A HIGH THROUGHPUT MULTIPLEXED PLATFORM FOR MONITORING PROTEOLYSIS IN THE CLASSICAL SECRETORY PATHWAY – SEARCH FOR NOVEL ANTIVIRALS

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A High Throughput Multiplexed Platform for Monitoring Proteolysis in the
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ABSTRACT OF THE THESIS

A High Throughput Multiplexed Platform for Monitoring Proteolysis in the Classical Secretory Pathway – Search for Novel Antivirals

by

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RNA Viruses such as Human Immunodeficiency (HIV) and Dengue Virus (DenV) cause devastating diseases, including Acquired Immunodeficiency Syndrome (AIDS) and Dengue Hemorrhagic Fever. With 2.5 and 50 million new infections yearly, respectively, unsuccessful attempts at vaccine development and resistance to current treatment, all beg for novel antivirals. However, new methods and platforms are required to increase the chances for their discovery.

The classical secretory pathway is essential for biological functions and is utilized for the transport of proteins to the cell surface and/or extracellular matrix. Within the secretory pathway resides an array of enzymes that modify proteins into their mature and active forms. Viruses, as well as other pathogens, hijack such enzymes for their own benefit. While HIV and DenV are distant viruses, both rely on the cellular protease Furin within this compartment for maturation of their viral envelopes. Importantly, blockade of Furin processing leads to non-fusogenic virions, making it an ideal drug target.

In order to monitor proteolysis within the natural milieu of the secretory pathway, an assay with a robust and quantitative read-out was developed. The assay relies on a complex scaffold molecule targeted to the Endoplasmic Reticulum for transport to the cell surface. A substrate is flanked by the FLAG and HA tags fused to the N-terminus of the scaffold. In such a way, in the absence of proteolysis both tags are presented on the cell surface but only one tag (HA) is presented if proteolysis occurs. Tag presentation can be subsequently detected by fluorescent-coupled antibodies in a robust and quantitative manner through flow cytometry and/or microscopy techniques.

The assay described has been adapted to monitor processing of the HIV envelope and DenV pr-M proteins. It has been further calibrated in a 96-well plate format for flow cytometry, to demonstrate both robustness and repeatability, critical for high throughput screening. In addition, varied intensities of a stably expressed fluorescent protein were used for genetic barcoding through retroviral technology. Genetic barcoding was exploited to further develop the platform in a multiplexed format to assay multiple substrates in the same well. A robust platform such as the one described here will expand high throughput capabilities in search for novel antivirals and can additionally be adapted to other substrates in the secretory pathway.
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CHAPTER 1

INTRODUCTION

THE CLASSICAL SECRETORY PATHWAY

The classical secretory pathway is critical for normal cell function as it is utilized for the transport of many proteins and other cargo to the cell membrane or for their secretion into the extracellular matrix (ECM, see the Appendix for abbreviations) [1–3]. The pathway is a highly regulated system that can involve up to one third of the proteome and plays a vital role in the formation of the ECM, signaling pathways, immunity, and differentiation [1–3].

In order to reach the secretory pathway, proteins are targeted to the Endoplasmic Reticulum (ER), and travel through the Golgi and Trans-Golgi Network (TGN) where they can be co- and post-translationally modified into mature forms prior to their final destination; insertion within cellular membranes or secretion [1,4]. Protein modifications include glycosylation, myristoylation, palmitoylation, and importantly, processing of protein precursors into mature forms through proteolysis [1,5–7]. Proteolytic processing within the secretory pathway relies on a wide array of ER/Golgi/TGN resident cellular proteases, including signal peptidases (SP), signal peptide peptidases (SPP), and proprotein convertases (PC) [8–10]. While SPs and SPPs remove signal peptides/sequences required for the targeting of proteins to the ER and secretory pathway, PCs cleave proteins or remove inhibitory domains leading to conformational changes and the activation of proteins into their mature forms [1,8,9]. ER/Golgi/TGN resident proteases play an essential role in normal function, and mis-regulation of these enzymes has been attributed to the development of cancer, Alzheimer’s, and other diseases [11,12].

FURIN AND THE PROTEIN CONVERTASE FAMILY

The PCs are a family of nine serine proteases primarily responsible for the conversion of precursors into mature bioactive molecules within the secretory pathway [13]. The majority of PCs cleave proteins adjacent to conserved basic residues [9,11,13,14]. Four members (Furin, PC5/6, PACE4, and PC7) of the PC family are ubiquitously expressed and
are necessary for the activation/inactivation of receptors, ligands, enzymes, and/or growth factors [13,15]. Further, the family is a highly complex system in which each member exhibits unique, redundant, complimentary, and opposite functions [13]. Elucidation of the convoluted role of PCs in the secretory pathway and cellular biology should provide novel insights to the understanding and treatment of disease.

Furin, the first discovered and best characterized member of the PC family, has been shown to be required for a number of various cellular processes (see Table 1). Despite the diversity of function, Furin substrates are relatively conserved and contain a structural motif consisting of a core domain flanked by two flexible polar regions [9,16,17]. The core region contains the canonical highly basic amino acid recognition sequence R-X-K/R-R which fits within the catalytic domain of Furin and determines its binding affinity [16–18]. Cleavage occurs immediately after the terminal arginine (termed the P1 position) which is required for processing [16–18]. The two flexible regions facilitate access to the core domain, suggesting that proper secondary and tertiary structures are necessary for exposing the core recognition site for Furin-based processing [16,17].

In addition to normal cell function, Furin-based proteolytic processing is further exploited by many pathogens; protozoan, bacterial and viral alike [9,11,19-21]. Importantly, Furin/PC proteolysis has been shown to be required for the propagation of many human viral pathogens including Human Immunodeficiency Virus (HIV), Ebola, Influenza, and Dengue virus (DenV), among others [5,21,22,23].

**Viral Infection and the Secretory Pathway**

Many viruses exploit the secretory pathway for both entry and exit from the host cell. One such example is HIV, the etiological agent of Acquired Immune Deficiency Syndrome (AIDS). Currently, more than 34 million are living with HIV/AIDS and approximately 2.5 million individuals are newly infected annually [24,25]. Additionally, HIV/AIDS results in the death of roughly 1.7 million each year [24,25]. Although tremendous progress has been accomplished in treatment, HIV/AIDS still remains a devastating disease. Furthermore, toxic effects of current treatments together with the appearance of drug resistant HIV strains, begs for novel drugs as well as assays to facilitate their discovery.
Table 1. Notable Furin Substrates

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Function</th>
<th>Sequence</th>
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<tbody>
<tr>
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<td></td>
<td></td>
</tr>
<tr>
<td>HIV-1 gp160</td>
<td>Envelope/viral entry</td>
<td>PTKAKRRVVQREKR AVGIGA</td>
</tr>
<tr>
<td>HIV-2 gp140</td>
<td>Envelope/viral entry</td>
<td>PKRYSSAPVRNKRK GFVLG</td>
</tr>
<tr>
<td>Dengue Virus pr-M</td>
<td>Envelope/viral entry</td>
<td>GTCTTTGEHRKREK RVGIGA</td>
</tr>
<tr>
<td>Influenza A Virus HA</td>
<td>Envelope/viral entry</td>
<td>ATGPRNVQPRRKKK GLFGAK</td>
</tr>
<tr>
<td>Human Papilloma Virus 16 L2</td>
<td>Minor Capsid</td>
<td>MRHKRSAKRTKR ASATQL</td>
</tr>
<tr>
<td>Ebola Virus (Zaire) GP</td>
<td>Envelope/viral entry</td>
<td>GVAGLITGGRRTTRR EAIVNA</td>
</tr>
<tr>
<td><strong>Bacterial</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><em>Shigella dysenteriae</em> Shiga Toxin subunit A</td>
<td>Toxin Activation</td>
<td>LILNCHHHASRVAR MASDEF</td>
</tr>
<tr>
<td><em>Bacillus anthracis</em> Anthrax Toxin Protective Antigen</td>
<td>Toxin Activation</td>
<td>PELKQKSSNSRKKK STSAGP</td>
</tr>
<tr>
<td><em>Corynebacterium diphtheria</em> Diptheria Toxin</td>
<td>Toxin Activation</td>
<td>YMAQACAGNRVRK SVGSSL</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> exotoxin A</td>
<td>Toxin Activation</td>
<td>HLPLETFRHRQPR GWEQLE</td>
</tr>
<tr>
<td><strong>Eukaryotic</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Human β Amyloid Precursor Cleaving Enzyme 1 (BACE1)</td>
<td>Enzyme Processing</td>
<td>SGLGGAPLGLRLP ETDEEP</td>
</tr>
<tr>
<td>Human Matrix Metalloprotease 2 (MMP2)</td>
<td>Structural</td>
<td>DLQNTIETMKPR CGNPDV</td>
</tr>
<tr>
<td>Human Insulin-like Growth Factor 1 (IGF1)</td>
<td>Signaling</td>
<td>LKPASKSARVQAR HTDMPK</td>
</tr>
<tr>
<td>Human Transforming Growth Factor β (TGFβ)</td>
<td>Signaling</td>
<td>YRLESQTNRRKCR ALDAAY</td>
</tr>
<tr>
<td>Human Vascular Endothelial Growth Factor C (VEGF C)</td>
<td>Signaling</td>
<td>KLDVYRQVHSIIRR SLPATL</td>
</tr>
</tbody>
</table>

**Note.** Substrates include viral, bacterial, and eukaryotic with a wide array of functions. Sequences for Furin recognition shown [12,16,17,22,26,27].

While HIV primarily utilizes the cytosol for the viral life cycle, the HIV envelope (Env) protein is a notable exception. Comprised of two subunits, glycoprotein 120 (gp120) and glycoprotein 41 (gp41), Env is required for viral entry into the host cell, primarily T-cells [28,29]. In order to enter, gp120 first mediates binding to the primary host receptor CD4 necessary for subsequent binding to co-receptors CXCR4 or CCR5 [28–30]. Receptor binding of gp120 causes a conformational change which exposes the fusion peptide of gp41, required for fusion with the cell membrane and entry of the viral particle into the cell [5,28,29,31–34].

In an infected cell, Env is translated as a precursor which is targeted through the secretory pathway [33]. After trimerization and glycosylation, Env is post-translationally cleaved by Furin/PCs in the TGN into mature, gp120 and gp41 active subunits, which remain non-covalently associated [5,27-29,32,35]. Ultimately, the mature gp120/41 complex is embedded in the cell surface from which progeny virions bud and accordingly acquire Env. Furin-based processing of Env is absolutely required for proper conformational changes that
occur upon receptor binding. Thus, the blockade of Env processing in the secretory pathway results in production of non-fusogenic progeny virions [32,35,36].

Another prime example of viral exploitation of Furin/PCs and the secretory pathway is maturation of the pre-membrane (pr-M) proteins of Flaviviruses [23]. Flaviviruses are a genus of the Flaviviridae family, which includes important human pathogens such as DenV, West Nile virus (WNV), Yellow fever virus (YFV), and Japanese Encephalitis virus (JEV) [22,23,37,38]. Of particular note, the arthropod-borne DenV currently infects 50-100 million individuals each year, leading to Dengue Fever, or more lethal forms Dengue Hemorrhagic Fever and Dengue Shock Syndrome [39]. Additionally, the World Health Organization estimates that 40 percent of the world’s population is at risk of DenV infection [39]. Further, DenV has been implicated for use as a bioterrorism weapon and is now considered an emerging pathogen in the United States [39]. Importantly, the Flaviviridae family heavily relies on the host secretory pathway for their propagation.

As one of the features of the life cycle of Flaviviridae family, the DenV proteome is first translated into a polyprotein of which both viral and host proteases process it into mature and active proteins [38]. In order to exit the cell, the virus utilizes the secretory pathway, where it exploits cellular enzymes to cleave capsid-associated proteins. The most relevant example is pr-M, which is cleaved, according to published literature, by Furin [37,40]. Flaviviral pr-M proteins function primarily to prevent premature fusion mediated by the envelope protein (E), which is dependent on a low pH, upon exit from the host cell [22]. Accordingly, pr-M acts as a molecular chaperone for E and the conversion of pr-M into the mature membrane protein (M) occurs rather late within the TGN, due to the low pH in this vesicle compartment of the secretory pathway [22,40]. Similar to HIV, the processing of pr-M into M is necessary to generate fusion competent viral progeny [37,38].

It is well established that DenV pr-M is inefficiently processed by Furin/PCs, which appears important for its life cycle [22,37,40–43]. Remarkably, the rate of pr-M processing is highly calibrated and thus an increase or decrease can lead to a decrease in viral propagation [41–43]. In contrast to DenV, the pr-M boundaries of related Flaviviruses, such as JEV, WNV, or Tick-borne Encephalitis Virus (TBEV), have been shown to be efficiently cleaved during their viral lifecycle [23,41]. The existence of both efficiently and non-efficiently processed pr-M viral particles in the viral lifecycle, though interesting, has further
complicated the efforts in vaccine development against DenV [43]. Thus, elucidating the complex and convoluted role of pr-M processing in DenV infection will aid in the development of antivirals and vaccines against this devastating virus.

In summary, many viruses exploit cellular machinery to convert proteins into mature forms. Viral reliance, i.e. HIV and DenV, on Furin/PC processing and the secretory pathway for entry and exit, provides an intriguing drug target for blockade of viral infection.

**MAIN GOAL: DEVELOPMENT OF A ROBUST ASSAY TO MONITOR PROTEOLYSIS IN THE CLASSICAL SECRETORY PATHWAY**

Previously, the discovery of Furin/PC competitors has been hampered by the limited tools available. Common methodologies to detect processing/cleavage by PCs involve time-consuming techniques such as Western blotting for assessment of cleavage products [44]. Current high-throughput assays rely on fluorogenic substrates and do not represent the natural cellular milieu [45]. Several biochemical assays, while high throughput, are not only not performed in the cellular context but are not in the proper cellular compartment; i.e., the ER/Golgi/TGN. Moreover, non-cellular assays cannot account for cytotoxic effects of drugs under development. While addressing many of these issues, a cell based assay that relies on secreted alkaline phosphatase, while invaluable for drug discovery, does not monitor enzymatic activity on a single cell level and does not couple cell with phenotype [46]. Thus this assay does not allow for pinpointing cells within a population, which is crucial for the screening of endogenously expressed targeted peptide libraries or cells expressing cDNA or siRNA libraries [47,48]. Assays as the one described by Hobson et al, while cell-based, is limited to cell surface cleavage and relies on a rather complex design [49]. In addition, these assays are often not easily adapted to multiplexed platforms.

Therefore, the primary goal is to develop an *in vivo/ex vivo* cell-based assay that quantitatively monitors processing and/or transport to the cell surface at the single cell level, which can aid in drug discovery as well as help answer biological questions. The assay should thus have a robust and quantitative read out and be easily adaptable to high-throughput screening (HTS) and multiplexing. As proof of principle, the assay will be adapted to monitor well-characterized HIV Env and DenV pr-M processing by Furin/PCs, in part due to their relevance and impact on human health.
CHAPTER 2

MATERIALS AND METHODS

CONSTRUCTION OF VECTORS AND PLASMIDS

Appropriate lentiviral plasmids pTRIPZ used for shRNA-based knockdown were acquired from ThermoScientific (Pittsburgh, PA). TRIPZ plasmids contain genetic information for inducible expression of the shRNA and turbo Red Fluorescent Protein (tRFP) under a Tetracycline responsive element. Further, they contain a puromycin resistance cassette under the constitutive Ubiquitin Chain Promoter for Eukaryotic selection upon transduction.

Assay constructs/vectors were engineered as follows. The construct pBluescript.FLAG-HIV-1Env-wt-Lyt2. was created by digesting the previously constructed pBMN.FLAG-SBP-Citrine-Lyt2.i.Blasticidin, which contains a Kozak sequence and Prolactin signal sequence with Xhol/NotI restriction enzymes and ligating the resulting FLAG-SBP-Citrine fragment into pBluescript SK+ (Stratagene, Santa Clara, CA) digested with Xhol/NotI. The HIV-1 Env-wt sequence was amplified from the HXB2 strain of HIV-1 with a forward primer containing a HindIII site and a reverse primer containing an EcoRI site. The product was ligated into pBluescript.FLAG-SBP-Citrine-Lyt2 digested with HindIII/EcoRI restriction enzymes to create pBluescript.FLAG-HIV-1Env-wt-Lyt2. A HA tag was inserted by first amplifying the HA tag using a forward primer, which contains an EcoR1 site, and a reverse primer, which contains a BglII site, Glycine Linker, and MfeI site. The PCR product was ligated into pBluescript.FLAG-HIV-1Env-wt-Lyt2, utilizing the EcoRI site. Additional substrates were engineered utilizing overlapping primers to amplify the appropriate DNA sequence and subsequently digested with HindIII and EcoR1 and were ligated into pBluescript.FLAG-HIV-1Env-wt-HA-Lyt2 cut with HindIII/EcoR1. Primers used to amplify the appropriate substrates are listed in Table 2. The DenV Type 2 New Guinea isolate was used as reference for DenV pr-M substrates. Accession number NC 001563 was used as reference for WNV pr-M substrate [37].
Table 2. Primer Sequences

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Primer Sequence</th>
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<tbody>
<tr>
<td>HIV ENV-wt</td>
<td>fwd 5'-TATATAAAGCTTAAGAGAAGATGTTGTCAGAGAGAAAA-3'</td>
</tr>
<tr>
<td></td>
<td>rev 5'-TATAGAATCCAAAGCTCTCTATTCCCACGTCT-3'</td>
</tr>
<tr>
<td>HIV ENV-mut</td>
<td>fwd 5'-TATATAAAGCTTAAGAGAAGATGTTGTCAGAGAGAAAAAAGCCAGTGGG-3'</td>
</tr>
<tr>
<td></td>
<td>rev 5'-TATAGAATCCAAAGCTCTCTATTCCCACGTCT-3'</td>
</tr>
<tr>
<td>DenV pr-M wt</td>
<td>fwd 5'-TATATAAAAGCTTTGTACCACCACAGGAGAACACAGAAGAGAAAAAAGC-3'</td>
</tr>
<tr>
<td></td>
<td>rev 5'-TATAGAATCCACATGTGGAACGAGTGCCACTGCTTTTTTCTCTTCT-3'</td>
</tr>
<tr>
<td>DenV pr-M mut</td>
<td>fwd 5'-TATATAAAAGCTTTGTACCACCACAGGAGAACACAGAAGAGAAAAAAGC-3'</td>
</tr>
<tr>
<td></td>
<td>rev 5'-TATAGAATCCACATGTGGAACGAGTGCCACTGCTTTTTTCTCTTCT-3'</td>
</tr>
<tr>
<td>WNV pr-M wt</td>
<td>fwd 5'-TATATAAAGCTTTGCACAAAAACTCGGCATTCCCGTCGAAGC-3'</td>
</tr>
<tr>
<td></td>
<td>rev 5'-TATAGAATTCATGTGGAACGAGTGCCACTGCTTTTTTCTCTTCT-3'</td>
</tr>
</tbody>
</table>

Note. Sequences of the primers used to amplify corresponding DNA for the appropriate substrate.

Constructs were transferred into retroviral pBMN based transfer vectors by digesting the pBluescript versions of the vectors with XhoI/NotI restriction enzymes and ligating the product into the vectors pBMN.i.Blasticidin and/or pBMN.i.Zeoicin utilizing the XhoI/NotI restriction sites. A map of the constructs is shown in Figure 1.

![Figure 1. Map of assay constructs. The basic scaffold within the retroviral vector includes the prolactin signal sequence (SS), FLAG tag, putative substrate and HA tag fused to the transmembrane domain (TM) of Lyt2, the murine homolog of CD8a. Putative substrates include HIV Env, DenV pr-M, and WNV pr-M. Mutant non-cleavable versions for HIV Env and DenV pr-M included.](image)

CELL MAINTENANCE

Human T-cell line SupT1 was obtained from the American Type Culture Collection (ATCC, Manassas, VA). Cells were maintained in complete RPMI 1640 media supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), glutamine (2 mM), penicillin G (100 units/mL), and streptomycin (100 µg/mL). Phoenix GP
and HEK293T cell-lines (Nolan Lab, Stanford University, CA) were maintained in Dulbecco’s Modified Eagle’s media supplemented with 10% fetal bovine serum (Gemini Bio-Products, West Sacramento, CA), glutamine (2 mM), penicillin G (100 units/mL), and streptomycin (100 µg/mL).

**ANTIBODIES AND REAGENTS**

Anti-FLAG antibody was obtained from Sigma Aldrich (St. Louis, MO). Anti-HA, anti-mouse IgG Alexa Fluor 488, anti-rabbit IgG Alexa Fluor 647, anti-mouse IgG Alexa Fluor 555 and anti-rabbit Alexa Fluor 488 were obtained from Cell Signaling (Beverly, MA). Furin Inhibitor I was obtained from EMD Millipore (Darmstadt, Germany).

**VIRAL PRODUCTION AND TRANSDUCTION**

For the production of Moloney Murine Leukemia virus (MLV) based retrovirus, a 10cm² plate of Phoenix GP cells at 50% confluence was transfected with 3µg of the packaging vector (pBMN plasmids) and 3µg of a vector expressing the Envelope glycoprotein of the Vesicular Stomatitis Virus (pCI-VSVg) by mixing the plasmids in 125µl of serum free DMEM with 30µg of Polyethyleneimine (PEI) (linear, MW 24000; Polysciences, Inc, Warrington, PA).

For the production of HIV based lentivirus, a 10cm² plate of HEK293T cells at 50% confluence were transfected with 3µg of the packaging vector (pTRIPZ plasmids), 3µg of pCI-VSVg, 1.5µg of Viral Protein R-expressing vector (pRSV-VPR), 0.8µg of HIV transactivator (TAT) expressing vector, 0.8µg of HIV regular of expression of virion proteins (REV) expressing vector, and 0.8µg of HIV GAG-POL expressing vector by mixing the plasmids in 125µl of FCS-free DMEM with 30µg of PEI (linear, MW 24000; Polysciences, Inc, Warrington, PA). The TAT, REV, and GAG-POL expressing vectors were kindly provided by Dr. Kenneth Marcu at Stony Brook, NY.

For both MLV- and HIV-based viral production, media (DMEM with 10% FCS, Pen-Strep, L-Glutamine) was replaced 24 hours post-transfection and viral supernatant was collected 48 hours after transfection and filtered through 0.45 micron PTFE filters (Pall Corporation). The supernatant was used to spin-infect naïve HEK293T or SupT1 cells in a six or twelve-well plate format. Briefly, viral supernatant was mixed with polybrene (5mg/mL final concentrations) and added to the cells, the mixture was plated in a six or
twelve-well plate and spun at 1500 x g, 32°C for 80 min in a hanging bucket rotors centrifuge (Becton Dickinson). 24 hours post-infection, fresh media was added to cells.

For TripZ based transduction and knockdown, HEK293T cells were selected 2 days post-transduction with puromycin dyhydrochloride (Santa Cruz Biotechnology, Inc., Dallas, TX) at 2.5µg/ml. Media was replenished every 2-3 days until stable populations were isolated. Doxycycline (Dox), obtained from Sigma Aldrich (St. Louis, MO) was added at 2µg/ml for induction of shRNA.

**FLOW CYTOMETRY AND SORTING**

Cells were pelleted and incubated with mouse anti-FLAG (Sigma Aldrich, St. Louis, MO) and rabbit anti-HA (Cell Signaling, Beverly, MA) at 1:400 dilution for 20 minutes and then washed with phosphate buffered saline (PBS). Cells were then incubated with anti-mouse Alexa Fluor 488 and anti-rabbit Alexa Fluor 647 (Cell Signaling, Beverley, MA) antibodies at 1:200 dilutions for 20 minutes and washed with PBS. For staining and analysis of 96-well plates, cells were incubated with anti-FLAG and anti-mouse AlexaFluor 488 at 1:2500 dilutions. PBS was subsequently added to dilute excess antibody. If necessary, Propidium Iodide (PI) was added at 1µg/ml to measure cytotoxicity. Flow Cytometry and sorting were performed on a BD FACSCanto with 488nm and 633nm lasers and/or FACS Aria with 488nm and 633nm lasers. Data was collected on FACSDiva 6.1.1 and analyzed using FlowJo 7.6.5 or FCS Express 4 Plus.

**FLUORESCENT MICROSCOPY**

SupT1 cells were plated at 1x10⁶ cells per well on a twelve-well plate and treated with DCK or DMSO control. Cells were collected and spun down for immunostaining in suspension. The cells were incubated with mouse anti-FLAG (Sigma) and rabbit anti-HA (Cell Signaling) at 1:200 for one hour and then washed with PBS. The cells were then incubated with anti-mouse Alexa Fluor 555 and anti-rabbit Alexa Fluor 488 (Cell Signaling) for one hour and then washed with PBS. After antibody staining, cells were resuspended in 20µl of fluorescence-preserving media and mounted on slides with cover slips. The imaging was performed using Zeiss Axio Observer D1 with an attached Zeiss MRc camera (Zeiss, Oberkochen, Germany) and analyzed using ImageJ software.
**Real-Time Quantitative-PCR**

Quantitative real-time PCR (qRT-PCR) was performed as follows. Total RNA was extracted using TRIzol (Life Technologies, Grand Island, NY), purified and was treated with the RNeasy clean-up kit (Qiagen, Valencia, CA, USA). The purified RNA (400 ng) was reverse transcribed using qScript (Quanta Biosciences, Gaithersburg, MD) and qRT-PCR was performed using the CFX96 (Bio-Rad, Hercules, CA) and SYBR Green. The forward primer 5’-GCAAAAGCGACGGACTAAACG-3’ and reverse primer 5’-TGGAGACCACAATGCGGT-3’ were used to amplify Furin. GAPDH was used as a reference gene. Relative expression levels were compared before and after induction of shRNA with Dox with the formula (1 + amplification efficiency)\(^{\Delta(\Delta CT)}\) and the experiment was done in triplicate.
CHAPTER 3

RESULTS

DEVELOPMENT OF THE ASSAY AND ESTABLISHMENT OF CLONAL POPULATIONS

The assay relies on the expression of an engineered protein scaffold that travels to the cell surface. The scaffold contains the prolactin signal sequence at its amino terminus to ensure that it is targeted to the ER, the first step required for transport through the classical secretory pathway (see Figure 1). Downstream of the signal sequence, a cassette containing the FLAG and HA tags, flanking a putative protease substrate, was introduced (see Figure 1). Finally, the transmembrane domain (TM) of the murine CD8a glycoprotein receptor homolog Lyt2 was added at the C-terminal tail of the fusion (see Figure 1). A previously engineered Lyt2 TM tagged with the FLAG epitope was demonstrated to travel to the cell surface (data not shown), providing the appropriate scaffold for the assay. A four glycine linker was introduced between the HA tag and TM to increase flexibility.

The engineering of the scaffold, in such a way, ensures proper localization of the substrate within the luminal face of the ER/Golgi/TGN, as it travels through the secretory pathway. If transport is jeopardized, no tags will be detected on the cell surface (see Figure 2 [27], pathway 1). In the event of cleavage the FLAG-tag is released and only the HA tag can be detected on the cell surface (see Figure 2 [27], pathway 2). Conversely, if protease recognition/cleavage is blocked the FLAG-tag is retained and both tags travel to the surface (see Figure 2 [27], pathway 3). Tag presentation can subsequently be monitored through staining with fluorescently coupled antibodies and analyzed by flow cytometry and/or microscopy based techniques.

In order to demonstrate robust distinction between cleaved and non-cleaved events, 17 amino-acids comprising the gp120/gp41 boundary of the HXB2 HIV-1 T-tropic prototypic strain were used as substrate to corroborate the utility of the assay, and thus serve as proof of principle. The Env boundary, which is known to be cleaved by Furin and similar PCs, was inserted in-frame between the FLAG and HA-tags (see Figure 1).
Figure 2. Depiction of assay design. Surface recognition through fluorescently labeled antibodies against HA and FLAG as analyzed by flow cytometry. Pathway 1: No transport – neither FLAG nor HA is expressed on the surface. Pathway 2: Cleavage – The FLAG tag is dissociated from the scaffold resulting in surface expression of HA tag only. Pathway 3: No cleavage – The FLAG tag remains associated with the scaffold resulting in surface recognition of both FLAG and HA tags.


The Env boundary-bearing scaffold construct was introduced into a MLV-based retroviral vector used in consequent experiments for stable expression in mammalian cells [48]. A known non-cleavable recognition sequence containing an arginine-to-serine mutation, at the P1 position, was used as control [20]. It is important to mention that this mutant (mut) serves as control for the assay and not to prove Furin specificity. Wild-type (wt) and mutant boundaries are referred to as Env-wt and Env-mut, respectively (see Figure 1). Retrovirally transduced adherent HEK293T cells were analyzed by flow
cytometry and sorted based on HA cell surface expression, ensuring that proper transport to the cell surface occurred within the purified population. This population was clonally sorted into 96-well plates and amplified to create assay-expressing cell lines. HEK293T clones were then analyzed by flow cytometry following staining with anti-HA, anti-FLAG or both antibodies. A secondary allophycocyanin (APC)-coupled antibody was used to detect HA (HA-APC) and a fluorescein isothiocyanate (FITC)-coupled antibody was used to detect FLAG (FLAG-FITC). The analysis showed a dramatic distinction between Env-wt and Env-mut expressing cells. While HA-APC staining was robust with both cell lines (78-93%), it was FLAG-FITC positive only with Env-mut (81% versus 1%) (see Figure 3 [27]). This result was further corroborated by double staining, with 2% of cells co-expressing FLAG and HA in Env-wt cells in contrast to 75% in Env-mut cells (see Figure 3 [27]). Complete abrogation of the Env-mut boundary cleavage nicely corroborates that cleaved and non-cleaved events can be discriminated based on FLAG surface expression.

A similar result was obtained with non-adherent SupT1 T-cell line clones, chosen as a cell type that better mimics the natural milieu of HIV infection. While 98% of Env-mut cells stained for both FLAG and HA tags, only 0.9% Env-wt cells were double-positive (see Figure 4 [27]). Importantly, while Env-wt clones lost most of FLAG surface expression, both Env-wt and Env-mut were positive for HA (88-98%) (see Figure 4 [27]), demonstrating that both scaffolds travel to the cell surface, regardless of proteolytic processing.

**FURIN INHIBITION RECONSTITUTES FLAG SURFACE EXPRESSION**

In an attempt to further demonstrate the robustness of the assay it was important to prove whether a Furin/PC protease inhibitor can reverse the observed trend. For that purpose, SupT1 clones expressing Env-wt were analyzed by flow cytometry following incubation with increasing concentrations of the Furin inhibitor decanoyl RVKR chloromethyl ketone (DCK), known to bind to the Furin catalytic site and block its activity [44]. FLAG surface expression was progressively recovered in the presence of 1µM, 10µM and 50µM DCK for 48 hours at 37°C, increasing from 0.8% at 1µM to 42% and 90% respectively (see Figure 5 [27]). Control DMSO treated cells showed no FLAG surface expression.
Figure 5. Flow cytometry analysis of the dose dependent inhibition of SupT1 cell lines. Top panels: Naïve cells, treated with 0, 1, 10, 50 μM DCK. Bottom panels: As above with cells expressing minimal HIV wild-type boundary. DMSO treated cells showed no FLAG surface expression (data not shown).


expression (data not shown). These results proved the assay to be robust and enable clear discrimination between cleaved and non-cleaved events.

To further corroborate the robustness of the assay and utility to imaging-based plate readers, the assay was analyzed by microscopy. For that purpose, tag surface expression of the same clones was further analyzed through fluorescence microscopy prior to and following incubation with 50μM DCK. Env-mut was used as control. As seen in Figure 6 [27], Env-mut clones are both green (HA-FITC staining) and red (FLAG-Cy3 staining), while clones expressing Env-wt are only green, corroborating the flow data. Importantly, when treated with DCK, FLAG staining is restored as detected by red fluorescence. These results confirm that the assay can be utilized for imaging-based techniques as well as flow cytometry.
Figure 6. Fluorescent microscopy of surface staining. SupT1 cells were stained with anti-FLAG-CY3 and anti-HA-FITC. Top Panels: Cell line expressing wild-type boundary. Middle Panels: Cell line expressing wild-type boundary treated with 50 µM DCK. Bottom Panels: Cell line expressing mutated boundary. BF: Bright Field. Source: Stolp ZD, Stotland A, Diaz S, Hilton BJ, Burford W, et al. (2013) A novel two-tag system for monitoring transport and cleavage through the classical secretory pathway - adaptation to HIV envelope processing. PloS One 8: e68835. doi:10.1371/journal.pone.0068835.

ADAPTATION TO HIGH-THROUGHPUT SCREENING

In order to facilitate drug discovery, it is advantageous to adapt the assay to high-throughput platforms. Therefore, to expand the assay’s capabilities, the staining procedure was calibrated for 96-well plates. In attempt to facilitate ease of analysis and preparation of samples, as well as decrease cost of antibodies for large scale screens, the staining procedure was further calibrated to avoid washing of cells. Washing of cells is often necessary to discard excess antibody and increase signal to noise ratios. In such a way, a highly calibrated small amount of antibody can be used with minimal non-specific/background staining, without washing. It is important to note that this adaptation likely relies on antibodies with high affinities for their epitopes.

Accordingly, 96-well plates containing SupT1 Env-wt cells treated with DMSO or 50µM DCK were stained for FLAG-FITC, without washing, to determine if the assay and read-out were suitable for high-throughput analysis. When analyzed for mean fluorescent intensity (MFI), the plate treated with DCK had a value of 419.65 with a standard deviation
of 30.34 (see Figure 7). Conversely, cells with DMSO vehicle control had a lower value of 123.76 with a standard deviation of 12.20 (see Figure 7). In order to assess robustness, repeatability and reproducibility of assays, Z-factors are calculated with values between 0.5 and 1.0 deemed excellent for HTS and analysis, where 1.0 is the ideal assay. When calculated based on MFI values, the calibrated staining yielded a Z-factor of 0.57 (see Figure 7). Similarly, when plates were analyzed for percent positive for FITC, cells treated with DCK were on average 65.79% positive with a standard deviation of 3.8% (see Figure 7). However, cells treated with vehicle control yielded on average 3.85% positive with a standard deviation of 1.61% (see Figure 7). Further, when calculated based on percent fluorescence, the calibrated staining yielded an impressive Z-factor of 0.74 (see Figure 7). In addition, it is likely that with the use of robotics the Z-factor will most probably increase. As such, the assay can readily be used for HTS and drug discovery.

Figure 7. Flow cytometry analysis to demonstrate adaptability for HTS. 96 well plates analyzed by flow cytometry for FLAG surface expression. SupT1 cells expressing HIV Env-wt treated with and without inhibitor (50 µM DCK) were stained for FLAG without washing, and assessed for repeatability and reproducibility. Z-scores were calculated for MFI (upper) and %FITC (bottom).
High-throughput and high-content analyses produce large quantities of data. In order to streamline data analysis, flow cytometry analysis software can quickly generate heat-maps of 96-well plates. Heat-maps can be used to rapidly sift through large datasets to pinpoint hits. As such, hits can then be validated using secondary and tertiary assays. To demonstrate such data analysis, a preliminary screen was performed on a chemical library, kindly provided by Dr. Clemencia Pinilla and Torrey Pines Institute for Molecular Studies (TPIMS). The combinatorial library was supplied with over 1 million chemicals on a 96-well plate and with up to 200,000 chemicals in a single well. In this format, hits can be isolated through subsequent deconvolution-based screens. The library was screened at 0.25% final concentration of DMF solvent. SupT1 Env-wt cells were incubated with the library and subsequently stained with FLAG-FITC as well as PI to measure cytotoxicity. Positive and negative controls were included in columns 1 and 12 (see Figure 8). As such, heat-maps with a green-to-red scale were generated for both FITC MFI and cytotoxicity, where green indicates a negative value and red indicates a positive value (see Figure 8).

![Figure 8. Heat-maps of a preliminary screen. Screen of TPIMS compound library in 96-well format. SupT1 Env-wt cells were incubated with compounds and stained with FLAG-FITC and PI. A green-to-red scale was used for the analysis. Left panel: red indicates high levels of cytotoxicity while green indicates low levels of cytotoxicity. Right panel: red indicates FLAG positive while green indicates FLAG negative. Controls are in columns 1 and 12.](image_url)

While further experiments are necessary to determine appropriate concentrations to screen each well and compound, the preliminary screen, along with Z-factor calculations, provides a proof-of-concept that the assay is suitable for HTS and drug discovery. Further, the assay is readily adaptable to larger scale, single compound per well screens. The assay is
also currently being adapted to high-throughput microscopy based techniques through collaboration with Memorial Sloan Kettering Cancer Center (MSKCC) (data not shown).

**ADAPTATION TO A MULTIPLEXED PLATFORM**

To further expand the utility and high-throughput capabilities, the assay can be adapted to a multiplexed platform. Multiplexing allows for the analysis of multiple samples at the same time. In order to multiplex, the power of genetic engineering to barcode individual cells with genes encoding fluorescent proteins was exploited [50,51]. Through the use of retroviral technology, cells were engineered to express different fluorescent proteins to establish distinguishable populations within a mixture [50,51]. Cells were sorted and amplified based on different intensities of fluorescent proteins [50,51]. In such a way, three distinct populations of cells expressing different intensities of td Tomato: negative, dim, and bright can be separated (see Figure 9 [50,51]). Thus, genetically barcoded SupT1 cells, recently developed in the laboratory, were engineered to express different substrates in the context of the assay [50,51]. To that point, the HIV Env-wt boundary was assigned into negative td Tomato cells, HIV Env-mut into dim td Tomato cells, and DenV pr-M wt into bright td Tomato cells [50,51]. When the samples were mixed and stained for FLAG-FITC and HA-APC, a mixed population of single (HA only) and double (FLAG and HA) positive was detected (see Figure 9 [50,51]). However, when analyzed in the PE channel for td Tomato, it was possible to track back and decode which populations gave distinct phenotypes [50,51]. In this case, the HIV Env-mut (dim td Tomato) provided a non-cleaved phenotype as previously seen, while both HIV Env-wt (negative td Tomato) and DenV pr-M wt (bright td Tomato) demonstrated a cleaved phenotype, as expected (see Figure 9 [50,51]). Thus, the assay is readily adaptable to a multiplexed platform and monitor cleavage of multiple substrates in the same sample.

The utility of the assay can be expanded by adapting the multiplexed platform to high-throughput analysis. To that point, multiplexed cells were incubated with and without DCK inhibitor in a 96-well format, as previously described. As a mix, FLAG positive populations were readily isolated. Without inhibitor, the HIV Env-mut (dim td Tomato) was
Figure 9. Adaptation to multiplexed platform through genetic barcoding. A. A mixed population of SupT1 cells expressing td Tomato at different intensities (left dot panel). B. Barcoded assay cells were stained with FLAG-FITC and HA-APC. The HA-single positive and the HA and FITC-double positive populations were gated and analyzed in the PE channel to determine their barcoded identity. Source: Smurthwaite CA (2013) Fluorescent genetic bar-coding for biological applications: Adaptation for monitoring HIV-1 PR variants in mammalian cells [Thesis]. San Diego State University. Available: http://scholarworks.calstate.edu/handle/10211.10/4968. Accessed 4 January 2014.

FLAG Positive while HIV Env-wt (negative td Tomato) and DenV pr-M wt (bright td Tomato) were negative, as expected (see Figure 10). Upon addition of DCK, HIV Env-wt cells reconstituted FLAG surface expression (see Figure 10). However, interestingly the DenV pr-M wt cells remained negative for FLAG suggesting that Furin is not the enzyme responsible for cleaving DenV pr-M (see Figure 10).

**ELUCIDATING BIOLOGICAL MECHANISMS AND PINPOINTING SPECIFICITY**

To address the lack of FLAG surface recovery in the DenV pr-M wt cells upon treatment with 50 µM Furin inhibitor, higher concentrations of inhibitor were tested. Surprisingly, even in the presence of 100 µM and 150 µM DCK, in which cells began to show cytotoxicity, DenV pr-M wt cells failed to recover FLAG expression, while HIV Env-wt cells showed robust recovery (data not shown). In addition, genomic DNA from DenV pr-M wt cells was isolated and verified for correct insertion and sequenced to eliminate the
Figure 10. High-Throughput analysis of multiplex. Non-fluorescent, dim and bright td Tomato expressing SupT1 cells are used to express different substrates for the assay that monitors cleavage in the secretory pathway. Non-fluorescent cells contain the HIV Env-wt boundary. Dim td Tomato cells contain the HIV Env-mut boundary. Bright td Tomato cells contain the DenV pr-M boundary.

possibility that mutations occurred in the engineered retrovirally inserted sequence. Sequencing proved this was not the case. The results thus suggest that Furin is not involved in the cleavage of the DenV pr-M boundary, at least in the context of the assay within SupT1 cells.

Preliminary transfections into adherent HEK293T cells, showed that DenV pr-M exhibited a distinct phenotype when compared to HIV Env-wt (see Figure 11). Two independent analyses of DenV pr-M exhibited a unique pattern in which low HA expressing cells are FLAG negative, while high HA expressing cells are FLAG positive (see arrows in Figure 11). This phenotype was absent in HIV Env-wt (see Figure 11). This specific pattern is most probably due to over-expression of the substrate within the secretory pathway, which will most likely overwhelm the pathway and the enzyme that recognizes/cleaves the DenV pr-M boundary. It is well established that DenV pr-M is inefficiently processed which may be in accordance with the observed phenotype [43]. Nonetheless, these results highly
Figure 11. Analysis of transfections into HEK293T Cells. Cells were transfected with HIV Env-wt, HIV Env-mut, and DenV pr-M constructs and stained for FLAG-FITC and HA-APC. Two independent experiments were performed: top and bottom panels. Arrows in DenV pr-M plots highlight the unique pattern.

suggest that the protease that recognizes and cleaves HIV Env is different than that cleaves DenV pr-M, at least in the context of the assay.

In order to further investigate specificity of substrate recognition and proteases involved in the process of cleavage, the assay was coupled with RNA interference experiments. For that purpose, HEK293T cells expressing the assay were infected with lentiviral inducible shRNA particles and subsequently selected with puromycin. After stable populations were selected, Furin (shFurin) or non-silencing (shNon) negative control shRNAs were induced with Dox and analyzed 48 hrs later. A significant knockdown of Furin mRNA was obtained, as quantified by qRT-PCR (see Figure 12). Further, cells were stained with FLAG-APC and analyzed by flow cytometry. Upon knockdown of Furin, approximately 60% of HIV Env-wt cells recovered FLAG surface expression 48 hrs post induction (see Figure 12). Conversely, FLAG surface expression was not seen in DenV pr-M expressing cells (see Figure 12). Tracking FLAG recovery over time after shFurin induction showed that nearly 80% of HIV Env-wt cells were FLAG positive by day 3 post induction (see Figure 12). Impressively, when gating based on tRFP expression as a marker for shRNA induction, nearly all HIV Env-wt cells recovered FLAG surface expression following Furin knockdown (analysis not shown). However, once again, DenV pr-M cells did not
Figure 12. Knockdown of Furin in HEK293T cell lines. Cells were transduced with lentiviral shRNA particles and selected by puromycin. A. Cells were analyzed after induction with doxycycline by qRT-PCR for Furin. B. Flow cytometry analysis after staining with FLAG-APC for induced and non-induced shRNAs. C. Flow cytometry of FLAG surface recovery over time comparing HIV Env-wt and DenV pr-M.

respond to Furin knockdown at any time point, suggesting that another protease rather than Furin is cleaving the pr-M boundary.

In order to further elucidate DenV pr-M processing, more relevant control constructs are needed. As such, a DenV pr-M mut was engineered with a deletion of the P1 arginine, which is required for Furin/PC based cleavage (see Figure 1). Additionally, a construct containing the pr-M boundary of related WNV was developed for comparison (see Figure 1). It is known that while the viruses are related, DenV exhibits inefficient pr-M cleavage, while WNV exhibits robust pr-M cleavage, with important biological significance for viral infection in vivo [41]. Transfection into HEK293T cells, revealed that the DenV pr-M mut demonstrated a robust non-cleaved phenotype, while the DenV pr-M wt exhibited a similar unique pattern as seen previously (see Figure 13). This suggests that while Furin may not be the protease recognizing this boundary, the putative protease still requires the P1 arginine for cleavage. Further, WNV pr-M exhibited a robust cleaved phenotype when compared to DenV, corroborating published data. Nonetheless, the rate of cleavage and pattern displayed remained distinct from HIV Env-wt, as once again high HA expressing cells showed low levels of FLAG expression. The preliminary transfections support the notion that there is a distinct pattern of cleavage between HIV Env and Flaviviral pr-M boundaries. However, stable expressing clones are needed to further elucidate the mechanisms involved.
Figure 13. Transfection of additional substrates into HEK293T cells. Cells were stained for FLAG-FITC and HA-APC and subsequently analyzed by flow cytometry. Arrows indicate unique pattern.
CHAPTER 4

DISCUSSION

CONCLUSION

A novel in vivo cell-based assay that can discriminate between cleaved and non-cleaved events that occur in the ER/Golgi/TGN apparatus was developed. The engineering of a scaffold protein that utilizes the classical secretory pathway and travels to the cell surface was proven to be the perfect tool to monitor transport and cleavage events known to occur in the ER/Golgi/TGN compartment. The cleavage of the minimal gp120/gp41 HIV-1 envelope boundary (Env-wt) between the HA and FLAG tags was used to validate the efficacy of the cell based assay and was readily assessed in a very robust and straightforward manner. A single arginine-to-serine point mutation within the gp120/gp41 HIV-1 boundary (Env-mut) completely abrogated cleavage, as demonstrated by strong FLAG surface expression. The robustness of the assay was further demonstrated with Env-wt, where a complete loss of FLAG surface expression was observed. Importantly, in the presence of the known Furin/PC protease inhibitor DCK, a total reconstitution of FLAG surface expression was observed, corroborating that the loss of FLAG was specifically due to the cleavage of the substrate by Furin/PCs while traveling to the cell surface. While the minimal Env boundary is not intended to mimic the complex three dimensional and trimerization requirements of the HIV Env that occurs in vivo, it was intended to prove the utility of the assay. The results clearly demonstrate that the assay permits the assessment of Furin-mediated processing. Further, while the assay does not address enzyme specificity, it is intended to monitor cleavage in a robust manner, it does suggest Furin-based processing, as shown by the complete abrogation of cleavage in the presence of a single point mutation that disrupts the well-conserved Furin recognition/cleavage site (see Table 1). Nonetheless, enzyme specificity is easily investigated by utilizing the assay in conjunction with RNA interference-based technologies or knockout cell-lines.

The knockdown of Furin in HIV Env-wt cells resulted in FLAG surface recovery similar to when treated with DCK. These results demonstrate that the assay when coupled with knockdown or knockout technologies can pinpoint proteases that specifically cleave the
substrate engineered in the assay. In this case, the results support literature in the notion that Furin processes the HIV Env boundary.

**Adaptability to Other Substrates**

While the HIV envelope gp120/gp41 boundary is a well-recognized target of Furin/PCs, it was chosen as proof of principle in part due to the impact of HIV-1/AIDS. The simplicity of the assay allows for its adaptation to any other substrate of choice, provided cleavage occurs within the ER/Golgi/TGN compartment. The assay can thus provide a platform for monitoring the cleavage of proteins from viral, bacterial or eukaryotic origin, processed and/or cleaved during transport from or residency within the ER/Golgi/TGN compartment. Table 1 shows, as example, some of the known Furin substrates that could be studied with the assay. However, this assay system targets all the enzymes that cleave within the luminal face of the ER/Golgi/TGN compartment, including non-Furin PC and SP family members, or the β-site amyloid precursor protein cleaving enzyme (β-secretase), critical for the onset of Alzheimer’s disease [10,12,17].

To proof ease of adaptability, the assay was engineered to monitor DenV pr-M processing by Furin. Cell lines expressing the DenV pr-M boundary in the context of the assay exhibited a cleaved phenotype, but interestingly, did not respond to DCK treatment or knockdown of Furin. The results suggest that Furin is not involved in DenV pr-M cleavage. Due to the complexity of PCs, it is possible that this is cell-type dependent and/or that another PC, other than Furin, processes DenV pr-M. Nonetheless, it is established that DCK often inhibits other members of the PC family, which should then have resulted in the recovery of FLAG. This suggests that perhaps a non-PC protease cleaves DenV pr-M. It is important to note that deletion of the P1 arginine in the pr-M cleavage site reversed the phenotype to be non-cleaved in the context of the assay. This implies that cleavage still occurs at the same residue and that the putative protease is likely to be of serine-type. However, in order to pinpoint the enzyme involved, these results need to be replicated in a more natural context of DenV infection, such as hepatocytes. The assay can then be coupled with RNAi technologies to knock down all proteases within the secretory pathway in order to pinpoint the putative protease. In addition, it remains to be seen whether Furin can be conclusively eliminated as the protease that processes pr-M in viral infection.
Nonetheless, the assay was further able to distinguish known substrates that are cleaved efficiently and inefficiently. Despite similar amino-acid sequences, the WNV pr-M and DenV pr-M boundaries showed a remarkable distinction in cleavage efficiency, which correlates with published data [25,41–43]. These results further highlight the utility of the assay to elucidate complex biological questions such as Flaviviral pr-M cleavage.

Utility for Drug Discovery

The assay represents an *in vivo* cell-based platform for drug discovery as it can be exploited for the screen of inhibitors/competitors against the process of cleavage of any substrate of interest and/or against the activity of the enzymes and their co-factors responsible for cleavage. The robustness and simplicity of the assay is an asset for drug discovery screening. The cell-based platform can utilize flow cytometry and/or microscopy to assess the activity of proteolytic enzymes that reside or function in the secretory pathway. As the phenotypic outcome is coupled to the cell, the assay allows for the sorting and amplification of single cells, and as such the assay is distinguishable from other existing assays. The coupling of the phenotype to the individual cell, in contrast to assays that rely on secretion of enzymatic/fluorogenic substrates, can be exploited to screen endogenously expressed/targeted peptide libraries or cells harboring cDNA or siRNA libraries. Naturally, the assay allows for the screening of chemical compound and/or combinatorial libraries as well. The robust and quantitative results obtained with the HIV-1 Env boundary make the assay an attractive method to screen for Furin competitors against Env recognition and/or cleavage to discover novel anti-viral compounds targeted against HIV. Moreover, as the versatility of the assay allows the replacement of the chosen substrate, the assay can be used for drug discovery against a wide array of targets involved in diseases that rely on the secretory pathway for transport and/or cleavage.

Further, the assay was shown to be adapted to a multiplexed platform. In such a way, the assay can monitor multiple substrates at the same and as such expand the utility of HTS. In addition, a multiplexed platform can allow comparison and/or analysis of the effectiveness of inhibitors against similar substrates. An ideal multiplex in the context of the assay would be for instance, the analysis of pr-M cleavage across all four DenV serotypes or other members of the Flavivirus genus.
Assessment of Transport through the Classical Secretory Pathway

In the assay, the HA tag is used to evaluate proper transport to the cell surface, while the FLAG tag is used to evaluate cleavage. As such, while these results focused on the presence or absence of FLAG on the cell surface as a correlate of substrate cleavage, the assay could also focus on the absence or presence of the HA tag for the analysis of factors strictly required for transport or affecting the secretory process with no role in proteolytic processing. Thus, the assay can be used in conjunction with siRNA-based screens to identify regulators and effectors involved in transport.

Adaptability to Other Tags

The assay is based on the HA and FLAG independent tags, chosen for the availability of widely utilized, affordable fluorescent-coupled antibodies. The assay should be adaptable to any tag or fluorescent protein of choice, provided they are recognizable on the cell surface by flow cytometry or microscope imaging-based techniques and can be engineered to flank both sides of a putative substrate. For instance, a fluorescent protein such as green fluorescent protein (GFP) could substitute for the FLAG tag, increasing versatility of the assay for flow-cytometry-microscopy imaging-based coupled technologies, where GFP localization would serve as biosensor for processing. Similarly, the laboratory has previously reported an in vivo cell based assay for HIV protease activity based on the inducible activation of a GFP expression cassette in cell nuclei [52,53].

In conclusion, the versatile and robust in vivo cell-based assay combines both cleavage and transport. It can thus be exploited to study the proteins and factors that either affect or are required for protein cleavage-processing/maturation in the ER/Golgi/TGN compartment. On the other hand, as the assay relies on a scaffold protein that travels to the cell surface, it can be used to learn about the proteins and factors that are required for transport to the cell surface.

FUTURE DIRECTIONS: DRUG DISCOVERY AND STUDY OF FLAVIVIRAL PR-M PROCESSING

The assay, as adapted to HTS and multiplexing, will be used to screen preliminary chemical libraries such as the Prestwick library, which contains FDA approved drugs. While
this screen is intended as proof-of-principle to demonstrate screening capabilities, the assay is ultimately intended to screen large scale libraries such as those involved in the Molecular Libraries Program of NIH.

In order to elucidate on the results of DenV pr-M, it is important that the results be replicated in the context of viral infection, independently of the assay. Nonetheless, the assay will be adapted to the four serotypes of DenV as well as clinically relevant strains provided from Dr. Amilcar Tanuri (Universidade Federal do Rio de Janeiro, Brazil). Further, the assay will be adapted to study pr-M across all relevant Flaviviruses. Once adapted, the assay will be used to screen siRNA libraries as well as chemical libraries. Further, it is intended that large scale studies will be coupled with a bioinformatics approach through Dr. Rob Edwards of Computational Sciences (San Diego State University).

The assay coupled with the aforementioned technologies/screens should elucidate complex biology on the secretory pathway and viral infection.
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REFERENCES


APPENDIX

LIST OF ABBREVIATIONS AND ACRONYMS
**AIDS**: Acquired Immunodeficiency Syndrome

**APC**: Allophycocyanin

**BD**: Becton Dickinson

**BF**: bright field

**cDNA**: Complementary DNA

**cm**: centimeter

**DCK**: Decanoyl RVRK chloromethyl ketone

**DMEM**: Dulbecco’s Modified Eagle Medium

**DMF**: dimethylformamide

**DMSO**: dimethyl sulfoxide

**DNA**: Deoxyribonucleic Acid

**Dox**: Doxycycline

**E**: Flaviviral envelope protein

**ECM**: Extra Cellular Matrix

**ER**: Endoplasmic Reticulum

**FDA**: Food and Drug Administration

**FITC**: Fluorescein isothiocyanate

**FSC**: Forward Scatter

**fwd**: forward

**GFP**: Green Fluorescent Protein

**gp120**: HIV glycoprotein 120

**gp41**: HIV glycoprotein 41

**HA**: Hemagglutinin

**HEK 293T**: Human Embryonic Kidney

**HIV**: Human Immunodeficiency Virus

**hrs**: hours

**HTS**: High throughput screening

**Lyt2**: Mouse CD8a

**JEV**: Japanese Encephalitis Virus

**M**: Flaviviral membrane protein

**MFI**: Mean fluorescence intensity
**mL**: milliliter
**MLV**: Moloney Leukemia Virus
**min**: minutes
**MSKCC**: Memorial Sloan Kettering Cancer Center
**mut**: mutant
**ng**: nanogram
**NIH**: National Institute of Health
**nM**: nanomolar
**nm**: nanometer
**PC**: Proprotein convertase
**PCR**: Polymerase chain reaction
**PE**: Phycoerytherin
**PEI**: Polyethylenimine
**Phoenix GP**: Phoenix gag-pol
**PI**: Propidium Iodide
**pr-M**: Flaviviral pre-membrane protein
**REV**: HIV regular of expression of virion proteins
**rev**: reverse
**RNA**: Ribonucleic Acid
**RNAi**: RNA interference
**RPMI-1640**: Roswell Park Memorial Institute 1640 media
**shFurin**: small hairpin RNA specific for Furin
**shNon**: non-silencing small hairpin RNA using as negative control
**shRNA**: small hairpin RNA
**siRNA**: small interfering RNA
**SP**: signal peptidase
**SPP**: signal peptide peptidase
**SS**: signal sequence
**TAT**: HIV transactivator
**TBEV**: Tick-Borne Encephalitis Virus
**TGN**: Trans Golgi Network
TPIMS: Torrey Pines Institute of Molecular Studies

tRFP: turbo Red Fluorescent Protein

µg: microgram

µl: microliter

µM: micromolar

Vpr: Viral protein R

VSVg: Vesicular Stomatitis Virus glycoprotein

WHO: World Health Organization

WNV: West Nile Virus

wt: Wild type

YFV: Yellow Fever Virus