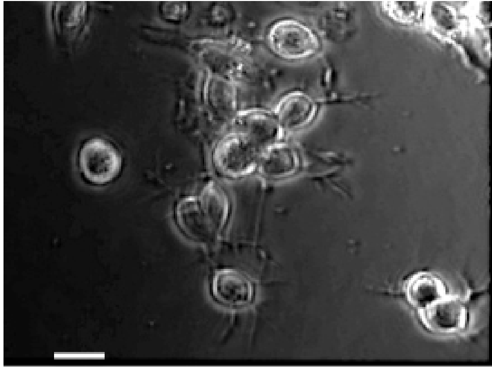
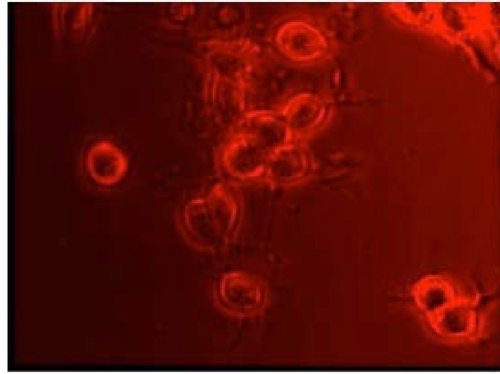
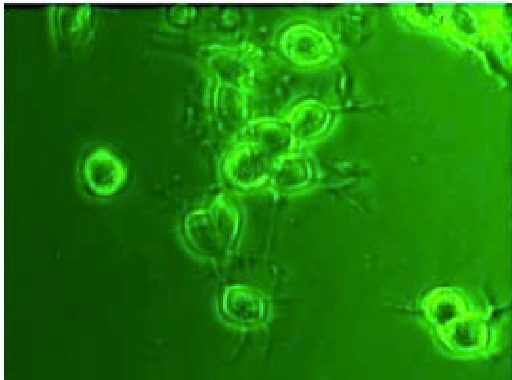
A. 

B. 

C. 

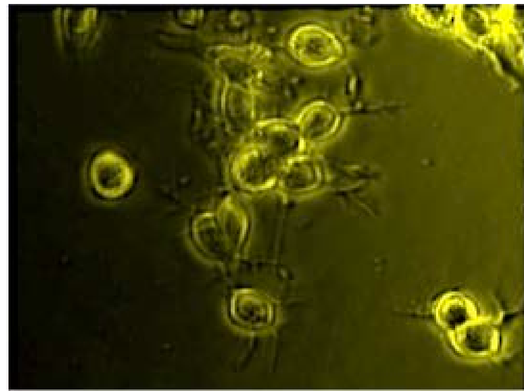
D. 

Figure 9. Immunofluorescence imaging of N2A cells in 5% serum. Image taken at day 4 after seeding. Imaging of N2A cells grown without co-culture of HEL-30 cells. A) Phase contrast image. B) Neurite outgrowth stain. Only neuronal cells are able to take up the stain into the neurites and fluoresce red. C) Viability stain. Only live cells are able to take up the stain and thereby fluoresce green. D) Merge image of the viability stain and neurite stain. The reason why all the cells stain green and therefore are viable is because the cells are first washed before the stain in applied and the washing would remove any dead cells since dead cells become non-adherent. Scale bar represents 20 µm.

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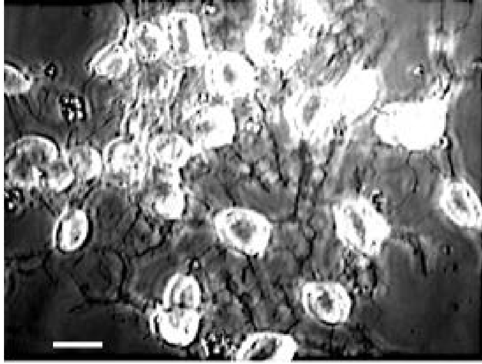

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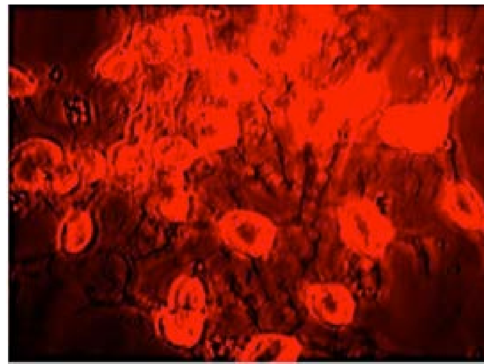

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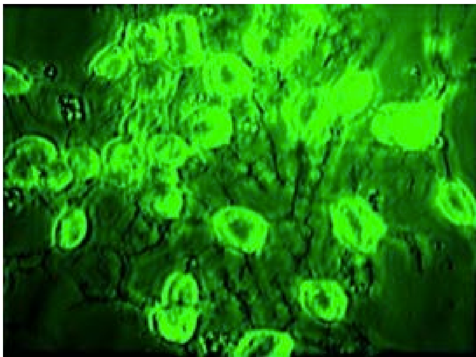

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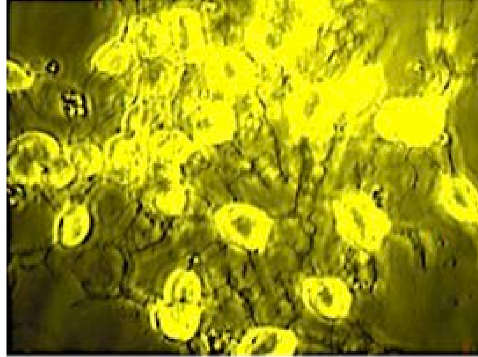

Figure 10. Imaging of N2A cells grown in 5% serum with the co-culture of HEL-30 cells. Image taken at day 4 after seeding at objective magnification 40x. A) Phase contrast image. B) Neurite outgrowth stain. Only neuronal cells are able to take up the stain into the neurites and fluoresce red. C) Viability stain. Only live cells are able to take up the stain and thereby fluoresce green. D) Merge image of the viability stain and neurite stain. Scale bar represents 20 μm.

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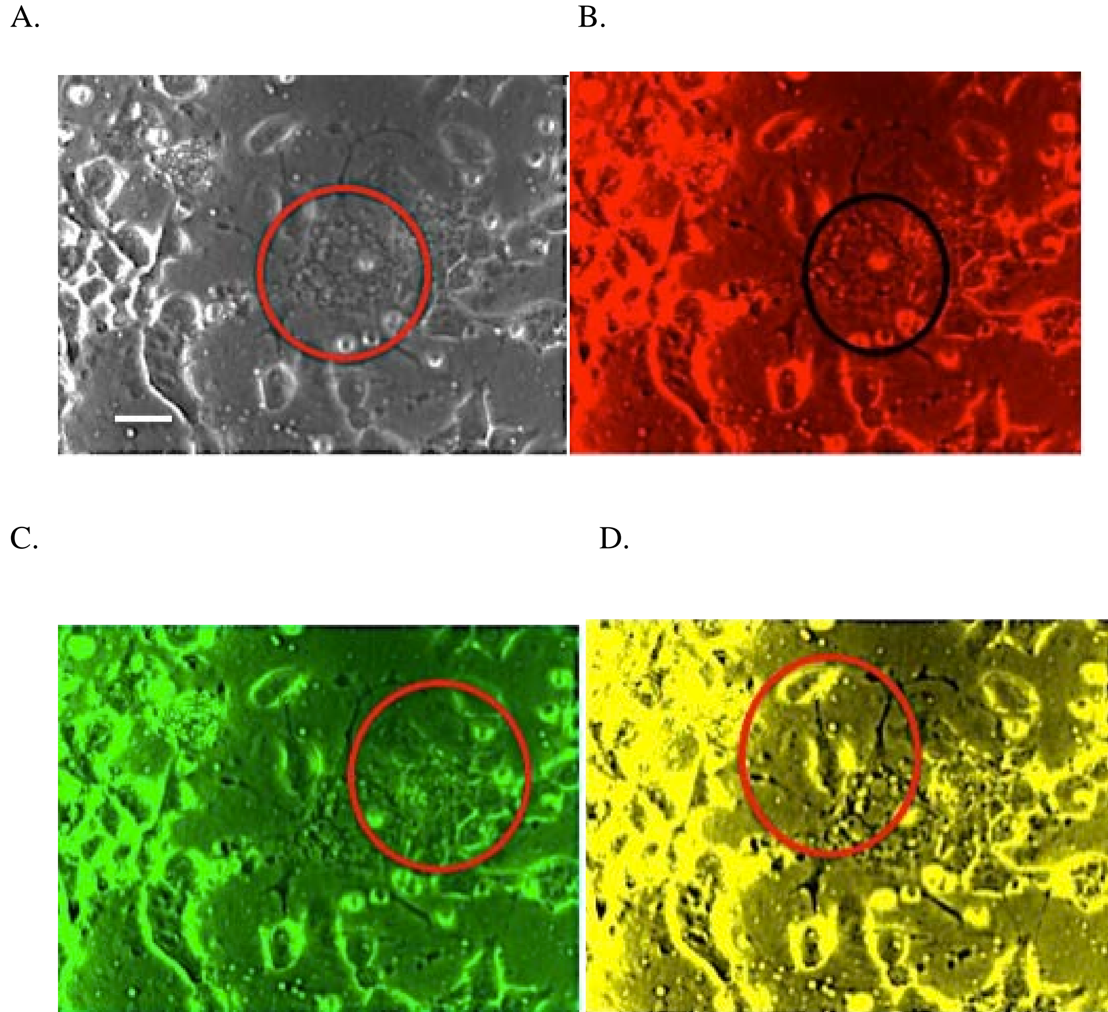

Figure 11. Imaging taken on day 4 after seeding of HEL-30 cells co-cultured with N2A cells grown in 5% serum. Circles outline an image of a cell gorwing underneath the level of the HEL-30 cells, which is that of an N2A cell and the neurites protruding upward through the pores. A) Phase contrast image. B) Neurite outgrowth stain. Only neuronal cells are able to take up the stain into the neurites and fluoresce red. C) Viability stain. Only live cells are able to take up the stain and thereby fluoresce green. D) Merge image of the viability stain and neurite stain. Scale bar represents 20 µm.

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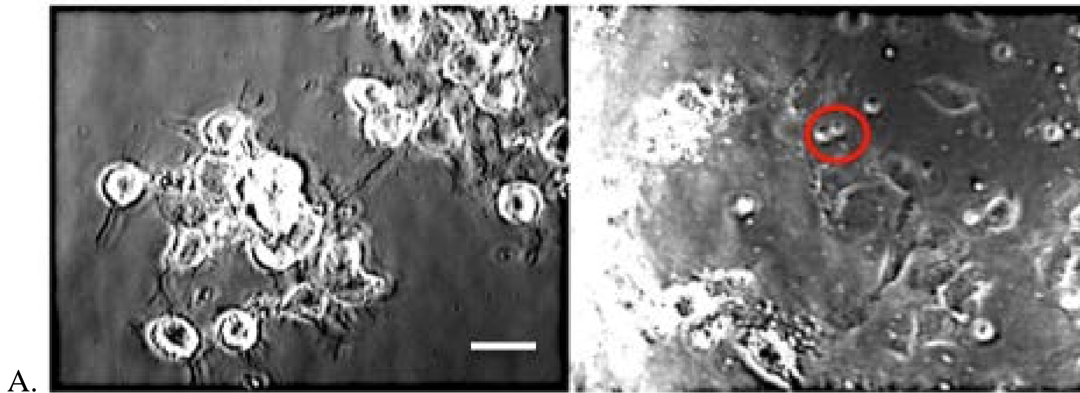
N2A level **HEL-30 Level**



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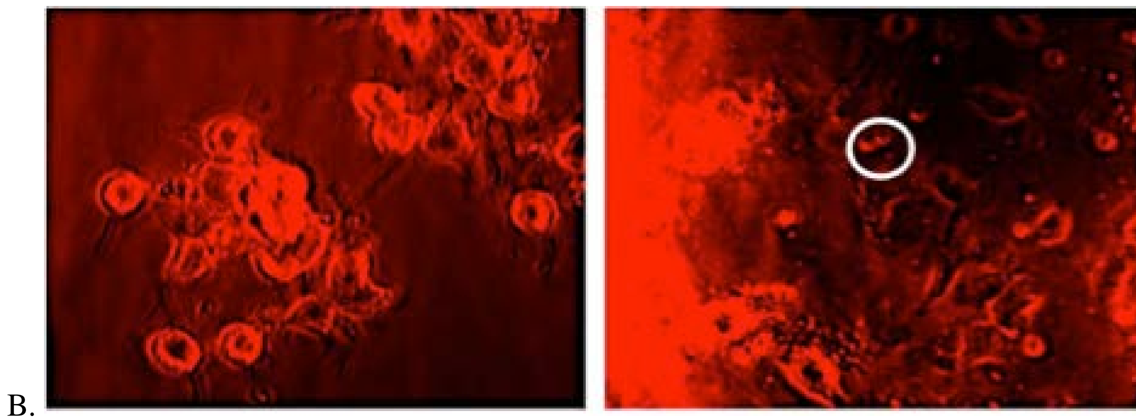

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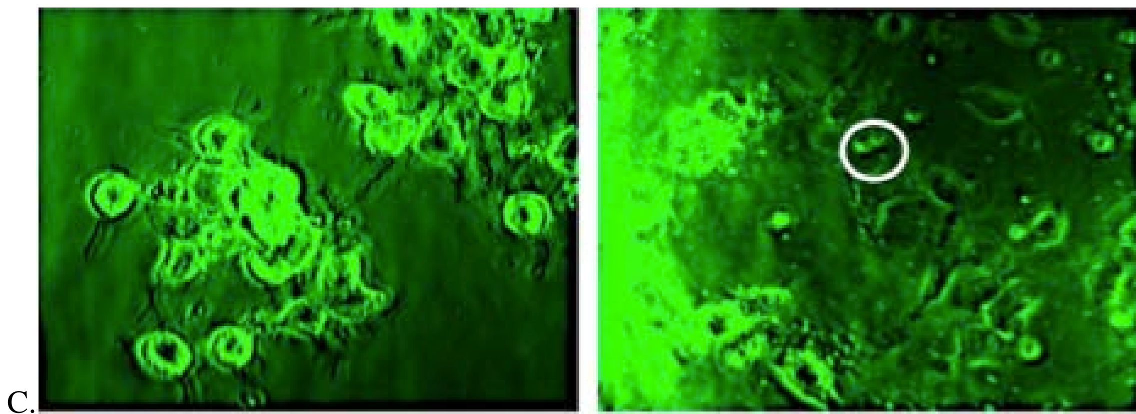

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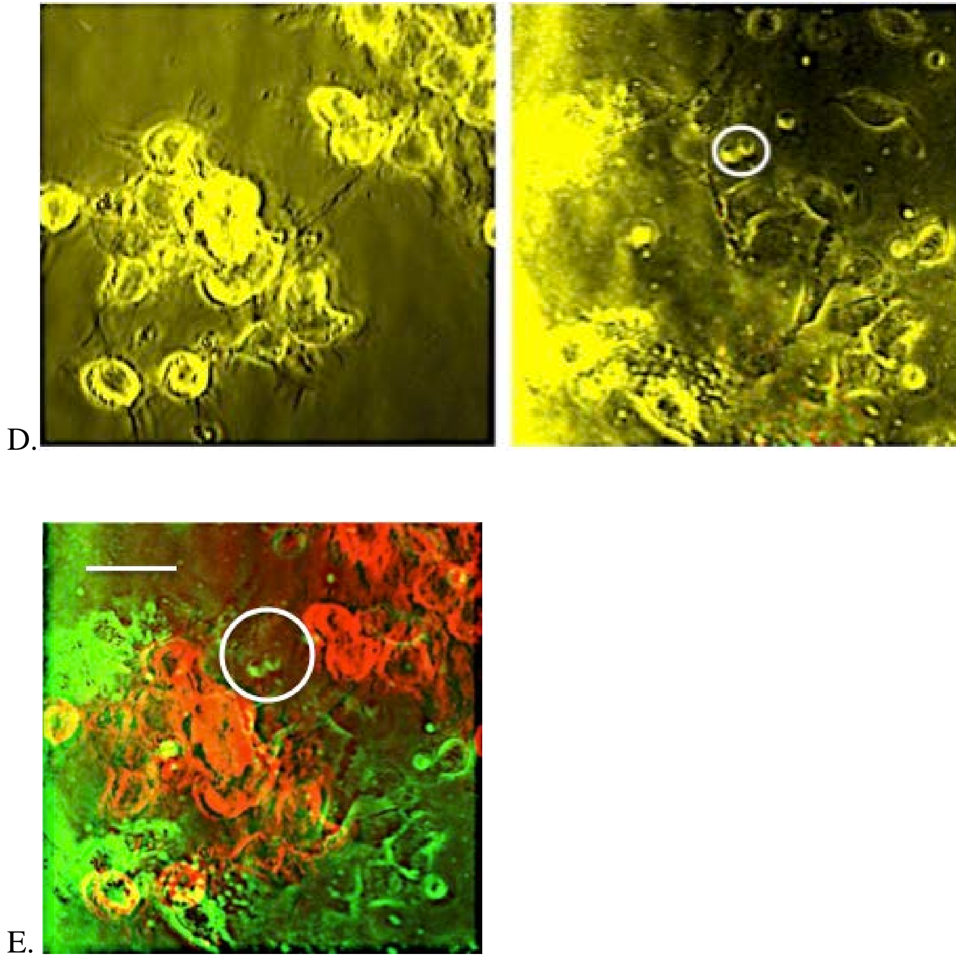

Figure 12. Overlay of images taken at day 4 after seeding. A side-by-side comparison of images taken from the N2A level and HEL-30 level on day 4 grown in 5% FCS serum without moving the microscope stage. Circle outlines a neurite in close proximity of an HEL-30 cell. There are at least 20 neurites total in the field. A) Phase contrast image. B) Neurite outgrowth stain. Only neuronal cells are able to take up the stain into the neurites and fluoresce red. C) Viability stain. Only live cells are able to take up the stain and thereby fluoresce green. D) Merge image of the viability stain and neurite stain. E) An overlay of two images, one taken from the N2A level and one taken from the HEL-30 level. Red image is from N2A level and green image is from HEL-30 level. Scale bar represents 20 µm.

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