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TITLE:

Single-Cell Characterization of Calcium Influx and HIV-1 Infection Using a Multiparameter Optofluidic Platform

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KEYWORDS:

high-throughout, optofluidic, live-cell imaging, HIV infection, calcium influx, purinergic receptor

SUMMARY:

Here, we present a protocol in which single cells are monitored for acute events and productive HIV-1 infection on a nanofluidic device. Imaging data define virus-host receptor interactions and signaling pathway dynamics. This is the first method for nanofluidic high-throughput longitudinal single-cell culture and imaging to study signaling kinetics and molecular interactions.

ABSTRACT:

HIV-1 causes a chronic infection that affects more than 37 million people worldwide. People living with human immunodeficiency virus (HIV) experience comorbidity related to chronic inflammation despite antiretroviral therapy. However, these inflammatory signaling has not been fully characterized. The role of early entry events on the activation of cellular signaling events and downstream gene expression has not been captured at the single-cell level. Here the authors describe a method that applies principles of live-cell fluorescence microscopy to an automated single-cell platform that cultures and images cells over user-customized time courses, allowing for high-throughput analysis of dynamic cellular processes. This assay can track single-cell live

fluorescence microscopy of early events that immediately follow HIV-1 infection, notably the influx of calcium that accompanies exposure to the virus and the development of productive infection using a fluorescent reporter virus. MT-4 cells are loaded with a calcium-sensitive dye and cultured in isolated pens on a nanofluidic device. The cultured cells are infected with an HIV-1 reporter virus (HIV-1 NLCI). A fluorescence microscope positioned above the nanofluidic device measures calcium influx over an 8-min time course following acute HIV-1 exposure. HIV-1 productive infection is measured in those same cells over a 4-day interval. Imaging data from these time courses are analyzed to define virus-host receptor interactions and signaling pathway dynamics. The authors present an integrated, scalable alternative to traditional imaging methods using a novel optofluidic platform capable of single-cell sorting, culturing, imaging, and software automation. This assay can measure the kinetics of events under various conditions, including cell type, agonist, or antagonist effect, while measuring an array of parameters. This is the first established method for nanofluidic high-throughput longitudinal single-cell culture and imaging: This technique can be broadly adapted to study cellular signaling kinetics and dynamic molecular interactions.

INTRODUCTION:

Chronic inflammation is a leading cause of HIV-associated early morbidities and mortality[1-3]. There are multiple mechanisms whereby HIV can activate inflammatory signaling, and recent evidence suggests a role for the P2X receptors in HIV entry which are calcium-gating adenosine triphosphate (ATP) receptors[3-10]. The P2X subtype of purinergic receptors (P2XR) may be important facilitators of this inflammation. However, the molecular mechanisms of HIV-P2XR interactions are largely unknown and may impact early and late HIV-1 viral life cycle steps. Defining the pathways and kinetics driving HIV-associated chronic inflammation is critical to advancing treatment options for people with HIV.

To assess whether HIV-1 directly agonizes P2X receptors, P2XR activation and HIV-1 infection must be measured in parallel. Assays of P2XR activity and HIV-1 infection have been independently established: Cellular calcium influx is an indicator of P2XR activation, and HIV-1 productive infection can be quantified by RNA abundance. Fluorescence detection of calcium influx is possible with the Fluo-4 calcium-sensitive dye, and HIV-1 infection can be visualized with the mCherry fluorescent reporter virus HIV-NLCI[11-15].

Because these indicators of P2X activation (acute cellular calcium influx) and HIV-1 infection (HIV-1 RNA synthesis) occur on different timescales (minutes versus days), there lacks a high-throughput method that allows for paired analysis of P2XR activation and HIV-1 infection. Standard high-throughput experimental techniques, such as flow cytometry, allow for the population analysis but cannot assess the relationship between acute and longitudinal events in single cells. Alternatively, single-cell imaging with standard fluorescence microscopy is low-throughput. These experimental limitations present a need for novel, high-throughput techniques to measure associations between acute and longitudinal cellular events directly.

An optofluidic system described is a novel platform capable of single-cell sorting and isolation, culturing, imaging, and software automation[16-19]. This system presents an integrated, high-

throughput alternative to the limitations of traditional imaging methods. The Beacon platform consists of a carbon dioxide (CO_2) and temperature-controlled incubator that supports cells contained on a chip. The chip possesses photosensitive transistors that generate an electrical gradient in response to targeted light. This resulting dielectrophoretic force is used to move individual cells across the nanofluidic chip to the desired regions. Cells are sorted into pens on the chip, which provide a barrier to isolate individual cells physically. Continuous laminar flow of growth media throughout the chip prevents cell migration from the pens while allowing for small-particle diffusion of nutrients and experiment-specific reagents. A fluorescence microscope sits above the chip. Software automation is used to capture images of the chip at the user-specified time point.

All cell characterization was performed using an optofluidic system for single-cell selection and manipulation. This system consists of integrated mechanical, microfluidic, and optical components that enable single-cell manipulation, assay, culture, and imaging. Cells are loaded and cultured on the disposable nanofluidic device consisting of 3,500 individual chambers (pens), each capable of holding sub-nanoliter volume. Cells can be positioned within pens using light-induced dielectrophoretic "cages" and cultured under temperature- and CO_2-controlled conditions. The microfluidics permit perfusion of media or buffers on the chip for cell culture or drug treatment. An actuated needle allows for the import and export of cells from incubated and shuttered well plates. The chip area can be imaged at 4x or 10x magnification in brightfield and fluorescent channels (including DAPI, FITC, TRed, or Cy5) to characterize cellular phenotypes or functional analysis. The entire system is automated using software that can be used for predesigned workflows or custom experiments.

Relationships between HIV-1 infection and P2XR have been studied, but a high-throughput procedure to directly characterize these interactions in parallel has not been reported. Here, the authors describe a methodology to study HIV-P2XR interactions through tracking acute calcium influx and subsequent HIV-1 productive infection at the single-cell level. Notably, this establishes a novel tool that allows for direct, high-throughput, longitudinal measurement of multiple targets in single cells.

PROTOCOL:

1. Preparation of cells for imaging

1.1 Prepare fresh Fluo-4 AM loading solution: Add 25 µL of 100x concentrated detergent solvent, then add 2.5 µL of Fluo-4 AM 1000x to a 1.5 mL tube. Vortex to mix.

1.2 Pipette 2.5 mL of culture media into the loading solution and invert to mix. Protect from light.

1.3 Centrifuge 2 x 10^6 MT-4 cells at 500 x *g* for 3 min.

1.4 Remove media and resuspend the pellet in 2 mL of the prepared Fluo-4 AM loading solution. Protect from light.

1.5 Pipette resuspended cells into a 35 mm Petri dish. Incubate at 37 ˚C for 15-30 min, then incubate for an additional 15-30 min at room temperature.

1.6 Transfer the cell suspension to a centrifuge tube. Centrifuge cells at 500 x *g* for 3 min and remove the supernatant consisting of the loading solution.

1.7 Resuspend the cell pellet by pipetting in 1 mL of the culture medium and centrifuge at 500 x *g* for 3 min to wash.

1.8 Resuspend the cell pellet by pipetting in the culture medium at a concentration of 2 x 10^6 cells/mL (minimum 50 µL). Protect from light.

2. Optofluidic system preparation, cell loading, and cell penning

2.1 Prepare a chip with the wetting solution, which facilitates cell penning.

2.1.1 To do this, load centrifuge tubes containing 2 mL of wetting solution and 50 mL of DI water onto the instrument with a new optofluidic chip and run the **Wet Chip** function. This function will automatically flood the chip with the wetting solution through the system fluidics, incubate the chip at 50, ˚C and flush the chip with water 3 times.

2.1.2 Once water flushing is done, flush the chip with 3 cycles of 250 µL of culture media.

2.2 Supplement the cell suspension from step 1.8 with 1:100 F-127 detergent solute before the loading to reduce the likelihood of cells sticking to the chip channels.

2.3 Use the instrument export needle to import cells from a 1.5 mL centrifuge tube using the **Load** operation and **Small Volume Import** for a 5 µL cell package volume.

2.4 Pen cells using an optimized optoelectronic positioning (OEP) voltage of 4.3 V and 5 µm/s cage speed. Penning occurs using OEP. Accomplish penning using the **Autopen** function, which will autodetect single cells, surround them with an OEP cage, and move them into a nearby pen. If cells of interest remain following auto-penning, use the **Manual pen** function to select target cells (with a mouse click) and a destination pen (with a subsequent mouse click).

2.5 Once penning is complete (either when the **Autopen** function completes or when the desired number of penned cells has been attained), flush the chip with 3 cycles of 250 µL of culture media to clear any remaining unpenned cell from the chip.

3. Single-cell infection and functional imaging

3.1 After penning cells, obtain fluorescent images of cells in FITC, Texas Red, and DAPI channels to measure the baseline Fluo-4, mCherry, and autofluorescence.

3.2 Infuse HIV-1 into the microchip at a concentration of 13 ng of HIV-1 NL-CI per 2 x 10^6 cells by slowly pipetting the suspension into the chip through the export needle.

3.3 Immediately after HIV-1 addition, repeatedly obtain images in the FITC and DAPI channels over a 10-min time course.

3.4 Obtain images in the Texas Red and DAPI channels at 1-, 2-, 3-, and 4-days post-infection (DPI).

4. Analysis of single-cell functional imaging data with FIJI software

4.1 At each time point in each imaging channel of interest, use the FIJI **Point Selection** tool and the **Measure** function to measure each cell's Fluo-4, mCherry, and autofluorescence signals. Simultaneously measure the background fluorescence by calculating the mean fluorescence intensity (MFI) of the pen and chip containing each cell.

4.2 Control for the background fluorescence and autofluorescence by using the **Measure** function to find the MFI in a **Region Selection** and subtracting this value from the cell fluorescence value.

4.3 Normalize the background fluorescence of each chip image: Measure the MFI of the chip in each field. Then subtract the MFI of the chip with the least background fluorescence from all other chip MFI measurements.

4.4 Normalize the background fluorescence of each cell-containing pen: measure the MFI of each cell's pen in each field of view. Then subtract this value from the cell's raw MFI for each field.

4.5 Control for the cell autofluorescence: Subtract the day 0 and day 4 DAPI MFI of each cell from the day 0 and day 4 mCherry MFIs.

REPRESENTATIVE RESULTS:

Figure 1 illustrates the format of the chip and raw imaging data acquired through the fluorescence microscope. The identification and clustering of uninfected and infected cells via mCherry measurement are shown in **Figure 2**. These clusters are analyzed for calcium influx kinetics in **Figure 3**, which demonstrates early calcium influx in HIV-infected cells. **Figure 4** shows a significant positive correlation between calcium influx and mCherry fluorescence [20].

FIGURE LEGENDS:

Figure 1: Chip loading and raw imaging data. Schematics of loading cells into pens on the chip using opto-electropositioning (**A**). Calcium influx is quantified by detecting the Fluo-4 calcium-

sensitive dye in the FITC channel over an 8.5-min time course (**B**). HIV-1 productive infection is measured over a 4-day time course by detecting the mCherry reporter virus HIV NL-CI in the Texas Red channel (**C**).

Figure 2: Representative HIV-infected cells positive for mCherry signals at day 4 post-infection. MT-4 cells were infected with the mCherry reporter virus HIV-1 NL-CI on the optofluidic platform, and mCherry signal was measured 4 days post-infection (dpi). Cells with a change in mCherry signal >40,000 MFI (where a clear breakpoint was noted) at 4 dpi were clustered into the "mCherry-high" population; cells with a change in mCherry fluorescence <40,000 MFI were clustered into the "mCherry-low" population. Statistical software was used to plot the mean values of mCherry fluorescence at 0 and 4 dpi. Statistical significance at 4 dpi was measured using an unpaired t-test of the means (mCherry-high=50235 MFI; mCherry-low=25017 MFI; $p<0.000001$). *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$

Figure 3: Representative HIV-infected cells having high rates of early calcium influx. MT-4 cells were infected with the mCherry reporter virus HIV-1 NL-CI on the optofluidic platform. Intracellular calcium was measured by Fluo-4 fluorescence repeatedly over a 9-min time course. Statistical software was used to plot the mean Fluo-4 values of the mCherry-high and mCherry-low cell clusters. Statistical significance was measured using unpaired t-tests at each time point. *=$p<0.05$, **=$p<0.01$, ***=$p<0.001$.

Figure 4: Representative intracellular calcium flux higher in mCherry-positive cells. MT-4 cells were infected with the mCherry reporter virus HIV-1 NL-CI. Intracellular calcium was measured by Fluo-4 fluorescence 9 min post-infection, and mCherry was measured 4 days post-infection. A simple linear regression was performed with statistical software.

DISCUSSION:

The described methodology to study the relationship between calcium influx and HIV-1 productive infection in single cells can be adapted to study intracellular calcium kinetics in response to other agonists or antagonists of interest. The preparation of cells for imaging is simple, minimally time-intensive, and reagents are available in convenient kits from widely used manufacturers. Fluo-4-based calcium measurement is well-described in the literature, and HIV-1 can easily be substituted for other viruses or compounds of interest to study on this platform. Different cell types and culture mediums can be used. Alternative or additional fluorescence channels are supported, and multiple fluorophores can be imaged simultaneously or sequentially.

Additionally, the procedure can be widely adapted to study fluorophore-labeled events in longitudinal time courses. Standard high-throughput methods, such as flow cytometry, do not allow for the paired study of discrete events over time courses. Traditional single-cell imaging through fluorescence microscopy is limited by throughput, cell migration across fields of view, and intensive time requirements. By combining fluorescence imaging with high-throughput and automated single-cell culture, this method allows for the elucidation of relevant molecular

kinetics, sequelae, and interactions at both short- and long-term time intervals. It is important to note that software and image analysis optimization is critical for each application.

The microscope is equipped with brightfield and Cy5, Texas Red, FITC, and DAPI fluorescence channels[16]. Available magnifications are 4x and 10x. This method is not currently suitable for investigations requiring more numerous fluorescence parameters or greater magnifications. Compounds of interest that will be infused into the chip for the cell exposure must exhibit diffusion to enter pens and reach cells. Access to a Beacon instrument may pose a cost barrier to investigators.

The optofluidic system has a wide scanning and stitching ability. The optical sorting on the chip has a high-efficiency variable depending on cell type, but automatic sorting efficiency is between 80-90%, and manual sorting can increase that efficiency to 100%. Each chip can accommodate 3,500 cells, and four chips can be run simultaneously, with a parallel workflow, resulting in high scalability. While the imaging workflow is reported by manual image analysis, more recent updates in the system allow for automatizing this. It is important to note that the data shown here are representative within the limitations of image threshold analysis, and optimization and automatization of image analysis are likely to result in more sensitive measures of both calcium influx and mCherry signal as indicative of HIV-1 productive infection.

The described protocol was established using MT-4 cells and HIV-1 as previously described[7, 21, 22]. When adapting the protocol to other cell lines or fluorescent targets of interest, optimization and validation are required. The kinetics of Fluo-4 efflux is cell line-dependent, so the cell type-specific dye retention should be confirmed to last at least if the intended calcium imaging time course. Validation of dye retention and agonist response on this optofluidic device can be completed by treating cells on the chip with the growth medium containing 10 µM ionomycin, which should induce potent cellular calcium influx and Fluo-4 signal in the FITC channel. Similar controls and validations should be conducted for any agonists/antagonists to be infused into the chip to ensure proper concentration and diffusion to cells. For single-cell analysis, cells should be prepared as a single-cell suspension to facilitate the sorting of individual cells into pens. Like traditional fluorescence microscopy, fluorescence exposure times and time points measured should be optimized to each respective target of interest.

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DISCLOSURES:

R.P.S. is an employee of Sema4. The other authors have no conflicts of interest to disclose.

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(Figure 1)

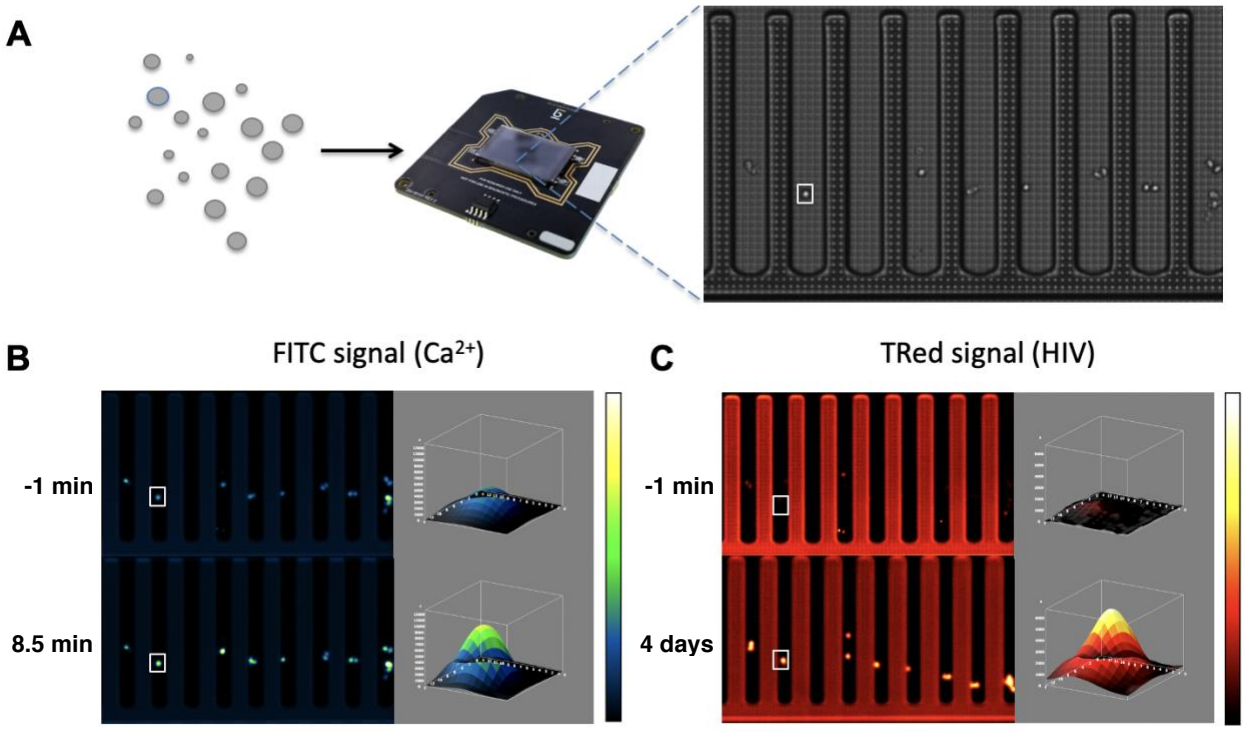


Figure 2

HIV-infected cells are mCherry-positive at 4 days

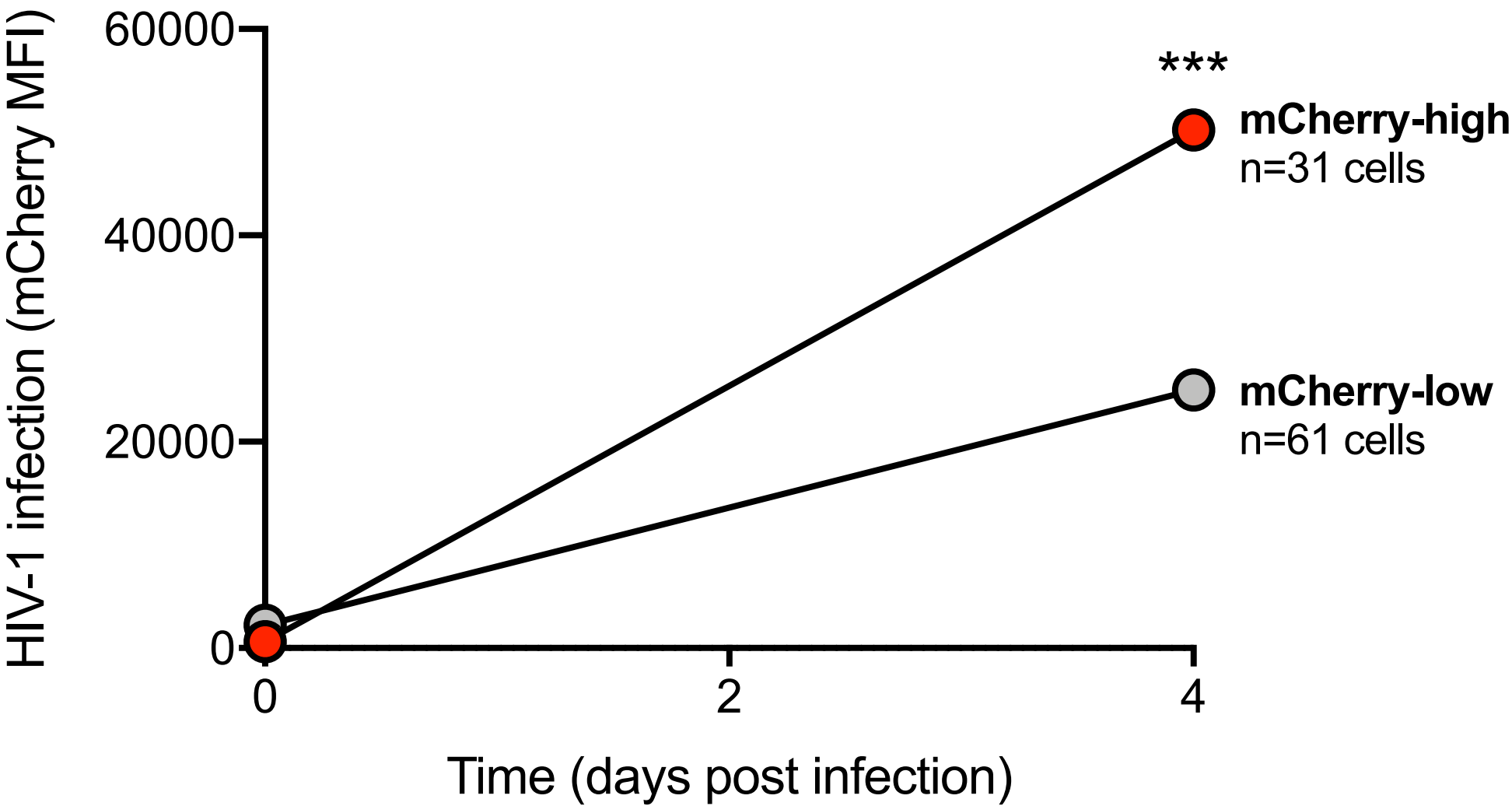


Figure 3

HIV-infected cells have high rates of early Ca^{2+} influx

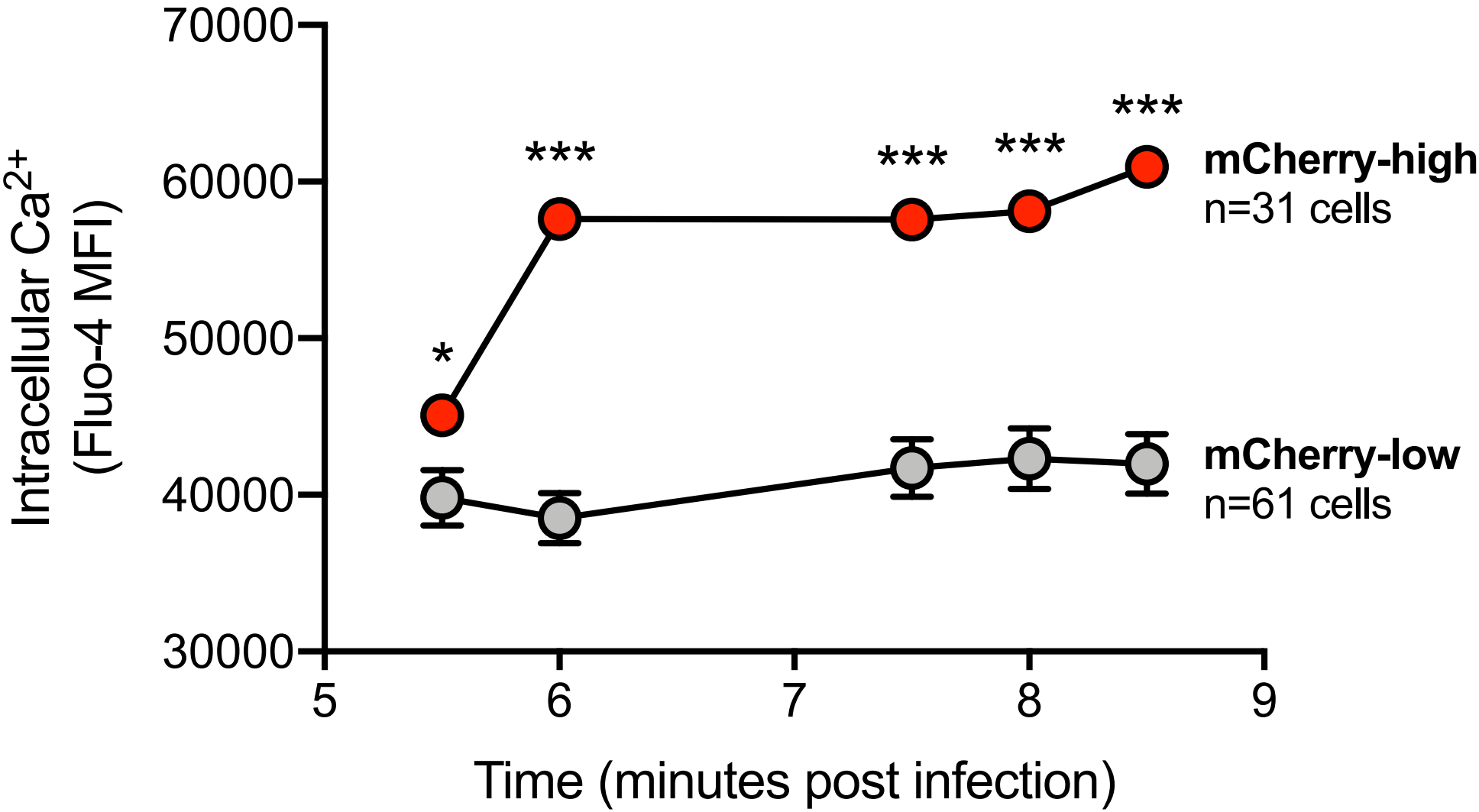
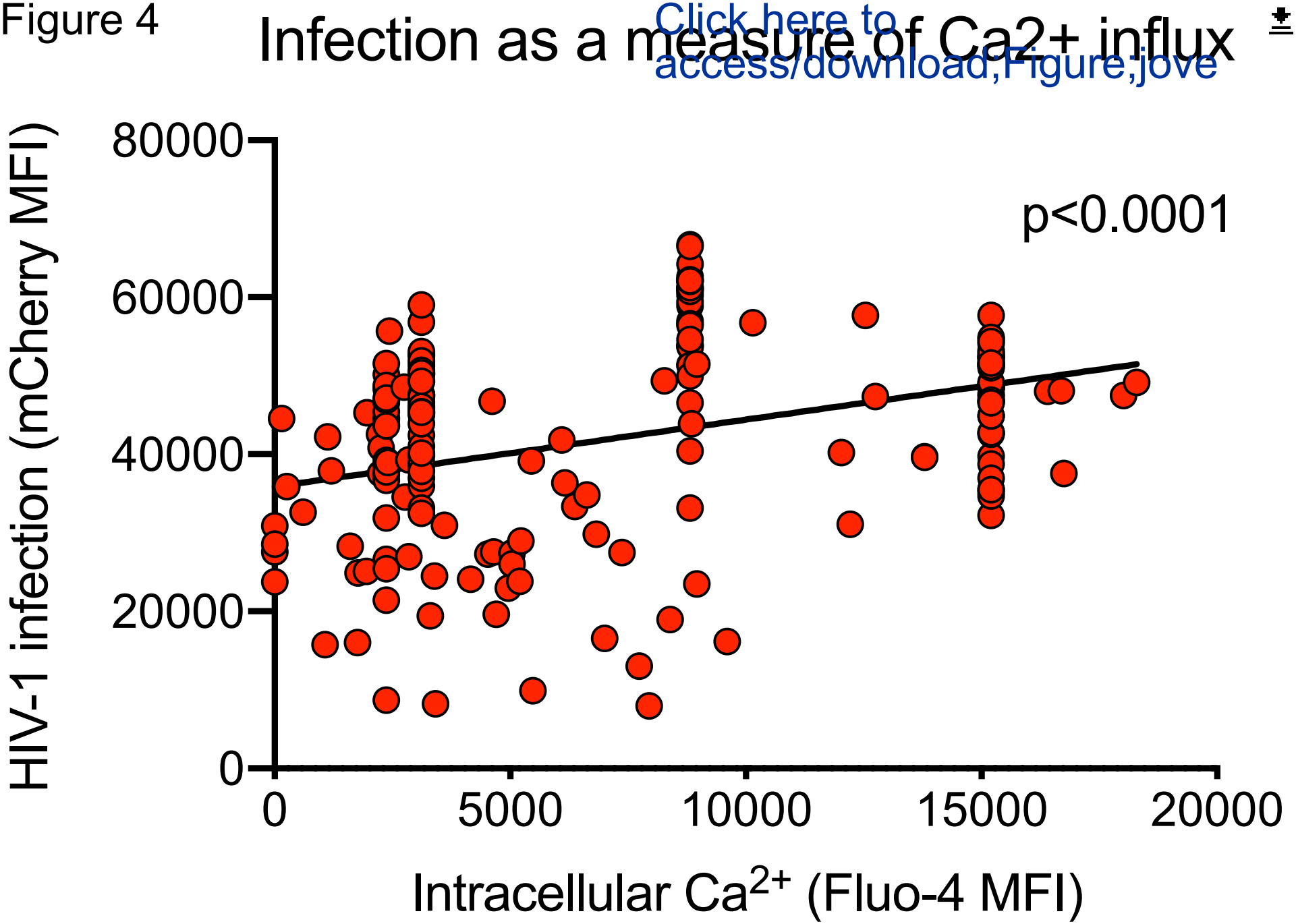


Figure 4



Name of Material/Equipment	Company	Catalog Number	Comments/Description
Beacon Optofluidic System	Berkeley Lights		
Fetal Bovine Serum	Gibco		
FIJI	Open-source software		PMID: 22743772, 22930834
Fluo-4 Calcium Imaging Kit	Thermo Fisher	F10489	
HIV-1 $NLCI_{NL4-3}$	Laboratory of Benjamin Chen		PMID: 28148796
Hyclone Pennecillin Streptomycin s	GE Healthcare Life sciences		SV30010
MT-4 cells	NIH AIDS Reagent		ARP-120
OptoSelect 3500 chip	Berkeley Lights		
Pipettor Tips	Denville Scientific	P3020-CPS	
Prism 9.0.0	GraphPad		
RPMI-1640 Medium	Sigma-Aldrich	R8758	
Serological Pipettes	Fisher Brand	13-678-11E	
Tissue Culture Hood	Various models		
T75 flasks	Corning	3073	

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Jaydev Upponi, PhD
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April 30, 2021

Dear Dr. Upponi:

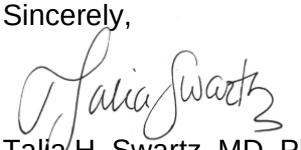
Thank you and the reviewers for your thoughtful suggestions to revise our manuscript entitled "A multiparameter optofluidic platform for single-cell characterization of early and late events in HIV-1 infection" for consideration as a Journal of Visual Experimentation Research Article.

We have made the requested edits in this resubmission and indicated the rebuttal to follow.

Many thanks for your input and looking forward to being in touch.

Thank you for your consideration and we look forward to your feedback.

Sincerely,



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Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

This has been done.

2. Please revise the following lines to avoid previously published work: 133-136, 139-143.

We have not included any previously published work.

3. Please revise the text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).

This change has been made.

4. Please define all abbreviations before use (MFI, etc.)

All abbreviations have been defined and a new section on abbreviations has been added.

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.
For example: Beacon, Berkeley Lights, Beacon OptoSelect, PowerLoad concentrate, Pluronics, Prism, etc.

This change has been made.

6. Please rephrase the Summary to clearly describe the protocol and its applications in complete sentences between 10-50 words: “Here, we present a protocol to …”

The new summary reads:
“Here, the authors present a protocol in which single cells are monitored for acute events and productive HIV-1 infection on a nanofluidic device. Imaging data define virus-host receptor interactions and signaling pathway dynamics. This is the first method for nanofluidic high-throughput longitudinal single-cell culture and imaging to study signaling kinetics and molecular interactions.”

7. Please adjust the numbering of the Protocol to follow the JoVE Instructions for Authors. For example, 1 should be followed by 1.1 and then 1.1.1 and 1.1.2 if necessary. Please refrain from using bullets or dashes.

This change has been made.

8. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

This change has been made.

9. Please replace “uL” and “uM” with “µL” and “µM” throughout the protocol, respectively.

This change has been made.

10. Line 190: How is penning performed?

“Penning occurs using optimized optoelectronic positioning (OEP).” This has been added to line 324-25.

11. Line 200: Please specify how is HIV-1 infused into the microchip. What is used for infusing?

“Infuse HIV-1 into the microchip at a concentration of 13 ng HIV-1 NL-CI per 2 x 10^6 cells by slowly pipetting the suspension into the chip through the export needle.” This has been added to line 334-35.

12. Please highlight up to 3 pages of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol. Remember that non-highlighted Protocol steps will remain in the manuscript, and therefore will still be available to the reader.

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13. Please ensure that the references appear as the following: [Lastname, F.I., LastName, F.I., LastName, F.I. Article Title. Source. Volume (Issue), FirstPage – LastPage (YEAR).] For more than 6 authors, list only the first author then et al. Title case and italicize journal titles and book titles. Do not use any abbreviations.

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14. For in-text formatting, corresponding reference numbers should appear as numbered superscripts after the appropriate statement(s).

This change has been made.

15. Figure 1: Please Maintain a single space between the numeral and (abbreviated) unit, except in cases of %, x, and ° (i.e., the degree sign; excluding temperature). Examples: 5 mL, t8.5 min, t4 days, etc. For time units, use abbreviated forms for durations of less than one day when the unit is preceded by a numeral. Do not abbreviate day, week, month, and year. Examples: 5 h, 10 min, 100 s, 8 days, 10 weeks

This change has been made.

16. Figure 2/3: Please define the units in the Y-axis.

This change has been made.

17. Figure 4: Please define the units in the X and Y-axis.

This change has been made.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:

This is a very interesting paper describing a method using optofluidic fluorescence microscopy for automated single-cell detection. This technique can be broadly adapted to study cellular signaling kinetics and dynamic molecular interactions. I believe this paper is worth filming a video for broad audience.

Thanks to the reviewer for this kind assessment.

Reviewer #2:

Manuscript Summary:

The authors present a new application of the Beacon Optofluidic System which involve simultaneously monitoring P2XR activity and HIV infection in individual cells. The P2XR activity is monitored using calcium sensitive dye whereas the HIV infection is monitored using a mCherry fluorescent reporter virus.

Major Concerns:

The Beacon opto-fluidic platform used here is interesting and may be useful for multiple different applications, in addition to HIV related studies. However, there are some questions that need to be addressed in this manuscript. The authors claim that "single-cell imaging with standard fluorescence microscopy is low-throughput". But it is unclear how exactly does the opto-fluidic platform increase the throughput of analysis. The authors should provide quantitative data to support this claim. The throughput will depend on the NA of the objective used, irrespective of the platform. Does the Beacon system have a wide scanning and stitching ability? A detailed description of the OptoSelect chip would be helpful, e.g. (1) what is the efficiency of positioning cells in individual wells?, (2) how many cells can be imaged at a time? etc. Why are there so few cells in image 1? It would be good to show the actual high throughout nature by imaging as many cells as possible. What is the sensitivity and limit of detection of the system for detection of Ca? Please describe as to how this technique is scalable, as claimed in the abstract? Another question is regarding the software. Is it automated (as mentioned in the abstract) or do the authors manually do the analysis using the ImageJ macros?

Thanks to the reviewer for these helpful questions and suggestions.

More detail has been added:

> The optofluidic system has wide scanning and stitching ability. The optical sorting on the chip has high efficiency that is variable depending on cell type, but automatic sorting efficiency is between 80-90% and manual sorting can increase that efficiency to 100%. Each chip can accommodate 3500 cells and four chips can be run simultaneously, with a parallel workflow, resulting in high scalability. While the imaging workflow is reported by manual image analysis, more recent updates in the system allow for automatization of this. It is important to note that the data shown here are representative within the limitations of image threshold analysis, and optimization and automatization of image analysis are likely to result in more sensitive measures of both calcium influx and mCherry signal as indicative of HIV-1 productive infection.

Reviewer #3:
Minor Concerns:
In this study, the authors reported their latest method article titled 'A multiparameter optofluidic platform for single-cell characterization of early and late events in HIV-1 infection' in the Journal of Visualized Experiments. They described a method that applies principles of live-cell fluorescence microscopy to an automated single-cell platform that cultures and images cells over user-customized time courses, allowing for high throughput analysis of dynamic cellular processes. This manuscript is correctly set up, and well-written. The manuscript can be accepted with minor revisions.
1. The abstract part can be shortened.
2. The introduction part's paragraphs should be rephased to connect each other.

Thanks to the reviewer for these helpful questions and suggestions. The abstract has been shortened and the introduction paragraphs have been rephrased to better connect to each other.

Reviewer #4:
Manuscript Summary:
This manuscript is describing a multiparameter optofluidic platform for single-cell characterization of early and late events in HIV-1 infection. The results are interesting and convincing. The developed platform provides a lot of visual information that can be further analyzed by researchers. There are many additional applications of this technology including understanding of cellular signaling kinetics and dynamic molecular interactions. Overall, this is an excellent piece of work.

Thanks to the reviewer for this kind assessment.