Utilizing homogeneous protein-protein interaction assays for virology research

A review of current applications

Abstract

Protein-protein interactions are important within various stages of the viral life cycle. Homogeneous assays are well suited to study these interactions and screen for inhibitors that could be used as anti-viral therapeutics. These types of assays have been used to look at protein-protein interactions involved in viral entry, replication, assembly and viral targeting of host cell defense mechanisms. Here we review recent publications and show the homogeneous assay set-ups used in a variety of different studies. We present the outcomes of each study and how the information gathered could lead to antiviral therapies.

Assay workflow

The first step in the viral life cycle is viral attachment to the surface of the target cell using both non-specific and specific interactions with cell surface receptors. The virus enters the cell via endocytosis, membrane fusion, or genome injection. Uncoating of the virus occurs where the viral proteins and genome are released and infect the host cell. Next, the viral genome undergoes replication and protein synthesis using the host cell machinery. Finally, the newly made proteins reassemble with the viral nucleic acid in the host cell and the new virions are released into the host organism (Villanueva et al.). Figure 1 shows various stages within the viral life cycle where protein-protein interactions play a key role. The protein-protein interactions at each of these stages could be used as targets for antiviral therapeutics.

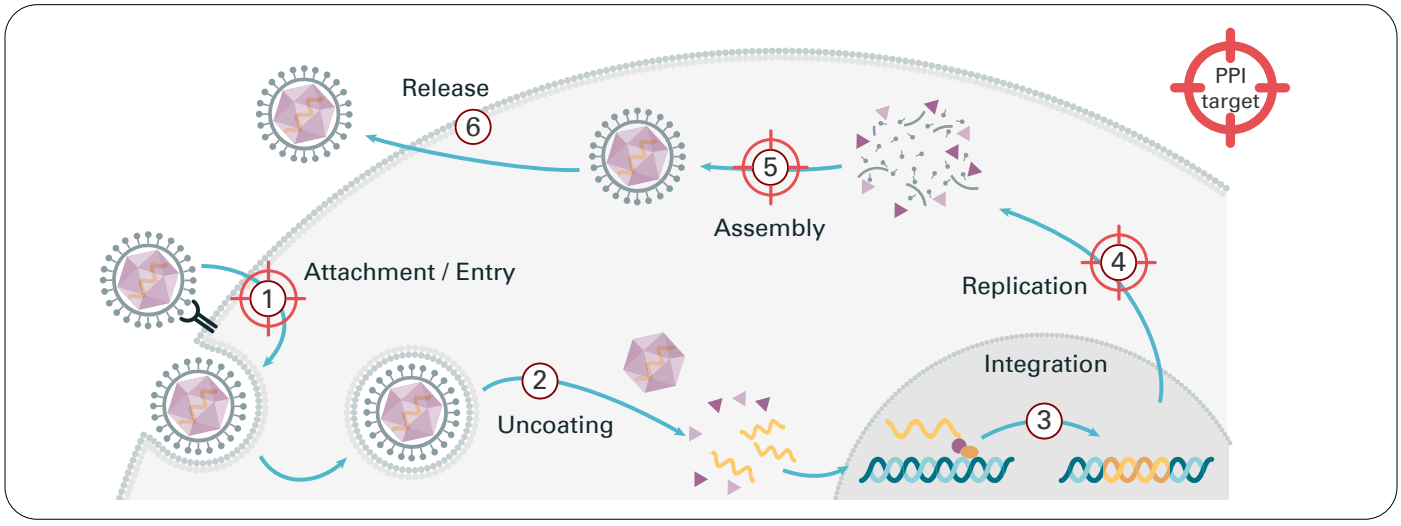

Figure 1: Generic viral life cycle diagram highlighting some of the protein-protein interactions that could be used as targets for antiviral therapies.

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Alpha and TR-FRET are two types of homogeneous technologies that have been used to probe protein-protein interactions and design drugs that target various stages of the viral life cycle. AlphaLISA® and AlphaScreen are bead-bead assay technologies that can be used to study a large range of biomolecular interactions in a microplate format. Alpha assays require two types of beads: Donor beads and Acceptor beads. Typically, each bead is conjugated to a protein or antibody used to capture one of the targets in the biomolecular interaction assay. When the two biomolecules interact, the Donor bead is brought into proximity of the Acceptor bead and excitation of the Donor bead results in a luminescent signal from the Acceptor bead. HTRF® (homogeneous time resolved fluorescence), a TR-FRET technology, can also be used to assess molecular interactions by coupling two biomolecules with either a donor (long-lived fluorescence) or an acceptor (short-lived fluorescence) fluorophore. When the biomolecules are in proximity, energy transfer between the two fluorescent dyes can be measured and is proportional to the number of biomolecules interacting. Both technologies can detect a broad range of affinities with dissociation constants (KD) ranging from picomolar to low millimolar and since the assays are homogeneous (no wash steps required), transient interactions can easily be measured.

Viral attachment and cell entry

One of the most commonly studied protein-protein interactions in virology is the viral protein-host cell receptor interaction. Blocking entry into the cell is one of the most effective ways to fight viral infection as evidenced by how the immune system creates antibodies that can block viral entry (Klimpel et al.). Once the host cell proteins that interact with the virus are identified, recombinant viral proteins and recombinant host cell proteins can be purified and used in high throughput homogeneous assays to show such interactions and identify inhibitors of the interaction.

The influenza hemagglutinin (HA) is a glycoprotein on the surface of the virus that binds the host cellular membrane at low pH (Kadam et al.). Omi et al. used a competition AlphaScreen protein-protein interaction assay to discover peptides that interfere with the binding between HA and a receptor mimic, α2–3-sialyllactose-PAA. They discovered a tetravalent peptide that targeted the HA protein for degradation. Broadly neutralizing antibodies (BnAbs) targeting the HA protein have also been used to develop therapeutics (Kadam et al.). AlphaLISA has been used to screen for small molecule alternatives to neutralizing antibodies (NAb) which use a similar binding mechanism. Kadam et al. used a competition assay with a neutralizing antibody mimic protein (NAb mimic) to screen for peptides that bind the HA protein in a similar fashion as the broadly neutralizing antibodies (Figure 2). Von Dongen et al. used a similar assay to screen for small molecules that could bind the same epitope as the broadly neutralizing antibodies. In both cases, the authors were able to identify lead drug candidates that could be used as antiviral therapeutics.

Homogeneous assays have also been used to study the mechanism of the viral protein recognition of the host cell receptor. Shang et al. used Alpha to show the isolated spike protein S1 N-terminal domain (NTD) of mouse hepatitis coronavirus (MHV) binds similarly as the S protein (without the transmembrane or intracellular domain) to the host cell receptor CEACEM1a. This suggests that the MHV S1-NTD is already primed to recognize and engage the receptor. This information is important in understanding the spike protein-receptor binding mechanism of action.

Studying the binding of viral proteins to host cell receptors of different species can aid in better understanding of the evolutionary changes that allow viruses to cross species. For example, MERS-CoV coronavirus originated in bats and has adapted to infect humans. In Yang et al., an Alpha homogeneous assay was set up using recombinant spike proteins (S1) from different coronaviruses and either the human or the bat host cell receptor protein (DPP4). Using this assay, they were able to show host protein species preferences of the different viral proteins and speculate on a mechanism of cross-species adaption. Figure 3 shows an example of how tagged viral proteins and tagged host cell receptor proteins can be captured by Alpha beads and used to detect the interaction.

In HIV, the capsid protein stability and integrity are essential in the early stages of infection (Villanueva et al). Specifically, previous studies have shown that capsid protein dimerization is critical for infectivity (von Schwedler et al..). In order to target early stage HIV infectivity, Thenin-Houssier and Zhang et al. both used HTRF to screen for inhibitors of the capsid C-terminal domain (CTD) dimerization. Zhang et al. looked for small molecule alternatives to the previously identified peptide inhibitor, CAI, by screening for inhibitors of the capsid-CAI interaction and identified the compound, TX-1918. Thenin-Houssier set up a slightly different HTRF screen looking for inhibitors of tagged capsid CTD dimerization (Figure 4). Of the large number

of compounds screened, Thenin-Houssier identified a compound, Ebselen, that showed inhibition of capsid dimerization with an IC_{50} of 46.7 nM and they demonstrated it inhibited HIV infectivity.

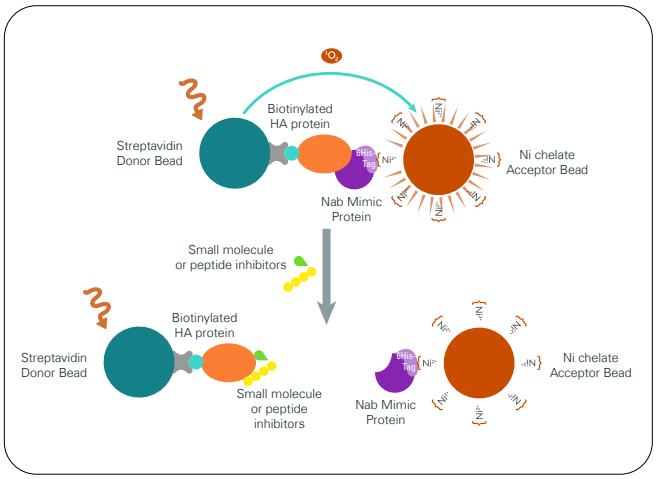

Figure 2: Alpha assay schematic for finding small molecules or peptides that mimic neutralizing antibody binding activity. Binding of small molecule or peptide blocks the protein-protein interaction and results in a loss of signal.

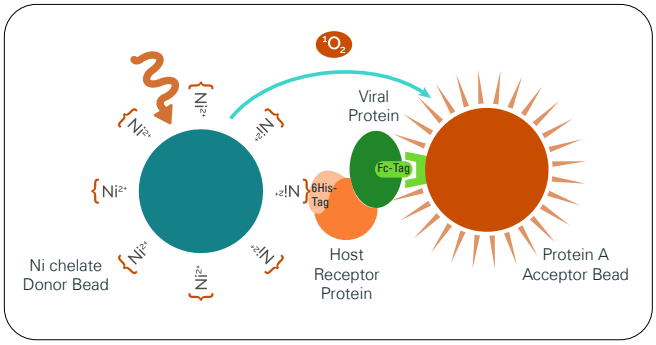

Figure 3: AlphaLISA assay schematic to measure viral proteins binding to host cell receptor proteins. In this schematic, the host cell receptor protein is tagged with a 6XHis-tag and is captured by the Ni chelate donor beads. The viral protein is tagged with an Fc-tag and captured by Protein A Acceptor beads. Upon binding, there is an increase in Alpha signal.

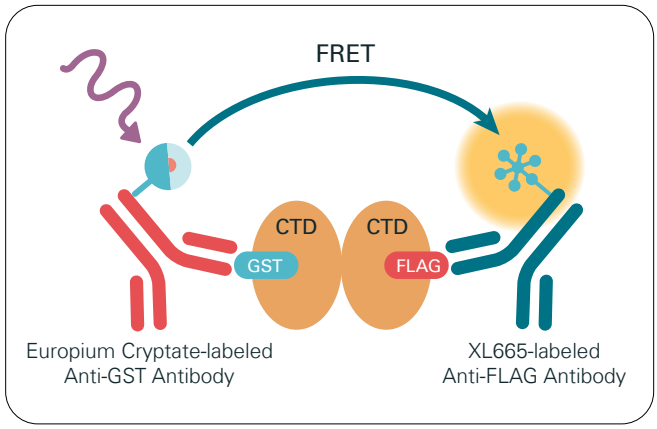

Figure 4: HTRF assay format – CA proteins C-Terminal Domains (CTD) are tagged with GST and FLAG peptide respectively. Anti-GST and Anti-FLAG antibodies are coupled with Eu+ cryptate (donor) and XL665 (acceptor) respectively. The dimerization of CTDs brings the donor and acceptor-coupled antibodies in proximity and triggers FRET.

Viral replication

Many antiviral therapies that inhibit viral replication target the DNA polymerase of the virus. This has had some adverse pharmacological effects and therefore, it has been important to look at other stages within the viral replication process. For example, as part of the human cytomegalovirus (CMV) replication process, the CMV terminase complex cleaves the viral DNA into unit lengths and aids in packaging the DNA with the capsid proteins. Ligat et al. used Alpha to show a conserved sequence within the CMV terminase complex that is critical for the interaction between two of the subunits: pUL56 (which has a critical role in DNA cleavage and packaging) and pUL89 (Figure 5A). A deletion or targeting mutation of this sequence disrupted the complex formation (Figure 5B) and blocked viral replication and propagation in a cell-based assay. They speculated that a peptide containing this sequence could be used as an antiviral therapeutic.

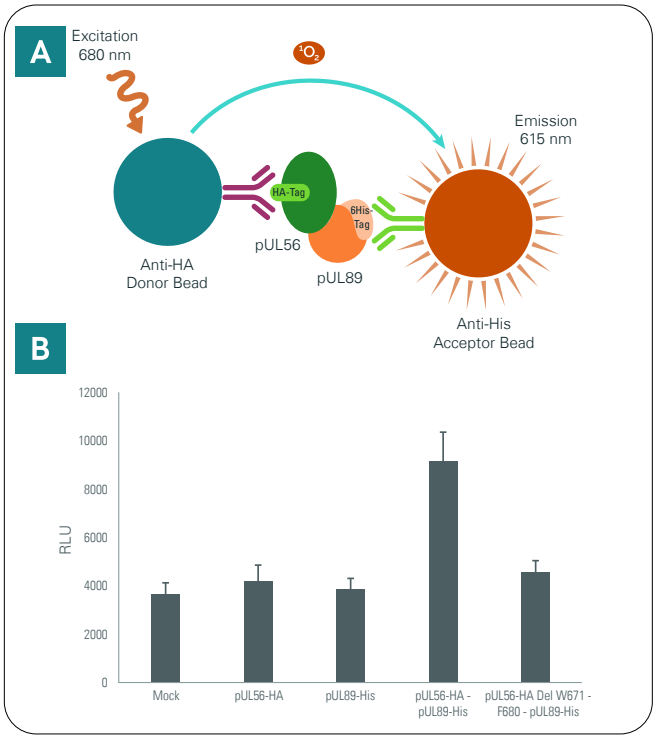

Figure 5: AlphaLISA assay for detecting pUL56 interaction with pUL89. A) AlphaLISA schematic. HA-tagged pUL56 was captured by anti-HA Donor beads and 6XHis tagged pUL89 was captured by anti-His Acceptor beads. When binding occurs, an increase in Alpha signal is seen. When a mutation in one of the proteins prevent binding, a decrease in signal is seen. B) The Alpha assay for the binding of full-length pUL89 with wild-type pUL56 (HA-pUL56) or a deletion mutant of pUL56 (HA-pUL56 Del W671-F680). To show specificity of the signal, each protein was tested in the absence of the other protein and an assay was also performed without any proteins (mock).

After viral entry into the cell, the viral proteins and nucleic acids are shuttled to the nucleus for replication. In the case of the alphavirus Venezuelan equine encephalitis virus (VEEV), this trafficking event is dependent on the viral capsid protein interaction with the host cell nuclear transporting protein importin $\alpha/\beta1$ heterodimer. Since there are no anti-VEEV therapeutics available, this interaction has been targeted as a potential way to inhibit viral replication (Thomas et al.). Thomas et al. performed a high throughput screen using Alpha to look for compounds that inhibit the importin $\alpha/\beta1$ and viral capsid protein interaction. They found a compound, G281-1564, that could inhibit VEEV replication at low micromolar concentrations.

Viral targeting of host cell defense mechanisms

Not only do viruses hijack the host cell machinery for the purpose of replication, they also have adapted to interact with other host cell proteins to suppress the host cell defense mechanisms. For example, in response to HIV infection, the host cell produces the APOBEC3G (A3G) protein. A3G is a cellular cytidine deaminase that represses HIV-1 replication by inducing G-to-A hypermutation in the viral DNA. In turn, HIV expresses the Vif protein which binds to A3G, resulting in its degradation via the 26S proteasome (Pery et al.). Pery et al. performed a high throughput screen of inhibitors of the A3G-Vif interaction using LANCE® TR-FRET. They started with a 307,520-compound library for their primary screen for inhibitors of the binding between the two proteins. They narrowed it down to 311 after dose-response and counter screening and used cell-based assays to identify compounds that could attenuate the Vif dependent degradation of A3G. Finally, they selected the compound N.41 which showed potent antiviral activity by increasing A3G in the cells.

As part of the host cell immune response, protein ubiquitination triggers type I interferon (IFN) responses which upregulate a variety of genes that restrict viral replication. In response to this, some viruses encode deubiquinase (DUB) domains that can remove ubiquitin from proteins that initiate the IFN response. Crimean-Congo hemorrhagic fever virus (CCHFV) encodes a cysteine protease of the ovarian tumor family (OTU) that acts as a DUB to repress interferon production. Scholte et al. used HTRF to confirm binding of a ubiquitin variant (UbV-CC4) to the CCHFV OTU domain as well as a mutated version of the OTU domain. They were able to show that the prolonged interaction of the OTU with UbV-CC4 interfered with the viral RNA synthesis. Their data suggest that the development of antivirals targeting the viral cysteine protease, OTU, may be an effective way to repress viral replication.

The host structural maintenance of chromosome 5/6 complex (Smc5/6) is a protein complex that has evolved to repress hepatitis B virus (HBV)transcription. In turn, HBV has adapted and expresses a protein (HBx) that binds the cellular DNA damage-binding protein 1 (DDB1) and redirects it to degrade the Smc5/6 complex. In Ramakrishnan et al., HTRF was used to characterize the binding between tagged recombinantly expressed HBx and DBB1. Using untagged proteins, they could disrupt the interaction (Figure 6A). Figure 6B (upper) shows the competition data with untagged HBx, untagged DBB1, SV5-V (another viral protein known to bind DBB1), and BSA. Hbox is a peptide containing the region of HBx that binds the DBB1 complex.

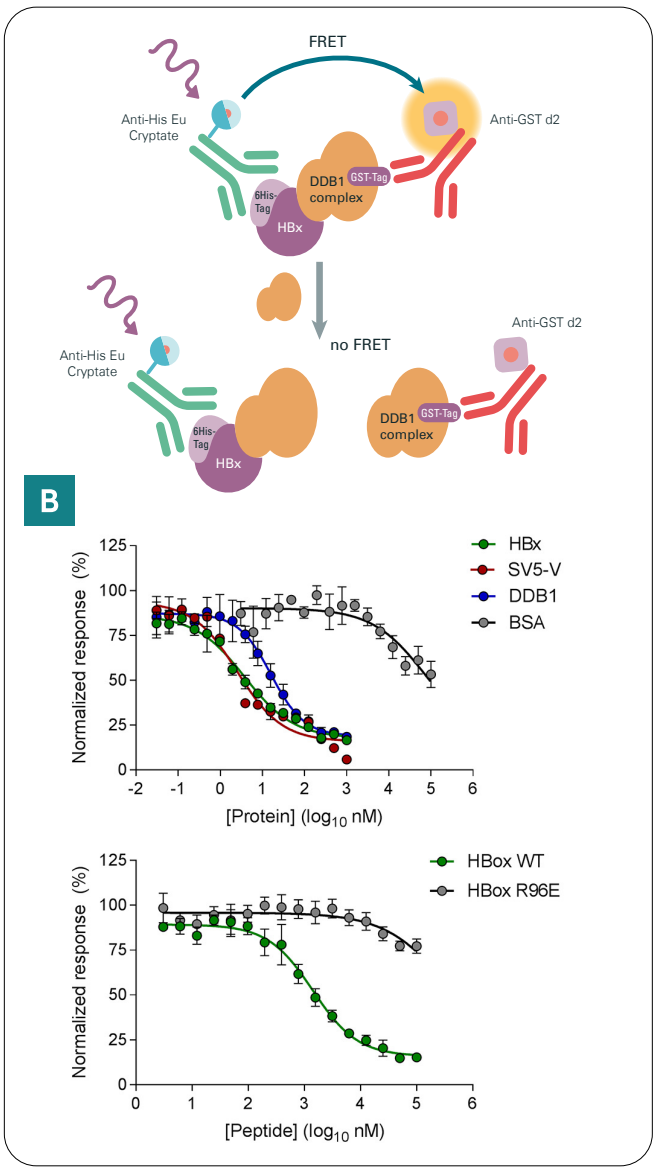

Figure 6: A) HTRF assay for detecting inhibitors of the HBx-DBB1 interaction. The HBx protein is tagged with a 6XHis tag and the DDB1 complex is tagged with a GST tag. Anti-His and Anti-GST antibodies are coupled with Eu+ cryptate (donor) and d2 (acceptor) respectively. The binding of the HBx to the DBB1 complex brings the donor and acceptor-coupled antibodies in proximity and triggers FRET. Then the untagged HBx, DDB1, SV5-V or peptides will interfere with binding resulting in a loss of signal.

B) Results from competition experiments. Competition of the TR-FRET signal in the presence of recombinant untagged HBx, SV5-V, DDB1, and BSA are shown (upper). TR-FRET analysis of the complex in the presence of WT HBx H-box peptide and a mutant Hbox peptide (lower).

Figure 6B (lower) shows competition of untagged wildtype HBox peptide compared with a mutant HBox (R96E) peptide. Various assays were used to identify key regions and residues that were required for the interaction between the viral protein HBx and DDB1. Together, this data demonstrates that HBx interacts with DDB1 in a manner that can be competitively inhibited and can be used to design antiviral therapeutics.

Viral assembly and maturation

Homogeneous assays have also been used to look at the HIV viral protease activity targeting virion maturation. In the later stages of the HIV viral life cycle, upon release of the immature viral particles, HIV maturation is initiated by proteolysis of the capsid proteins by a viral protease. Huang et al. designed an Alpha assay to screen 130 known protease inhibitors to see which inhibitors could suppress the precursor auto-processing of the HIV virions in crude cell lysates (Figure 7A). They were able to confirm multiple known protease inhibitors within the library capable of suppressing the auto-processing at low micromolar concentrations (Figure 7B). By inhibiting the formation of mature virions, they could decrease HIV infectivity. In addition, using AlphaLISA they were able to look at precursors carrying mutations that are known to cause resistance to current protease inhibitors and reproduce the expected resistance.

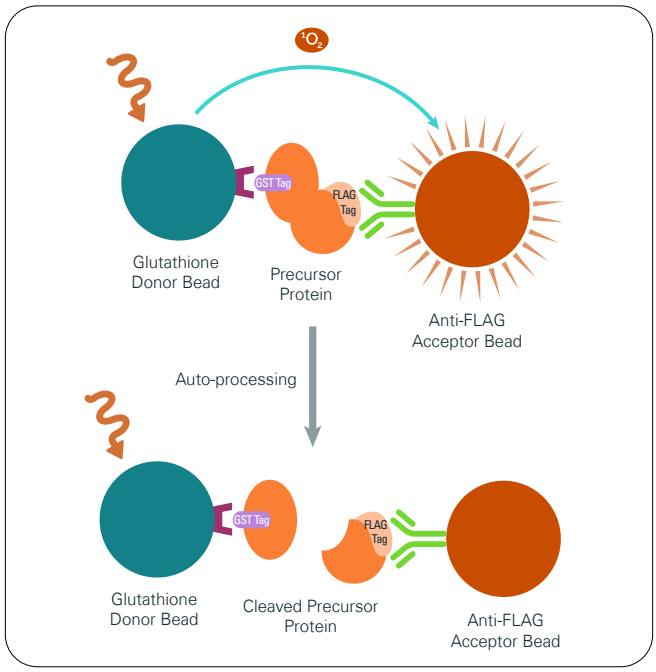

Figure 7: A) Example of AlphaLISA assay used for detecting viral protease activity. The precursor protein is both GST and FLAG tagged. The glutathione donor bead and anti-FLAG acceptor bead bind the protein bringing the beads in proximity. After cleavage, the beads are no longer in proximity, leading to a decrease in Alpha signal. Inhibitors will prevent cleavage and therefore lead to an increase in Alpha signal.

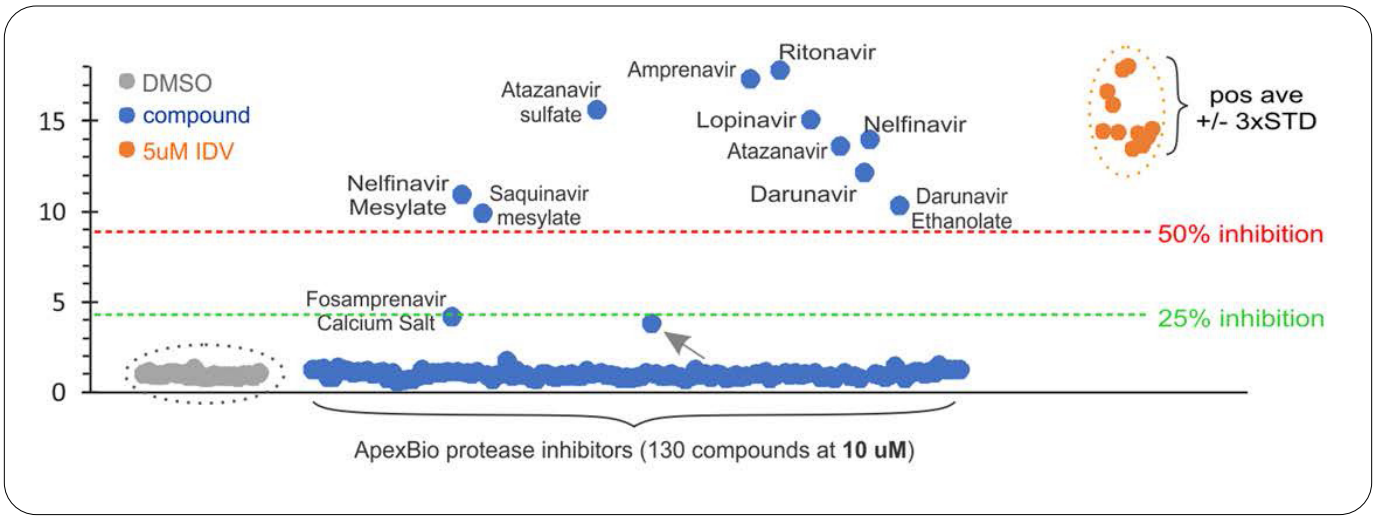

Figure 7: B) AlphaLISA screening of ApexBio protease inhibitor library. AlphaLISA screening at 10 µM with 0.1% DMSO. Y axis is the signal to background in the Alpha assay. Copyright: Huang L, Li L, Tien C, LaBarbera DV, Chen C. Targeting HIV-1 Protease Autoprocessing for High-throughput Drug Discovery and Drug Resistance Assessment. Sci Rep. 2019; 9(1):301. Figure reproduced with permissions conveyed through https://creativecommons.org/licenses/by/4.0/.

Conclusion

Homogenous assay technologies such as Alpha and TR-FRET are simple, fast, and robust methods for studying and screening for inhibitors of protein-protein interactions throughout the viral life cycle. The publications presented here demonstrate the versatility of these assays in discovering and designing the next generation antiviral therapeutics.

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