Real-time visualization of developing viral infection in fibroblasts

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This chapter discusses the experiences and challenges of a method of live cell imaging in the development of active fluorescently tagged viral infections in a Core facility from the bench through image analysis. First, details of the live cell system and components are presented. Then preparation of the cells and fluorescently tagged virus is discussed. Finally, the image acquisition and analysis of the resulting images and examine the strengths and weaknesses of this type of system are discussed.

Keywords live-cell; real-time; timelapse; virus; VZV; image analysis; microscopy; fluorescence

1. Introduction

The University of Pittsburgh Department of Ophthalmology Imaging Module is part of a federally funded core facility that provides support to primary investigators in the Department of Ophthalmology and collaborators from other departments. One of the challenges of a Core facility is in managing the variety of experimental conditions that must be addressed safely and effectively while still making it possible to conduct experiments. This chapter will discuss the conditions for live cell imaging of one cell type; human embryonic lung fibroblasts that have been infected with Varicella Zoster virus.

Live cell imaging in our facility is conducted on a fully automated inverted Nikon TE2000E epifluorescent/brightfield microscope with xenon excitation (Sutter DG4), DIC, and phase capabilities. The prior stage accepts a Bioptechs Delta T stage adapter that controls temperature, allows perfusion and CO_2 control. The system rests on a TMC vibration isolation table with an overhead rack. Image acquisition via a Photometrics Cascade 1K camera is automated with MetaMorph software which allows control of multiple wavelengths, stage positions, z-stacks, and time points. Data is recorded to the hard drive of the controlling computer during the experiment, and is transferred to a portable hard drive as soon as the experiment is completed.

The goal of this study was to develop a live-cell time-lapse microscopy system to visualize VZV infection of fibroblasts in culture, and to monitor expression of a fluorescently-tagged viral protein over time. For this, we utilized a recombinant VZV expressing an enhanced green fluorescent protein (EGFP)-tagged viral protein kinase. Future extensions of this system will facilitate the more specific assignment of VZV gene classes, and will enable accurate descriptions of VZV protein localizations during different stages of viral infection.

2. Varicella Zoster Virus background

VZV is a human herpesvirus that causes chickenpox, establishes life-long latency in sensory neurons associated with infected skin cells, and reactivates after a prolonged period in which no viral replication occurs to cause herpes zoster (shingles). VZVis closely related to herpes simplex virus type 1 (HSV-1), and all but five VZV genes have corresponding orthologs in the HSV-1 genome. This similarity has raised the assumption that VZV and HSV-1 utilize common molecular mechanisms for replication. However, differences in pathogenesis and increasing evidence indicating differences in viral protein functions suggest this is not entirely the case [7, 8, 15]. Consequently, a more comprehensive understanding of VZV replication is needed to fully understand what makes each virus unique.

Herpesvirus gene expression occurs in temporally distinct waves that are controlled by viral transcription factors. Temporal classification of HSV-1 gene expression has relied upon high multiplicity, synchronous infection of cultured cells with cell-free virus [5]. With VZV this cannot be similarly experimentally determined because high titers of cell-free VZV cannot be obtained. In cultured fibroblasts, VZV spreads by cell-to-cell contact, remains tightly associated with the outer surface of the infected cell membrane and infectious viral particles are not released into the culture medium. As a result, VZV-infected cell stocks must be maintained and used to establish low multiplicity infection. Thus, no VZV-infected culture is ever synchronously infected, but instead contains cells at multiple stages of infection. Cell-free, membrane-associated infectious VZV can be prepared from infected cell cultures by sonication [4] or by vortexing with glass beads [13]. However, even under optimal conditions, this is highly inefficient and kinetics of VZV protein expression can only be observed on a single-cell basis using indirect immunofluorescence of fixed cells [3]. A major drawback to this approach is the requirement of antibodies specific to the viral protein and the non-dynamic parameters. This is a major concern when viral proteins do not produce strongly reactive antibodies or have not been used as antigens. Live-cell visualization of fluorescently-tagged viral proteins eliminates the requirement of a strongly reactive antibody and may provide useful information regarding the dynamics of protein localization during infection by using live cell microscopy.

3. Temperature control and dishes

Temperature control is a critical component of long term, live cell imaging. Two major components must be considered; temperature in the room and temperature in the dish. Our live cell system is situated in a rather small room which is supported by a dedicated air conditioning system and temperature in this room is more stable than in the standard laboratory setting, but there is still some fluctuation as the system cycles on and off which can cause focal drift.This drift can easily ruin an experiment. In our facility the drift problem is exacerbated by the location of the air duct, which blows directly toward the microscope. Controlling this situation proved easier than might have been expected; the overhead rack on our air table provided the structure for a draped enclosure (Fig. 1). Our system is permanently draped across the top and on three sides, and during an experiment a partial drape is pulled down across the front of the system. This drape creates a micro-environment around the scope that is more stable than the room as a whole. The temperature within the enclosure is slightly warmer than the rest of the room because of the heat generated by the camera, and it is important that the front drape allow circulation from the sides and a space for heat to escape at the top. This adaptation allows the enclosure to remain cool enough to be safe for the camera, but stabilizes the environment around the stage and ameliorates focal changes. It is necessary to turn the system on and allow it to stabilize for several hours before

Fig. 1 Draping of microscope.

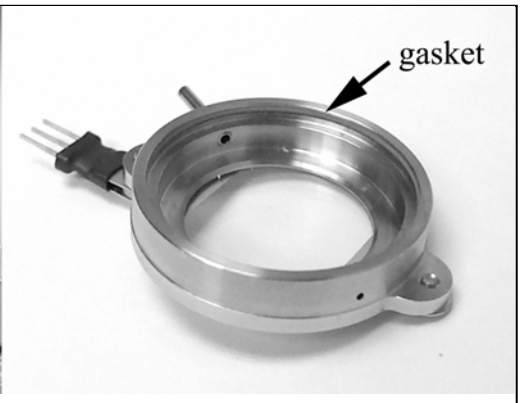

Fig. 2 Bioptechs dish with gasket installed.

beginning an experiment. Monitoring the acquisition during the experiment is necessary to make sure everything is running well and to confirm good focus. Isolation of lamp heat from the microscope by using a xenon lamp with a light guide also contributes to our success in controlling drift. MetaMorph 7.0 does allow on-the-fly focal corrections during timelapse acquisition should this be necessary because of cellular movement or drift.

The number of investigators, skill levels, variety of different cells, viruses, bacteria and solutions that are required for different experiments in our facility dictates that our system be simple, clean, safe and autoclavable as much as possible. The Bioptechs Delta T4 culture dish system provides a realistic and successful system for our needs. It allows investigators to grow cells in sterile 35mm dishes with 0.17mm (#1.5) coverslip bottoms which can be purchased by individual laboratories. These dishes can be sealed in the lab without transferring the cells and without manipulating plated coverslips as is required by most closed systems. The critical element that makes this system workable for long-term imaging is the heated lid with a gasket (Fig. 2), which makes this a "semi-closed" system. Without the gasket the T4 system will leak after about eight hours as the lid does not join the dish closely, and there is a spill hazard when installing the dishes on the stage. With the gasket the autoclaved T4 lid is sealed on the dish under a hood in the laboratory and we have system without leakage for over 120 hours with perfusion. Different lid designs are available to accommodate a variety of perfusion and/or CO_2 connections. The Delta T4 system heats the dish from below and also heats the lid, which helps control for temperature gradients within the dish. The system must be calibrated regularly to confirm accurate temperature readings.

Higher NA (oil) objectives require the use of objective heaters, lest the objective act as a heat-sink at the point of contact with the dish. Bioptechs objective heaters have been satisfactory with all of our objectives even if the sleeve cannot be removed. Calibration is critical; failure to calibrate the objective accurately at the oil interface can result in the objective acting as a secondary heating element, substantially raising the temperature in the dish, potentially damaging the objective and confounding the temperature control system of the dish.

4. Perfusion and pH

Perfusion is achieved with an Instech P720 peristaltic pump. For most applications an acceptable perfusion rate is about 3ml/hr. This pump would be inappropriate for a fully closed system because the irregularity of the flow of the pump would cause distortion when the coverslips flex and change the focus of the system. However, the semi-closed mini-chamber created by the DeltaT heated lid of the Bioptechs system is more forgiving than a true closed system and accommodates minor changes in pressure with a gas perfusion exhaust port; a tiny hole in the lid that allows excess pressure to escape the dish and avoiding distortion of the coverslip bottom. All of the components of the perfusion system that come in contact with media can be autoclaved.

Perfusion is called for in instances where the nutrient supply in the dish may become depleted during the experiment and/or in circumstances where the investigator wants to expose the cells to different solutions and then wash them out during the course of the experiment. Perfusion does increase the possibility of system failure and introduces larger amounts of media to the area around the microscope, so it should be used only when necessary, especially when perfusing infectious solutions. The hazard presented by perfusing Biohazard level 2 substances prohibits many shared facilities from supporting this kind of imaging. Initially we perfused during any long-term (6+ hour) experiment to control fluid level in the dish and maintain the pH of the media because direct connection of CO_2 (5% medical grade) to the dish dried the media out too quickly and the change in concentration of media could have influenced the outcome of the experiment. One way to address this was to bubble the 5% CO_2 into the media flask, and this works well but still requires perfusion. Another approach is to buffer the media with HEPES to maintain pH which in some cases can be used in lieu of CO_2. [16] There have been reports of HEPES toxicity during live cell experiments [17] so caution should be used with buffered solutions. Connecting humidified CO_2 directly to the dish is a very effective way to maintain experiments 8-36 hours, sometimes even 48 hours without perfusion. The Bioptechs system maintains conditions comparable to

those in the incubator, so the usual timepoint for changing the media of the dish maintained in the incubator can be used to gauge the duration that the dish can be maintained on the stage without perfusion. Removing the element of perfusion dramatically reduces the possibility of contamination by leakage or mechanical failure and is highly desirable especially when imaging infectious cultures. Our humidification setup is simple; CO_2 is bubbled into a sealed flask containing distilled water through a small gauge needle and the humidified CO_2 is pushed out the top of the flask via tubing connected directly to the dish.

During long-term acquisition when perfusion is necessary we drape the substage of the microscope as closely as possible to help contain media leakage in case of system failure. When using an air objective, a standard lab drape can be attached to the objective with a soft elastic band and the drape can extend from the top of the objective; when an objective heater is being used the drape has to be fixed at the bottom of the objective. This kind of draping also makes it easier to see if there is a leak by providing a white, absorbent field that would clearly show the media. Periodic checks around the edge of the dish with swabs to confirm that the gasket seal is intact are critical.

5. Safety from exposure to infectious pathogens

Research protocols for each project are readily available and are discussed clearly before beginning an experiment in the facility. For the study presented here, Bioptechs heated lids are transferred to VZV infected cultures grown in Bioptechs coverslip-bottom 35mm dishes and sealed in a laminar flow hood, and the dish is transferred to the microscope apparatus using latex gloves and secondary containment. Medium is recycled through the infected cells using a peristaltic pump and a closed tubing system connected to the Bioptechs lid. At the termination of the experiment, the infected dish, tubing and media are decontaminated with a solution of 10% bleach before being removed from the microscope stage. The tubing is rinsed thoroughly with de-ionized water and autoclaved, and the microscope stage is wiped down with a solution of 70% ethanol.

6. Cells, viruses and infections

VZV-permissive MRC-5 fibroblasts, which are a normal human diploid cell line (ATCC, Manassas, VA) were used for the studies described here, and were maintained as previously described [1]. For live-cell experiments, cells were trypsinized and plated to confluence (2×10^5 cells/dish) twenty-four hours preceeding additional manipulations. The VZV expressing EGFP-tagged ORF66 protein kinase (VZV.GFP-66) has been recently described [2]. Briefly, this virus encodes an EGFP-66 gene in place of the wild-type ORF66, so that the expression is regulated by the natural ORF66 promoter. Active VZV GFP-66 infections were established in confluent monolayers of MRC-5 cells using liquid N^2-frozen cell-associated stocks. When MRC-5 cells exhibited >80% EGFP fluorescence under UV microscopy, they were trypsinized and used to infect previously established MRC-5 cultures in 35mm dishes at a ratio of 1 infected cell to 100 uninfected cells. Infections were allowed to proceed for 24 hours before initiation of the time-lapse capture, at which time approximately 10% of cells exhibited EGFP fluorescence.

7. Controls before timelapse capture

Control experiments must be performed to confirm viability of the cells with the stage system and this preparation can save effort by identifying problems before launching into a full experiment. Cultures that have been successfully grown under laboratory conditions should be grown in Bioptechs dishes to ensure that the cells will adhere to the dishes. Some cell lines have difficulty transitioning from plastic dishes to glass bottom dishes. Often these problems can be overcome by flaming the dish, acid washing it, or coating it with some type of matrix [12]. Number of cells must also be calculated; a confluent monolayer is optimal for timelapse conditions; confluent cells tend to be healthier and to move less than non-confluent cells.

The effects of the heated stage and prolonged incubation under constant media exhange and CO_2 perfusion should be assessed for each experimental set-up. First, uninfected cells grown in dishes should be treated under the intended stage and perfusion and timing conditions without any time-lapse capture. Following the desired incubation period, the treated culture should be microscopically examined and compared with the untreated culture for changes in cellular morphology that could indicate apparatus-induced damage; we were particularly attuned to VZV induced and heat induced changes at the visualization points. If stage and perfusion conditions do not affect cultures, a similar control experiment including repeated UV exposure at the intended experimental intervals and duration should be performed to assess the effects of UV light. Cells should also be maintained and monitored off-stage for several hours after exposure to light to document any light toxicity that was not immediately apparent [16, 17]. Such preliminary controls will influence decisions about duration, frequency and wavelengths of light used during the experiment. It should be noted that further analysis of cell viability may be performed at the termination of each experiment, if required. For example, unfixed cells may be examined for uptake of a membrane impermeable DNA dye, such as propidium iodide or 7-AAD, which indicates either late-stage apoptosis or necrotic cell damage. In addition to testing the cells with the system, initial controls will also help to establish timecourse of the experiment including when in the course of infection to initiate imaging, how long to run, how often images should be acquired, when to infect and the level of fluorescence that can be expected. Often the preliminary experiments will define a point in the timecourse that marks a critical change and this knowledge will let the investigator narrow the period that the cells need to be imaged.

System controls Experimental goals need to be clearly defined before acquisition is begun; control images are required to accurately quantitate changes in fluorescence expression. Detailed discussion of system calibration for quantitation have been previously published [20]. Images in addition to the experimental images that are necessary for some kinds of quantitation include: *Digitizer offset*: the black level of camera. This value should never be zero (black) because zero levels indicate clipping of the image [11]. *Dark (Thermal) noise:* identifies hot pixels and charges that migrate to the CCD chip spontaneously. A single image of long exposure with no light available to the camera will provide an image of the dark noise in the system [9]. *Signal (Shot) noise*: Results from variation in the number of photons detected on any pixel with repeated measurements and provides a measure of the precision of the digital capture. Derived from several images taken in the same location with the same settings from a clear section of the sample [10, 14]. *Fluorescent sample (white image)*: acquired with uniform fluorescent slides [14]. *Background image for white image*: corrects for uneven illumination in fluorescent slide due to uneven lighting, camera defects, or dirt in the system. *Background image of sample*: corrects for uneven illumination in sample due to uneven lighting, camera defects, or dirt in the system. Shading correction images should also be acquired using the same acquisition settings that will be used in the experiment and should be acquired using a clear field in the sample itself [20].

8. Timelapse acquisition

Control experiments were performed as described, and no differences were observed between untreated cells and treated cells. For visualization of VZV infections and GFP-66 expression, VZV cultures were screened for green fluorescent plaques. Since it was not always clear in which direction the plaque was predominantly expanding, we imaged several developing plaques in time-lapse for each experiment to increase the likelihood of successful infection visualization, choosing central uninfected areas surrounded by VZV.GFP-66-infected areas. We obtained the most success in regions where extended EGFP-positive cellular processes were observed interacting with areas of cells in which no EGFP-expression was observed, and an example of this is shown in Fig. 3. At the initiation of the capture, the edges of VZV.GFP-66 plaques can be observed at the upper and lower image edges, with EGFP-positive processes from both sides extending into the center. We began to observe EGFP fluorescence in cells in the central area by 24-hours post-infection. Over time, differential GFP-66 expression was observed in

both the nucleus and cytoplasm, with some specific sub-nuclear and sub-cytoplasmic localized distribution.

These experiments were conducted with a 20X dry plan-apochromatic objective NA 0.45 and the correction collar adjusted for each sample. Wide-field illumination was provided with 175 watt Xenon lamp using a narrow EGFP bandpass filter (ex 480/20, em510/20). This acquisition mode was chosen

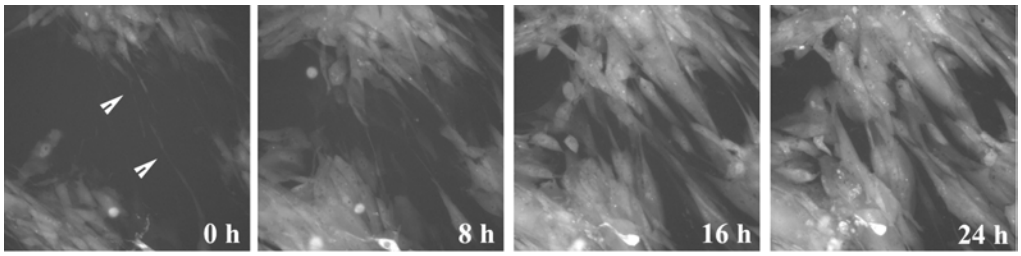

Fig. 3 Development of VZV.GFP-66 infection in MRC-5 cells. Time-lapse capture of EGFP fluorescence was performed every five minutes for twenty-four hours, and single monochromatic frames are shown with the corresponding time-points indicated at the lower right corner of each panel. The arrowheads at 0 hour demarcate extended EGFP-positive processes that were predictive of developing VZV infection.

over confocal because the signal in these samples is strong enough that out-of-focus fluorescence is not significant and the increased signal to noise ratio provided by the widefield system produces images more suited to quantitation [19]. The bottom of the bioptechs dishes were cleaned with Sparkle prior to imaging as was the objective lens. All images were acquired with room lights off and the monitor positioned away from the microscope. Samples were allowed to stabilize on the stage for 30+ minutes before acquisition began. Images were acquired every five minutes with 400ms exposure on a Cascade 1K camera with 1004x1002 pixel chip at 1x1 binning.

In order to accurately record a developing infection the final intensity of the infection must be used to calculate the initial exposure. Care must be taken that the exposure used remains in the linear range of the camera [20]. Preliminary experiments will define the final level of fluorescence that can be expected, and unless the ability of the cells to tolerate light dictates otherwise, the exposure time should be set slightly lower in case the signal comes up more strongly during the experiment. Our particular cell line is not particularly light sensitive, and we did not seen an increased death rate of these cells with the exposures we used. If light toxicity has been documented then the exposure times and capture period must be set to the minimum possible to control for this damage [19]. Overexposing the images at any point in the experiment will make it impossible to quantitate the increase in fluorescence.

9. Image analysis

Preliminary images for this study were acquired with a low power air objective, which limits the kind of analysis that can be performed. Also, while the acquisition of a single plane at each location limited the light required for the study, it also prohibits true deconvolution. Deblurring algorithms may be used on single 2D images to improve image appearance, but these images are non-quantitative. Nevertheless, the significant signal change over the course of the study provides strong general information about the transfer of the infection.

Prior to quantitation appropriate controls need to be applied to the image stacks, including background subtrations, flat fielding, and fluorescence flat fielding [6, 14]. With each of these adjustments, accuracy of the measurements must be considered and error should be propogated through any measurements that are produced as appropriate [20]. Linescan measurements can be used to quantify increase in fluorescence in a defined line, and histograms may be produced for a region of interest such as a specific cell or plaque. Image and region minimum and maximum values can be useful to give a general idea of the percentage of change over time.

10. Future investigations

In future studies, we will utilize live-cell time lapse visualization with dual-fluorescent viruses to create a road-map of specific markers for different stages of VZV infection, using expression and localization of fluorescent VZV fusion proteins. Our approach is improved over the current method of understanding the regulation of VZV gene expression because it allows accurate measurement of the timing of VZV protein expression and localization, it does not require the use of viral protein-specific antibodies in fixed cells, and it is more sensitive to subtle changes in VZV protein localization. Additional uses for this platform may include studying protein trafficking dynamics between organelles using photo-shiftable fluorescent fusion proteins, measurement of protein:protein interactions during infection with bi-molecular fluorescence, and dynamics of syncytia formation. A key improvement will be increased magnification to observe more detail in VZV-mediated effects on the host cell and to improve the resolution of the images. When multiple wavelengths are used the system will also be tested for chromatic abberation. High resolution images will require acquisiton of stacks, and at that time we will include correction for spherical abberation and also perform quantitative deconvolution.

System improvements: 1.Nikon has a new Perfect Focus system that controls for drift by registering the position of the dish relative to the objective with an infrared beam. Performance tests of this system were excellent, and this type of focal drift compensation presents a significant improvement in live cell imaging. Incorporating this equipment into our live cell system will resolve the problem of focal drift.

2. Phenol red-free media is more desirable for long term imaging because the inclusion of the dye can introduce background fluorescence [19, 16]. We anticipate using phenol-red free media whenever possible. 3. The quantity of CO2 required for the Bioptechs dishes is so low that it does not register on a standard regulator. Subsequent experiments are being conducted with a flowmeter (Concoa 565/560 series) attached in-line between the dish and the regulator to allow more control and precise CO_2 measurement.

Caveat: activities of fluorescent fusion proteins: There is a very real possibility that fluorescent protein epitope-tagging may interfere with the normal function of a viral protein, especially since EGFP expressed alone has the capacity to dimerize [21]. Therefore, it is essential to characterize the properties of the tagged viral protein with respect to the untagged version before expending a lot of time and effort. We have previously reported that VZV.GFP-66 grows with similar kinetics to its parent strain, pOka, and that GFP-66 maintained kinase activity in VZV infected cells [2]. This implied that the GFP-tag did not influence ORF66 function or negatively affect VZV replication. Thus, we concluded that VZV.GFP-66 behaved similar to wild-type VZV.

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