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Optimization of combination reporter viruses for microscopy and cell sorting

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ABSTRACT

Using a novel combination reporter virus that allows monitoring of both HIV fusion and viral LTR-driven Enhanced Green Fluorescent Protein (EGFP) expression, an indicator of productive viral infection, we have found that over ninety-percent of the cells that undergo fusion with HIV do not become productively infected. Additionally, the extent of resistance to productive infection by HIV differs between immune cell subsets. We aim to understand the cellular genes and proteins that restrict productive HIV replication.

Currently, we are using an LSR II flow cytometer-based assay that detects CCF2-AM dye cleavage, indicating membrane fusion, and EGFP expression, which indicates productive infection. This allows assessment of the susceptibility of different immune cell populations to HIV infection. We seek to optimize the combination reporter virus for cell sorting and high-resolution microscopy, which will enable novel approaches to studying factors regulating the susceptibility of cells to HIV infection.

A major obstacle was encountered due to the fact that fluorescent proteins that work well on the LSR II may not be optimal for cell sorters and microscopes. Furthermore, the CCF2-AM dye is incompatible with EGFP, leading us to measure fusion and EGFP, in parallel. We aimed to develop a non-EGFP combination reporter virus that will be compatible on all machines. From a compilation of seven different dyes, we identified mCherry as a dye that worked well on all instruments except the FACSAria, which lacks a compatible laser for the red dye. In future work, we hope to test viruses carrying mouse CD24 protein, which is detectable with anti-CD24 antibodies in a variety of fluorescent colors and would be compatible with the FACSAria.

INTRODUCTION

Shortly following the discovery of the acquired immunodeficiency syndrome (AIDS) in 1981, the human immunodeficiency virus (HIV) was identified in 1983. Since then, much has been discovered about HIV, including the structures of many of its proteins and the basic mechanisms of attachment and fusion to cells. Despite all we know all about HIV, a cure has still not been developed. The problem with developing a single drug that will stop HIV infection is that the virus has an extremely high mutation rate and is, therefore, highly recombinant. HIV is classified as a retrovirus, which means after entering a cell, its RNA is reverse transcribed into DNA, after which viral DNA is integrated into the cell's genome. After integration, the cell produces the proteins necessary for the virus to form and bud from the cell membrane to infect more cells (Figure 1). Through the process of reverse transcription, errors are made in the sequence of the newly produced, double-stranded DNA due to the lack of a proofreading enzyme (HIV and Pathogenesis of AIDS). This

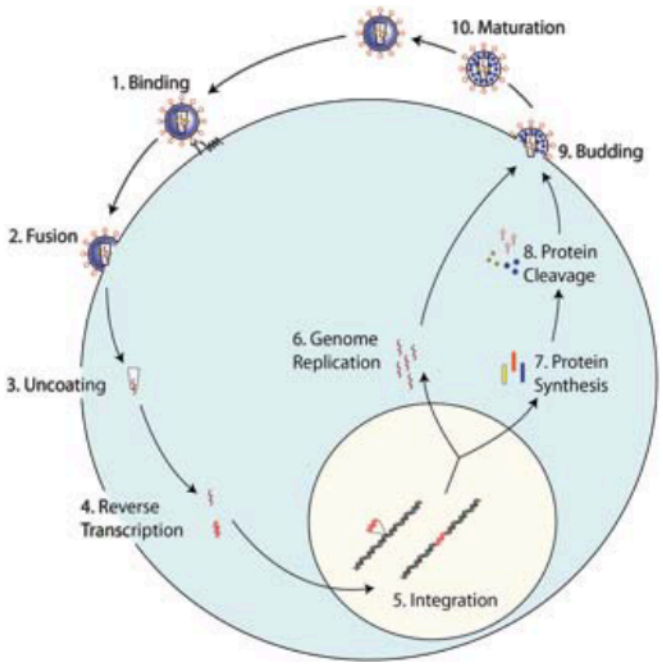

Figure 1. Viral life cycle. Assay measures fusion (step 2) and productive infection (step 7).

results in a tremendous diversity of viral sequences within a single infected individual. Thus, even when a drug works to stop certain strains of the virus, other strains of HIV are present in an individual that require different drugs to stop. Current drug therapies, therefore, consist of a cocktail of drugs that aims to target multiple stages of the viral life cycle simultaneously, slowing the emergence of resistance of virus.

While these therapies have been successful at slowing the progression of infection and extending the lifespan of patients, they do not cure the infection. A subpopulation of cells can become "latently" infected, wherein HIV integrates into the host cell's chromosomes, but does not produce viral RNAs or proteins. These latently infected cells are not sensitive to antiretroviral drug cocktails, allowing the virus to remain dormant for years. If a patient stops treatment, the virus inevitably returns and disease progression continues. Thus, lifelong therapy with antiretroviral medications is necessary.

Our laboratory is interested in the cellular factors that determine whether cells will become productively or latently infected once HIV has fused with the cell membrane. We are also interested identifying the exact subpopulations of immune cells that are targets for the virus.

Currently, we use a combination reporter virus assay that allows us to identify cells that have fused with HIV as well as determine whether or not these cells have progressed through the viral lifecycle to the point of viral protein production. By incorporating beta-lactamase (blam) into viral particles and loading target cells with the fluorescent blam sensitive substrate CCF2-AM, we can identify cells that have fused with HIV by observing changes in the optical properties of the substrate. When the virus fuses with and enters the cell, the beta lactamase in the viral particles reacts with the CCF2-AM dye present in the cells. This dye can then be excited at a specific wavelength, causing it to fluoresce, allowing us to determine if the virus has fused with the cells.

The most common fluorescent protein used is green fluorescent protein (GFP), isolated from the jellyfish A. victoria and from the sea pansy R. reniformis (Chalfie, 1995; Kar-Roy, et al., 2000). Since the discovery of this novel reporter protein, many others have been developed including dyes in red, blue, and yellow spectra (Ai, et al., 2007; Diéguez-Hurtado, et al., 2007; Marzylak, et al., 2007; Shaner, et al., 2005). These reporter proteins are used in HIV research as a marker for productive infection (Kar-Roy, et al., 2000). Because HIV is a retrovirus, it integrates itself into the cells' genetic material and utilizes the cells' machinery to produce more virus (HIV and the Pathogenesis of AIDS). Our reporter viruses also encode an enhanced green fluorescent protein (EGFP) gene driven by the viral LTR promoter, allowing us to identify in which viral proteins are being actively produced, an indicator for productive infection (Figure 1). In short, the cells that become productively infected will express a protein that fluoresces when stimulated at a specific wavelength. Using an instrument called an analytical flow cytometer (BD LSRII), we can identify subsets of CD4+ T cells that are susceptible to viral fusion and determine if they have become productively infected. The flow cytometer works on the principle of ordering cells into a single line through a column of solution. The dyes within the cells are then excited at certain wavelengths to induce fluorescence, which allows us to identify different substances present in the cell, depending on the dyes used. We aim to utilize this assay on machines other than the BD LSRII, including cell sorters (FACSAria and iCyt) and microscopes (Delta Vision). The cell sorter works similarly to the flow cytometer in that it can order cells and excite them. It can also separate the cells into subpopulations based on what wavelength at which it fluoresces. The flow cytometer allows us to characterize a sample, but opening up the assay to the other machines will allow for more in-depth analysis of our samples. For

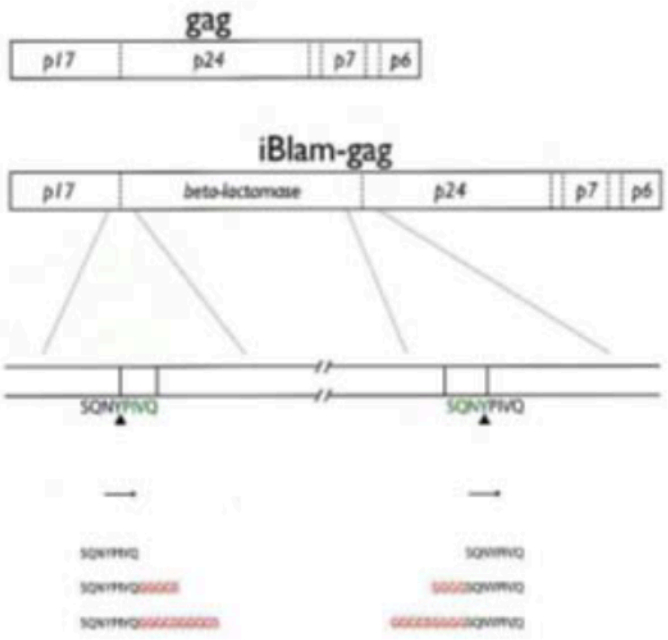

Figure 2. Beta-lactamase insert in the gag gene.

example, use of the cell sorter allows us to isolate populations of cells. Purifying these populations would provide the ability to analyze them by proteomics and RNA sequencing in order to identify factors regulating the progression of HIV through various stages of its life cycle. Furthermore, using this assay in conjunction with a microscope will allow us to see intact HIV target tissues, such as the lymph node or gastrointestinal tract, in order to better understand how HIV works in vivo. Ideally, we could adapt our assay for each machine and measure for CCF2-AM and EGFP, but every fluorescent dye and reporter gene has a specific wavelength of light that is optimal for excitation. Each dye and gene also has specific wavelength of light that is emitted upon energy release. One problem with our current assay is that the emission and excitation spectra of the CCF2-AM dye are extremely similar to that of EGFP, meaning that we cannot detect both simultaneously. Right now, we measure fusion using CCF2-AM on day one of infection and productive infection using EGFP on day 3 in order to avoid conflict, but in the future we hope to use a reporter gene other than EGFP in the assay so that we can measure fusion and productive infection simultaneously.

A second problem is that each instrument has different lasers and optical capabilities, so a reporter that works on the LSR2 may not be optimal–or even be detectable–on the other machines due to their unique laser configurations. This leads us to the purpose of this experiment: to evaluate which dye would be most compatible with each machine. We tested seven different dyes (EGFP, mCherry, Katushka, tagRFP, tagBFP, EYFP, and Cerulean) on each instrument. Our results suggest that mCherry is the ideal candidate for the adaptation of our current fusion and productive infection assay. It works well on all machines except the FACSAria, which lacks a suitable excitation laser. Further research may aim to test viruses encoding a mouse CD24 gene that is expressed similarly to EGFP and that is detectable with anti-CD24 antibodies available in a variety of fluorescent colors.

Figure 3. Instrument capabilities. *DeltaVision Microscope uses 7-channel LED illumination source, not lasers as the other instruments use.

Laser	LSRII	FACSAria	iCyt	DeltaVision Microscope*
355nm	x		x	
405nm violet			x	
407nm violet	x	x		
488nm blue	x	x	x	
561nm yellow-green	x		x	
633nm red	x	x		
640 HeNe			x	
UV	x			

The second part of this experiment was to construct a viral genome where the beta-lactamase gene is inserted into the gag region of HIV (Figure 2). In the current version of our combination reporter virus assay, we use a viral genome with beta-lactamase inserted into HIV's Vpr gene (blam-Vpr). Unfortunately, Vpr is only incorporated into HIV at about 50 to 100 molecules per particle. If we could insert blam into the Gag gene, it would be incorporated into the viral particles approximately 5,000 times, a 50-fold increase from where we are now. An increase in the amount of beta-lactamase present in virions would provide two significant advantages. First, it would allow us to detect cells that have undergone fusion with HIV for longer periods of time, possibly leading to simultaneous detection of fusion and reporter gene expression (using a reporter gene other than EGFP). Second, higher levels of blam in the viral particles would shorten the duration of time needed to see conversion of the CCF2-AM dye, potentially allowing us to monitor viral fusion in real-time. This is critical in microscopy of tissue specimens that typically have limited viability *ex vivo*.

Figure 4. Fluorescent reporter protein excitation and emission spectra.

Fluorescent Protein	Excitation Max (nm)	Emission Max (nm)
tagRFP	555	584
mCherry	587	610
Katushka	588	635
Cerulean	433	475
EGFP	488	509
EYFP	514	527
tagBFP	402	457

Hypothesis

According to the excitation maximums and the instrument optical capabilities, it appears that EGFP on the 488nm blue laser would be best suited for these three machines. To simultaneously measure fusion and productive viral infection, however, we need a different florescent protein. Any of the other proteins presented here may work well, but none will be optimal because 488nm laser is the only one that all three machines have. Therefore, each machine will not be capable of exciting any one dye, except EGFP. Additionally, none of the lasers exactly match up to the excitation maximums of the available proteins. Empirical testing of the fluorescent reporter genes will be required.

METHODS and MATERIALS

We began by growing up DNA from glycerol stocks through transformation in bacteria. A 500uL stock was set up and, after a 24-hour incubation period, was transferred to a 5mL stock and was left to incubate for another 24-48 hours. Upon completion of the incubation, a Qiagen plasmid purification kit was set up to isolate the plasmid DNA from the bacteria. The DNA isolated by this process was then used as an HIV core in combination with a viral envelope and blam-vpr DNA in a transfection with 293T cells. These cells incubated over a period of a few days to allow for ample production of the virus. The virus harvested from the transfection was then used to infect CD4+ T cells

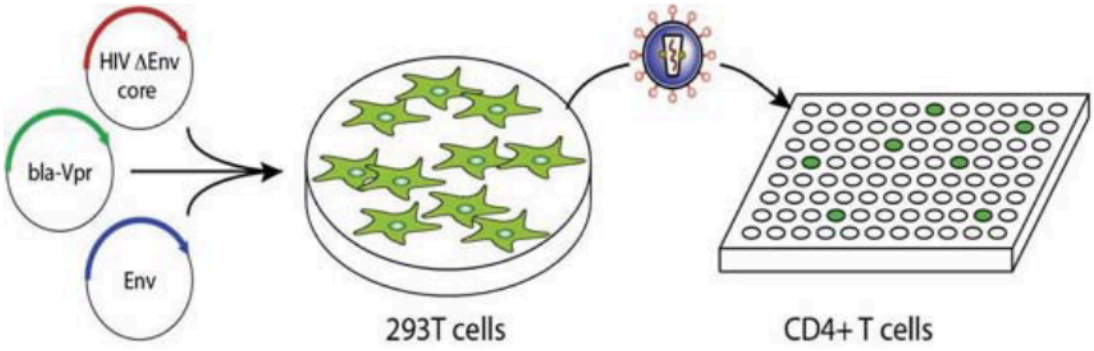

Figure 5. Pseudovirus production. Transfection and spinfection.

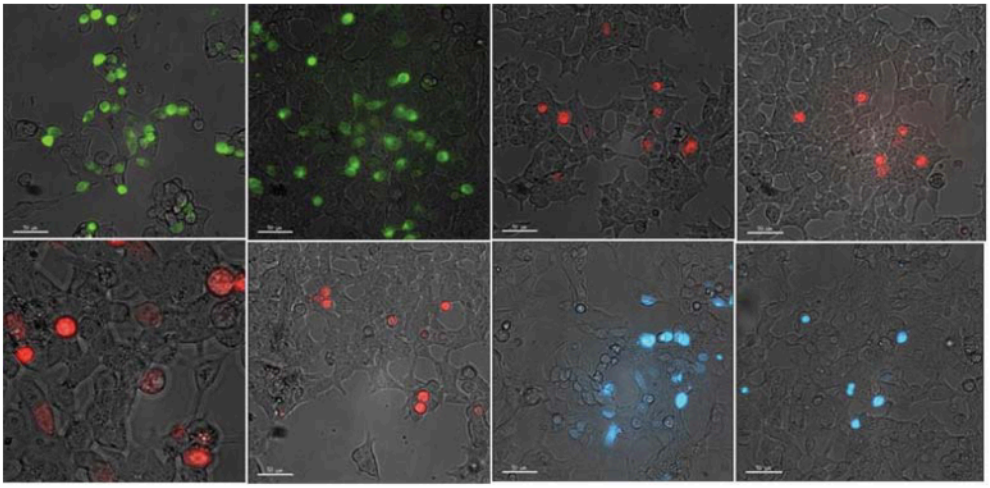

Figure 6. Images from DeltaVision microscope. Top row from right to left: EGFP day 1 after transfection, EYFP 1 day after transfection, Katushka day 1, and mCherry day 1. Bottom row from right to left: tagRFP(11) day 2, tagRFP(23) day 1, tagBFP day 2, and Cerulean day 2.

through spinfection. The CD4+ T cells were then left to incubate for a few days, and subsequently analyzed on an instrument such as the LSRII or FACSAria.

To work towards our goal of a better beta-lactamase construct, we used a series of PCR cloning techniques that would allow us to link each segment of our product (the gag p17 region, beta-lactamase, and the gag p24 region). Our aim was to fuse these three segments together and then introduce them into an HIV core vector using restriction enzyme digestion. Constructs were designed introducing blam to the p17 region and the p24 region with no linkers, one linker, and two linkers. Linkers were segments of nucleic acids that introduced space between the blam and p17 and p24 region respectively. These were used to allow more space for conformational shapes of the proteins formed. This was to ensure that the protein had enough room to get into their proper conformations.

RESULTS

All fluorescent proteins worked well on the DeltaVision microscope (Figure 6). EGFP and EYFP had the best rates of productive infection, but the brightness of all proteins worked well. EGFP and EYFP worked comparably well; all red dyes worked comparably well; and tagBFP worked better than Cerulean.

For LSRII data, the red peaks indicate the fluorescence emission of an untransfected cell population while the blue peaks indicate cell populations expressing

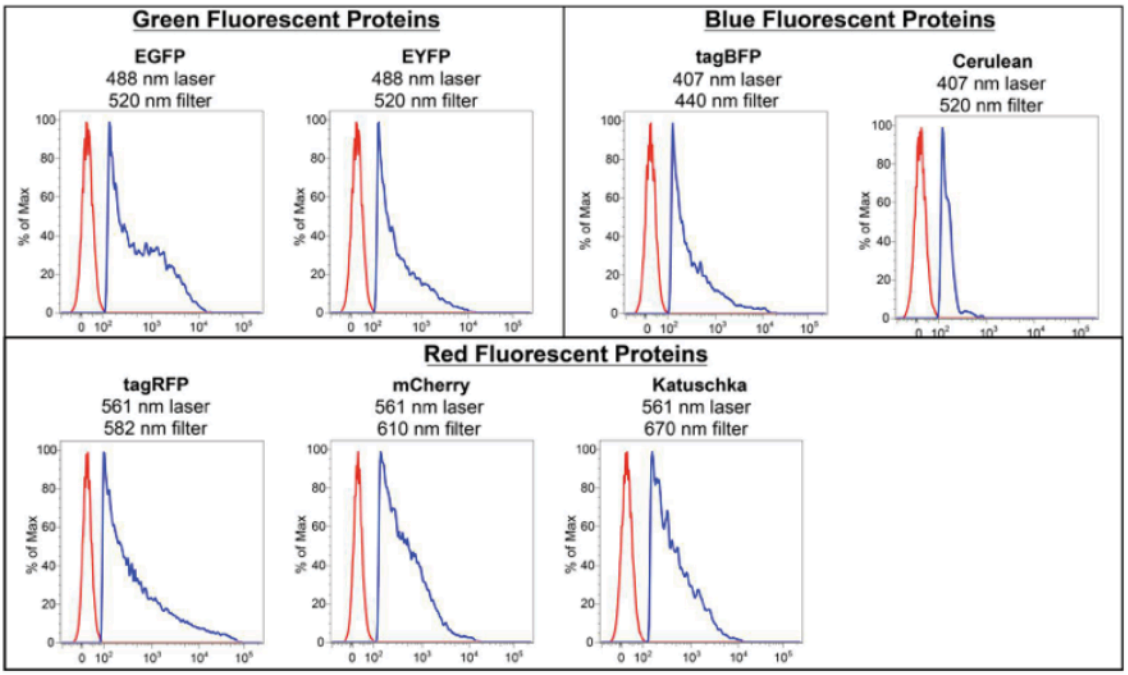

Figure 7. Untransfected cells (red) and cells expressing the fluorescent proteins (blue) utilized in our reporter viruses.

the reporter construct (Figure 7). EGFP worked better than EYFP; tagBFP worked better than Cerulean; and mCherry worked the best out of the red dyes, with Katushka as the second best of the red dyes. Overall, mCherry was the optimal dye for the LSRII, with what was quantitatively the strongest signal.

For the second part of the experiment, we successfully constructed cloning intermediates p17-blam and blam-p24 through overlap PCR, but were unsuccessful in generating a full-length p17-blam-p24 clone. We altered the annealing temperature and extension time, but could not get the desired product. Our next attempt was to try adding all three segments at once, which was successful, but we were unsuccessful in cloning the products into the backbone vector. After a series of attempts at the PCR process, we tried a different method, called Gibson Assembly, but those attempts were also unsuccessful. The Gibson Assembly Method required each segment of desired DNA as well as primers to be combined in a single isothermal reaction. The failures in cloning are likely due to the complex nature of this cloning project, the large size of the HIV core vector (14+kb), and the tendency of HIV to recombine.

CONCLUSIONS

From an array of seven unique dyes, we identified mCherry as a dye that worked well on all instruments except the FACSAria, which lacks a laser with an appropriate wavelength to excite red dyes. In future work, we hope to test viruses carrying the mouse CD24 gene. As with florescent reporter genes, mCD24 is produced following uncoating, reverse transcription, integration of HIV into the host genome, and viral LTR-driven gene expression. Unlike fluorescent reporter genes, the mCD24 molecule is not itself fluorescent, but is detectable with anti-CD24 antibodies that can be obtained in a variety of fluorescent colors. Several of these colors are compatible with the FACSAria cell sorter.

Furthermore, determining why the cloning of the beta-lactamase gene into the gag region of the viral genome was unsuccessful will hopefully lead to the development of a method that will allow the clone to work.

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