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Corresponding Author:	Kirill Gorshkov, Ph.D. National Center for Advancing Translational Sciences Rockville, MD UNITED STATES
Corresponding Author's Institution:	National Center for Advancing Translational Sciences
Corresponding Author E-Mail:	kgorshkov89@gmail.com;kirill.gorshkov@nih.gov
Order of Authors:	Bruce Tran
	Eunkeu Oh
	Kimihiro Susumu
	Mason Wolak
	Kirill Gorshkov
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TITLE:

High-throughput Confocal Imaging of Quantum Dot-Conjugated SARS-CoV-2 Spike Trimers to Track Binding and Endocytosis in HEK293T Cells

AUTHORS AND AFFILIATIONS:

Bruce Nguyen Tran[1], Eunkeu Oh[2], Kimihiro Susumu[2,3], Mason Wolak[2], Kirill Gorshkov[1*]

[1]National Center for Advancing Translational Sciences, 9800 Medical Center Drive, Rockville, MD 20850

[2]Optical Sciences Division, Code 5600, Naval Research Laboratory, 4555 Overlook Ave. S.W., Washington, DC 20375

[3]Jacobs Corporation, Hanover, MD 21076

Email addresses of co-authors:

Bruce Nguyen Tran (bruce.nguyentran@nih.gov)
Eunkeu Oh (eunkeu.oh@nrl.navy.mil)
Kimihiro Susumu (kimihiro.susumu.ctr.ja@nrl.navy.mil)
Mason Wolak (mason.wolak@nrl.navy.mil)
Kirill Gorshkov (kgorshkov89@gmail.com)

Corresponding author:

Kirill Gorshkov (kgorshkov89@gmail.com)

SUMMARY:

In this protocol, quantum dots conjugated to recombinant SARS-CoV-2 spike enable cell-based assays to monitor spike binding to hACE2 at the plasma membrane and subsequent endocytosis of the bound proteins into the cytoplasm.

ABSTRACT:

The development of new technologies for cellular fluorescence microscopy has facilitated high-throughput screening methods for drug discovery. Quantum dots are fluorescent nanoparticles with excellent photophysical properties imbued with bright and stable photoluminescence as well as narrow emission bands. Quantum dots are spherical in shape, and with the proper modification of the surface chemistry, can be used to conjugate biomolecules for cellular applications. These optical properties, combined with the ability to functionalize them with biomolecules, make them an excellent tool for investigating receptor-ligand interactions and cellular trafficking. Here, we present a method that uses quantum dots to track the binding and endocytosis of SARS-CoV-2 spike protein. This protocol can be used as a guide for experimentalists looking to utilize quantum dots to study protein-protein interactions and trafficking in the context of cellular physiology.

INTRODUCTION:

Fluorescence microscopy enables researchers to peer into the inner workings of the cell using specialized dyes[1], genetically encoded fluorescent proteins[2], and fluorescent nanoparticles in the

form of quantum dots (QDs)[3]. For the severe acute respiratory syndrome coronavirus of 2019 (SARS-CoV-2) global pandemic, researchers have employed fluorescence microscopy to understand how the virus interacts with the cell both at the plasma membrane and in the cytoplasm. For example, researchers have been able to gain insights into the binding of the SARS-CoV-2 Spike protein on the virion's surface to human angiotensin-converting enzyme 2 (hACE2) on the surface of human cells, subsequent internalization *via* fusion at the plasma membrane, and endocytosis of the Spike:hACE2 protein complex[4,5]. Great insights have also been gained into the SARS-CoV-2 egress from cells *via* the lysosome using cellular fluorescence imaging, a unique feature of coronaviruses previously thought to occur *via* traditional vesicle budding from the Golgi, as it is with many other viruses[6]. A mainstay of almost all aspects of biological research, the cellular fluorescence microscopy technique has necessarily advanced in its breadth and scope of applications from super-resolution imaging of whole animals to automated high-content multi-parametric imaging for drug screening. Here, automated high-content confocal microscopy is applied to the study of SARS-CoV-2 cell entry using fluorescent QDs conjugated to the viral spike protein.

High-content analysis of images generated by biological imaging platforms allows for greater extraction of valuable biological insights than single parameters such as whole-well intensity, that one would obtain using a multi-modal plate reader[7]. By separating the objects in a field of view using automated segmentation algorithms, each object or a population of objects can be analyzed for parameters such as intensity, area, and texture in each available fluorescence channel[8]. Combining many measurements into multivariate datasets is a useful approach for phenotypic profiling. When the desired phenotype is known, such as QD internalization in the form of puncta, one can use the measurements related to puncta such as size, number, and intensity to assess the efficacy of a treatment.

Cloud-based high content imaging analysis software can accommodate a large variety of instrument data outputs, including the high content imaging platform. By using a cloud-based server for image storage and online analysis, the user is able to upload their data from either the imaging instrument or from the network drive where the data is stored. The analysis portion of the protocol is conducted within the cloud software environment, and data can be exported in a variety of file formats for downstream data visualization.

The SARS-CoV-2 virus is composed of nonstructural and structural proteins that aid in its assembly and replication. SARS-CoV-2 spike has two domains called S1 and S2, with S1 containing the receptor-binding domain responsible for hACE2 interactions at the plasma membrane[9]. Spike has also been found to interact with other molecules at the plasma membrane that may act as co-receptors in addition to hACE2[10,11]. Throughout the spike protein sequence and particularly at the S1/S2 interface, there are protease cleavage sites that enable fusion at the membrane after the transmembrane serine protease 2 (TMPRSS2)[12]. Various recombinant SARS-CoV-2 Spike proteins have been produced from individual receptor binding domains, to S1, S2, S1 with S2, and whole spike trimers from multiple commercial vendors for use in research activities[13].

In this work, the surface of QDs was functionalized with recombinant spike trimers that contain a histidine tag (QD-Spike). The QDs produced by Naval Research Laboratory Optical Nanomaterials Section contain a cadmium selenide core and a zinc sulfide shell[14,15]. The zinc on the quantum dot surface coordinates the histidine residues within the recombinant protein to form a functionalized quantum dot that resembles a SARS-CoV-2 viral particle in form and function. The generation of the nanoparticles and protein conjugation was previously described using the QD-conjugated receptor binding domain[15]. This method describes the cell culture preparations, QD treatment, image acquisition, and data analysis protocol that can guide a researcher in studying SARS-CoV-2 Spike activity in the physiological context of a human cell.

PROTOCOL:

The HEK293T cell line used in this study is an immortalized cell line. No human or animal subjects were used in this study.

1 Cell culturing and seeding

1.1 Inside a sterile biosafety cabinet, wearing personal protective equipment (including lab gloves, lab coat, and safety glasses), prepare cell culture medium by supplementing Dulbecco's Modified Eagle Medium (DMEM) with 10% fetal bovine serum (FBS), 1% penicillin/streptomycin (P/S), and 250 µg/mL G418.

1.1.1 For 500 mL of media, add 443.75 mL of DMEM, 50 mL of FBS, 5 mL of P/S, and 1.25 mL of G418.

1.1.2 Filter through a 0.2 µm filter flask and maintain sterility to avoid bacterial contamination.

1.2 Inside a sterile biosafety cabinet, seed the interior 60 wells of a black, clear-bottom, poly-D-lysine coated 96-well plate with 20,000 cells in 100 µL per well of cell culture medium. Fill the outer 36 wells with 100 µL per well of phosphate-buffered saline (PBS).

1.2.1 To count the cells, add 2 µL of acridine orange and propidium iodide stain to 18 µL of cell suspension.

1.2.2 Load 10 µL of this solution into one side of a cell counting slide.

1.2.3 Repeat steps 1.2.1. and 1.2.2. to load both sides of the slide.

1.2.4 Place the slide into an automated cell counter.

1.2.5 Select the fluorescence counting protocol and press **Count**. Count both sides of the slide and calculate the average of the two live-cell densities.

1.2.6 To calculate the volume of cell suspension required, divide the total number of cells needed by the average cell density.

1.3 Inspect the wells after seeding under a light microscope to ensure that proper density and distribution have been achieved.

1.4 Incubate the plate overnight in a humidified incubator at 37 °C with 5% CO_2.

2 Treatment of cells with QD-Spike

2.1 Prepare 0.1% bovine serum albumin (BSA) by diluting in imaging media.

2.1.1 For 10 mL of imaging media, add 130 µL of 7.5% BSA and mix.

2.2 In a 12-well reservoir or assay plate, dilute the 440 nM QD-Spike (SARS-CoV-2, Isolate USA-WA1/2020) stock to 20 nM using 0.1% BSA in imaging media.

2.2.1 For 1 well of QD-Spike, add 2.27 µL of 440 nM QD-Spike to 47.73 µL of 0.1% BSA imaging media for a final concentration of 20 nM.

2.2.2 To generate a six-point 1:3 serial dilution (in triplicate), add 14.26 µL of QD-Spike to 285.74 µL of 0.1% BSA imaging media to make the highest concentration of 20 nM. Add 100 µL of 20 nM QD-Spike to 200 µL of 0.1% BSA media to make the second dilution of 6.67 nM QD-Spike. Repeat four times to generate the six concentration points.

2.3 Using a multi-channel aspirator, remove all spent media from each well. Using a multi-channel pipette, wash once with imaging media (100 µL/well).

2.4 Aspirate 100 µL of imaging media and add back 50 µL/well of QD-Spike solution.

2.5 Incubate the plate for 3 h in a humidified incubator at 37 °C with 5% CO_2. Continue to step 4.

3 Fixation and nuclei staining

3.1 Inside a sterile biosafety cabinet, wearing personal protective equipment (including lab gloves, lab coat, and safety glasses), prepare 4% paraformaldehyde (PFA) in 0.1% BSA imaging media.

3.2 Aspirate 50 µL of QD-Spike from each well and add 100 µL/well of 4% PFA.

3.2.1 Do not let the wells dry out. Use an automated multi-channel pipette to avoid drying of the wells (recommended).

3.3 Incubate for 15 min at room temperature.

3.4 Wash three times with 1x phosphate-buffered saline (PBS).

3.5 Prepare the deep red nuclear dye by diluting the 5 mM stock solution to 1:1000 dilution in PBS.

3.6 Aspirate out the PBS and add back 50 µL/well of diluted nuclear dye.

3.7 Incubate for 30 min at room temperature.

3.8 Wash three times with PBS.

3.9 Image the plate or seal using a plate sealer for imaging at a later date. Store the plate at 4 °C. Fluorescence should be stable for several weeks or more.

4 Acquisition set-up and imaging

4.1 Start the software for the imaging platform. Turn on the high content imaging platform, and the light on the status bar should be on. If the machine is not connected, the software will go into offline analysis mode.

4.2 Login into the imaging platform.

4.3 Create a new acquisition protocol.

4.3.1 Select **Plate Type** as 96-well clear bottom imaging plate.

NOTE: The plate selection in the instrument may vary and can be customized by loading the correct plate definitions that include plate dimensions such as height, width, and other distances to define well spacing and focal points.

4.3.2 Select **Optical Mode** as confocal and magnification as 40x water immersion.

4.3.3 Select **Binning** as 1.

4.3.4 Choose the channels and fluorescent excitation/emission wavelengths as described in steps 4.3.5–4.3.8. Take a snapshot when the settings are selected to view the image and ensure the settings are appropriate.

NOTE: Digital Phase Contrast (DPC) will allow visualization of live cells without a cell stain or fluorescent dye. It is not recommended for fixed cells.

4.3.5 Select the **Mode** for DPC such as High Contrast to produce well-defined cell bodies. Take a snapshot to confirm that this setting is appropriate, as seen in the image window.

4.3.6 Select the **FITC Channel** for use with the ACE2-GFP cell line that allows visualization of ACE2 trafficking within the cell.

4.3.7 To make a custom channel for the QD channel, select the triangle drop-down menu for the channel and choose the excitation in the 405 nm range and emission in the 608 nm range.

4.3.8 If cells were fixed and a deep red nuclear dye was used, select the far-red channel with emission greater than 630 nm to further delineate the cell body and act as a cell mask.

4.3.9 Select a well with a strong cytoplasmic QD signal to set the exposure time, laser power, and Z-height position. Follow steps from 4.3.10–4.3.13.

4.3.10 Check whether the default height produces an image where the cells are in the desired focal plane. If the endocytosed puncta are the target organelle, the Z-position will be lower than if the plasma membrane is the target organelle.

4.3.11 Choose an exposure time that produces a bright image with gray levels at least three-fold greater than the background signal. This is typically between 100 and 300 ms. At 20 nM, the gray levels should be approximately 6000 a.u. using an exposure time of 200 ms and laser power of 80%.

4.3.12 Check gray levels by right-clicking on the image produced with the Snapshot feature in each channel and selecting **Show Intensity**. Then, left-click on the object of interest or background to view the gray level for that pixel.

4.3.13 Adjust the laser power to fine-tune the intensity of the objects of interest.

4.4 Save the acquisition protocol.

4.5 Switch to **Run Experiment** and enter the **Plate Name**.

4.6 Run the experiment.

5 Data analysis

5.1 Import data onto the imaging software.

5.1.1 After acquiring all images, export measurements to the imaging software. This requires a server to be established and an account to be created. Create a screen for the data to be exported into the imaging software.

5.2 In the imaging software, select **Image Analysis** to begin building the analysis protocol.

5.3 Load the measurements from the imaging experiment by right-clicking on the file in the screens list and clicking on **Select**. The plate map of imaged wells and their associated fields should be visible at the bottom left-hand corner of the window.

5.4 Choose a well with bright cytoplasmic QD spots to begin image segmentation.

5.5 In the **Input Image Building Block**, select **Flatfield Correction**.

5.6 Add the next building block to the protocol by selecting **Find Nuclei**.

5.6.1 Here, use the DPC or far-red channel as the Nuclei marker.

5.6.2 Select the method that accurately segments the objects first, and then fine-tune the segmentation using the drop-down menu and adjusting the sliders.

NOTE: Good segmentation accurately defines the region of interest (ROI) for the nucleus, cell, and spots. Only the pixels that are positive for the marker should be captured within an individual ROI. Background signal should be excluded. If the background is captured in the ROI, the sensitivity of the method can be decreased, or the threshold of intensity can be increased to increase the stringency of the segmentation algorithm. This is applicable to all Find building blocks.

5.7 Next, add the **Find Cytoplasm Building Block** to identify the cytoplasm.

5.7.1 In this case, use the ACE2-GFP channel for cytoplasmic segmentation.

5.7.2 Select the method that best identifies the cytoplasm of each cell.

5.8 Next, add the **Find Spots Building Block** to identify the QD-Spike puncta.

5.8.1 Select the **Nuclei** as the ROI population.

5.8.2 Select the **Cell** as the ROI region. This captures the puncta within the entire cell.

5.8.3 Select the method that best identifies the QD puncta in each cell.

5.9 Once all the objects in the image have been segmented and identified accurately in this well, choose other wells to ensure the building blocks and settings can be generally applied to the other wells and other conditions.

5.10 Add the **Calculate Intensity Properties** for all segmented objects (nuclei, cytoplasm/cells, and spots).

5.11 Add the **Calculate Morphology Properties** for all segmented objects (nuclei, cytoplasm/cells, and spots).

5.12 Add the **Calculate Texture Properties** for all segmented objects (nuclei, cytoplasm/cells, and spots).

5.13 Define results by selecting the parameters for each population, including nuclei and spots. The number of objects can be used as an indirect measure of cell viability.

6 Export data

6.1 Click on **Batch analysis**. Wait for the analysis to finish before proceeding to the next step (step 6.2).

NOTE: The batch analysis allows the image analysis software server to load the analysis protocol using the selected measurements to analyze the data remotely.

6.2 Export the dataset to a local computer or network drive.

6.2.1 Connect the image analysis software to a helper application on the computer by downloading and opening a connection file.

6.2.2 Select the results file type (.txt, .csv, .html, or Native XML).

6.2.3 Choose the **File Folder** settings with the drop-down menu.

7 Analyze the data in a spreadsheet

7.1 Open the exported file. If it is a .csv file, save it as a .xls file format to enable the use of pivot tables. If the file path is too long, save the .xls file higher up in the folder hierarchy to avoid errors in saving.

7.2 Add columns to the spreadsheet that designate the conditions for each well. For example, the cell type, QD-Spike variants, QD-Spike concentrations, incubation time, etc.

7.3 Choose the **Pivot Table** function and build the table using the added conditions as **Rows**, and the measured parameters as **Columns**. Select the calculation for each parameter i.e., **Average**, **Standard Deviation**, **Median**, **Min**, **Max**, or **Count**.

7.4 Normalize the data to control samples, including unconjugated QDs as 0% and the highest concentration of QD-Spike as 100%.

NOTE: If assessing the efficacy of inhibitors such as neutralizing antibodies, normalize the data to media-only treated cells (100% efficacy) and QD-Spike without inhibitor (0% efficacy).

7.5 Plot the data in graphing software.

REPRESENTATIVE RESULTS:

Upon treatment, the QDs will be internalized as the nanoparticle will bind to ACE2 on the plasma membrane and induce endocytosis. Using an ACE2-GFP expressing cell line, translocation of both QDs and ACE2 can be visualized using fluorescence microscopy. Once internalized, the two QD and ACE2 signals show strong colocalization. From these images, image segmentation and subsequent analysis can be performed to extract relevant parameters such as spot count (**Figure 1, Figure 2B**). **Figure 1A** is a montage of cells treated with different concentrations of QD-Spike or media only as a control. Three channels were used, including digital phase contrast, FITC, and the custom QD channel with excitation at 405 nm and emission at 608 nm. DPC provides an image of the general cell shape. The DPC image provides an approximate indication of the entire cell morphology. The area of interest overlaps with the DPC signal and is sufficient for detecting internalized QDs. The FITC channel shows the ACE2-GFP changing localization and accumulating at regions that colocalize with QD-Spike. As the concentration of QD-Spike decreases, the QDs are no longer visible and the ACE2-GFP signal is similar to control. The merge channel demonstrates the colocalization of ACE2-GFP with QD-Spike. These objects are a mixture of spots and larger accumulations that can be segmented with the analysis software. Fine-tuning of the software analysis method used for image segmentation can be used to segment the objects of interest.

Here a concentration-response experiment with six different concentrations of QD-Spike was performed, starting at 20 nM, to determine optimal concentrations of QD (**Figure 2A**). QDs can be used as low as 2.22 nM and still show binding and internalization. However, it is recommended to use concentrations of 20 nM or higher to ensure a robust response.

During conjugation to the QD, protein aggregation may occur. The main issue with aggregates is that they will accumulate on top of cells and cause artifacts in the image segmentation step. Aggregates will not be able to enter cells, and the nanoparticles as a whole will no longer resemble a viral particle (**Figure 3**). Aggregates can be spotted in the QD solution as bright, clumped precipitates.

This assay can also be used to assess biologics, such as neutralizing antibodies that block viral entry. QDs were incubated with neutralizing antibodies (**Figure 4A**), starting at 30 µg/mL, for 30 min at room temperature before addition to cells. Antibodies raised against the reference Washington strain, SARS-CoV-2 RBD, were used. They blocked binding, internalization, and caused a reduction in the measured spot count compared to cells treated with QD-only (**Figure 4B**).

FIGURE AND TABLE LEGENDS:

Figure 1: High-content image analysis and segmentation of ACE2-GFP cells treated with QD_{608}-Spike. Representative images of accurate segmentation. First, nuclei are segmented from the channel containing the nuclear marker to create an ROI population. From the ROI population, an

ROI region outlining the cell is segmented using the ACE2-GFP channel. Lastly, QD_{608}-Spike spots are segmented from the Cell ROI region.

Figure 2: QD_{608}-Spike binds hACE2 and is endocytosed in hACE2-GFP HEK293T cells. (**A**) Image montage of hACE2-GFP (yellow) HEK293T cells treated with several concentrations of QD_{608}-Spike (magenta) or media-only. Digital phase contrast (cyan) was used to visualize the cell body. Scale bar: 20 µm. (**B**) High content analysis of QD_{608}-Spike Spot Counts normalized to 20 nM QD_{608}-Spike (100%) and the control (0%). N = triplicate wells. Error bars indicate standard deviation (S.D.).

Figure 3: Aggregated QD_{608}-Spike is not internalized into hACE2-GFP HEK293T cells. Image montage of hACE2-GFP HEK293T cells treated with 10 nM QD_{608}-Spike or media-only. Scale bar: 20 µm.

Figure 4: Neutralizing antibody blocks endocytosis of QD_{608}-Spike in hACE2-GFP HEK293T cells. (**A**) Image montage of hACE2-GFP (yellow) HEK293T cells treated with QD_{608}-Spike (magenta) preincubated with decreasing concentrations of neutralizing antibodies, using digital phase contrast (cyan) to identify cell bodies. Scale bar: 20 µm. (**B**) High content analysis of QD_{608}-Spike Spot Counts normalized to media-only control (100%) and 10 nM QD_{608}-Spike only (0%). N = triplicate wells.

DISCUSSION:

The method described in this article provides the necessary steps for imaging functionalized QDs in human cells using high-throughput confocal microscopy. This method is best suited for cells where endocytosis is the main route of viral entry rather than the activity of TMPRSS2 and membrane fusion, as it enables the study of SARS-CoV-2 Spike and hACE2 endocytosis. Because of the nature of the QD model and the C-terminal His-tag on the commercially available Spike trimer, any TMPRSS2 cleavage of Spike S1 and S2 domains would leave the quantum dot attached to the S2 domain only[12]. This may prevent internalization, given that the RBD is found in S1. Therefore, if the sequence of events at the cell surface were precise, where hACE2 is bound and then TMPRSS2 cleaves Spike, a negative signal with no internalization is expected.

As this protocol deals with imaging cellular processes, the cultured cells must be permissive to viral infection with the expression of hACE2. hACE2 may be transiently transfected into cells, or a stable cell line expressing hACE2 may be generated[17]. It is recommended to verify hACE2 expression and localization using immunofluorescence with antibodies against hACE2 unless the hACE2 has a fluorescent protein tag such as GFP that can be observed using microscopy. GFP-tagged hACE2 confers the added benefit of visualizing hACE2 trafficking following QD-Spike endocytosis. Some cell lines with endogenous hACE2 expression may be used, but this should be confirmed using immunocytochemistry. In some cases, the endogenous expression does not provide enough hACE2 binding partners for QD-Spike and may result in a signal that is poorly detected by high-throughput confocal microscopy.

One critical step in the protocol includes the procurement of high-quality QDs conjugated to the recombinant His-tagged SARS-CoV-2 Spike. The prerequisite for this is a properly purified protein that can be conjugated to the QDs without causing aggregation. Aggregated QDs will have several problems that prevent a successful experiment. Therefore, testing of QD-Spike using analysis tools (e.g., UV-visible spectroscopy, transmission electron microscopy, or dynamic light scattering) prior to full experimentation is highly recommended to preserve valuable resources if testing precious reagents[16]. During the labeling of QDs with spike, specific concentrations of QD and Spike are mixed to achieve a specific ratio of molecules. Only QD and Spike are mixed, and both solutions are highly purified. To assess the labeling of QDs, an acrylamide gel can be cast and loaded with QD alone as well as QD-conjugated to Spike, which results in heavier molecular weight bands.

The QDs used in this protocol have a fluorescence excitation/emission spectrum tuned to 608 nm emission following UV excitation (≤405 nm) since most QDs have high absorption near the UV range producing high photoluminescence[18]. This is an unconventional excitation/emission combination that requires a microscope to have customizable channels. Many traditional confocal microscopes can be set up with the right filters and laser lines to achieve this excitation/emission. Alternatively, excitation of the QD at the first absorption maximum, approximately 10 to 20 nm away from the emission peak (e.g., 592 nm for QD_{608} used here), will also be able to produce sufficient photoluminescence.

The cloud-based software used in this high-content analysis protocol uses a naming scheme that builds on previous steps. For example, the first objects that are segmented are nuclei, which create a population called Nuclei. Following this, the cell or cytoplasm can be identified as an ROI region within the population Nuclei. The terminology used in the image analysis software sets the name of the population of objects segmented using the nucleus building block as Nuclei. However, they do not necessarily have to be nuclei and can be cell bodies if no nuclear dye is available. This naming scheme can also be changed and customized within each building block output name.

Our protocol did not account for membrane interactions, but this could be done by adding an additional membrane stain that is independent of ACE2-GFP trafficking. To block nonspecific binding, 0.1% BSA is included in the assay media (DMEM + 0.1% BSA), and the cells are grown in 10% FBS as well. Mutant spike proteins could be used, but we were limited to commercially available spike proteins. In this case, a SARS-CoV-2 mutant non-ACE2 binding Spike protein was not available.

The QD nanoparticle method presents a powerful technology to study the binding and internalization of viruses that rely on Spike-mediated entry. This assay can be used for high-throughput screening in an analogous manner, as shown in **Figure 4**, to repurpose and identify potent antivirals that block cellular entry. While this protocol only demonstrated the use of QDs conjugated to the Washington WA-1 reference strain of SARS-CoV-2, this assay can be easily adapted to study the binding properties of newly emerging variants such as Alpha, Beta, Gamma, and Delta through the use of their respective spike proteins conjugated to QDs.

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DISCLOSURES:

The authors have no conflicts of interest to disclose.

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Figure 1

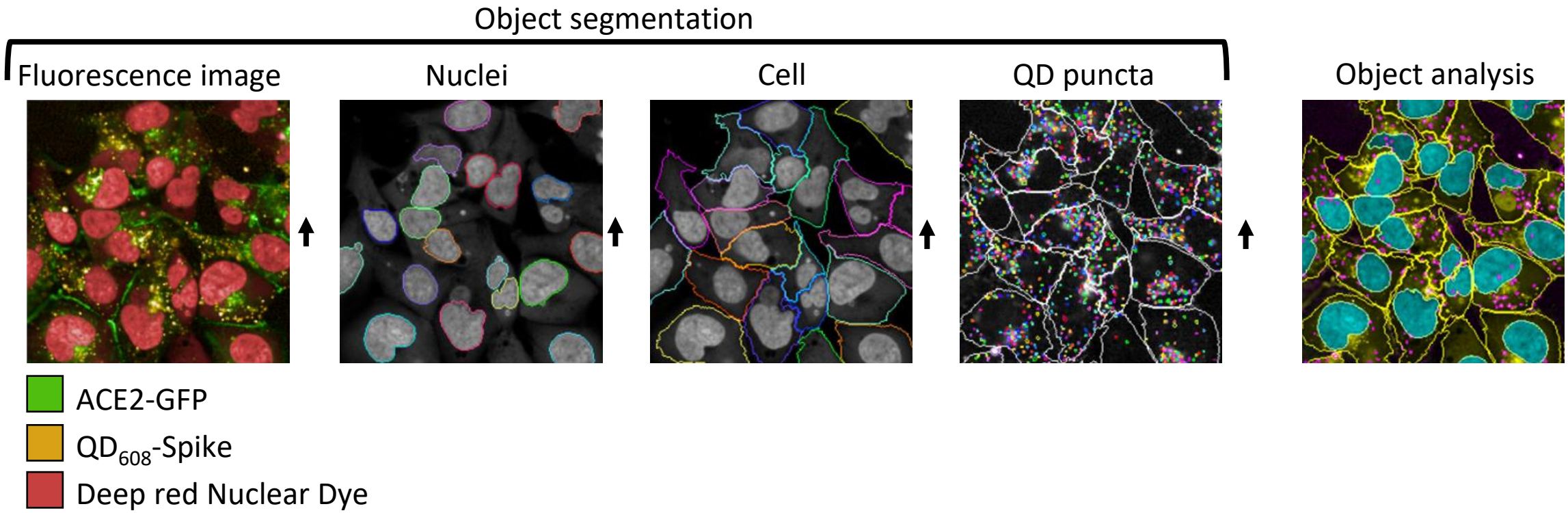
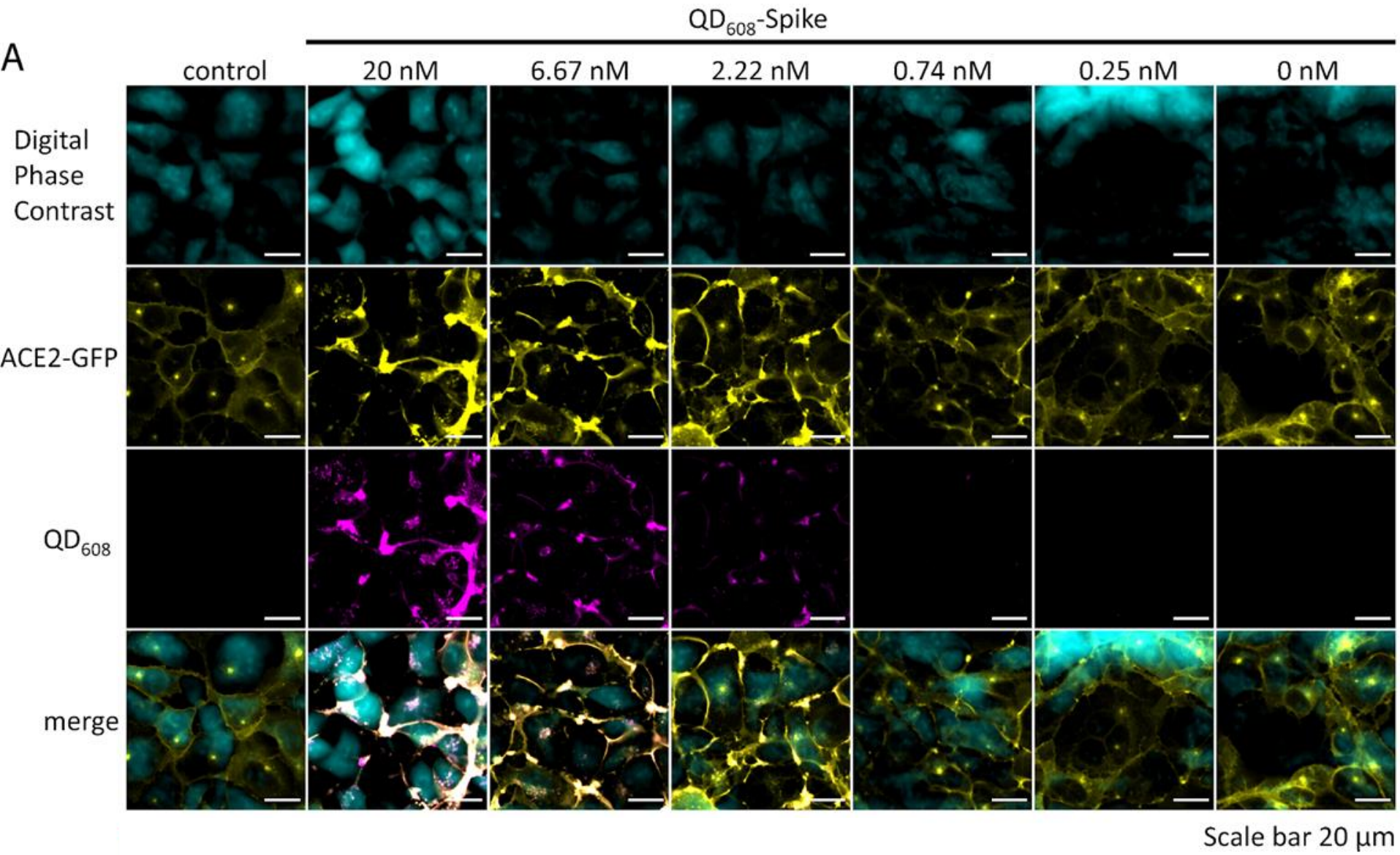


Figure 2



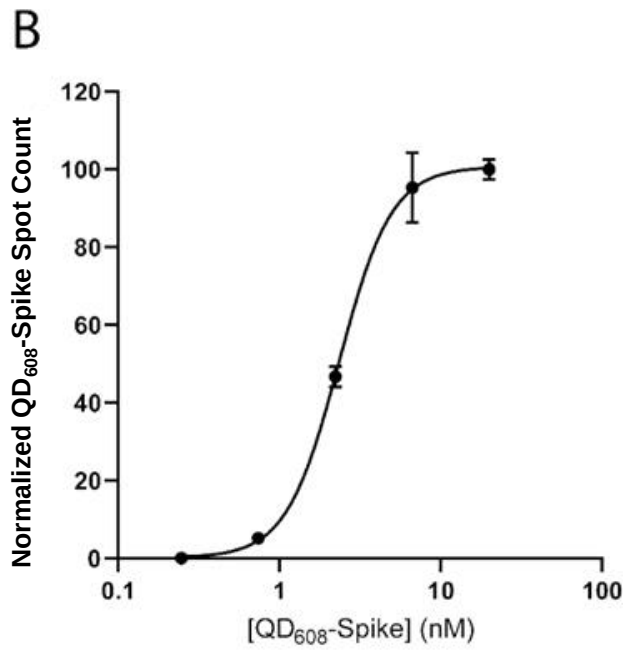


Figure 3

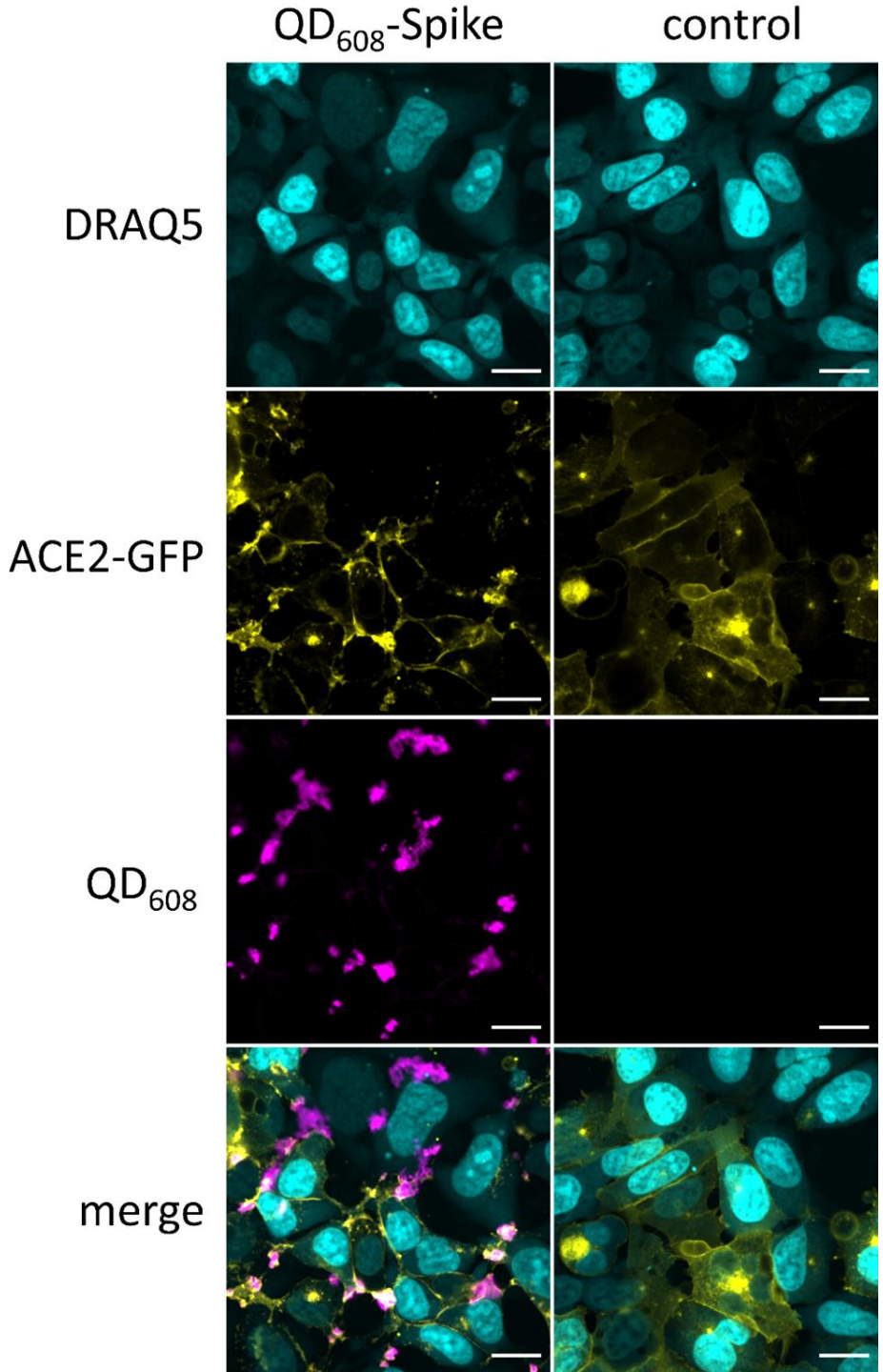


Figure 4

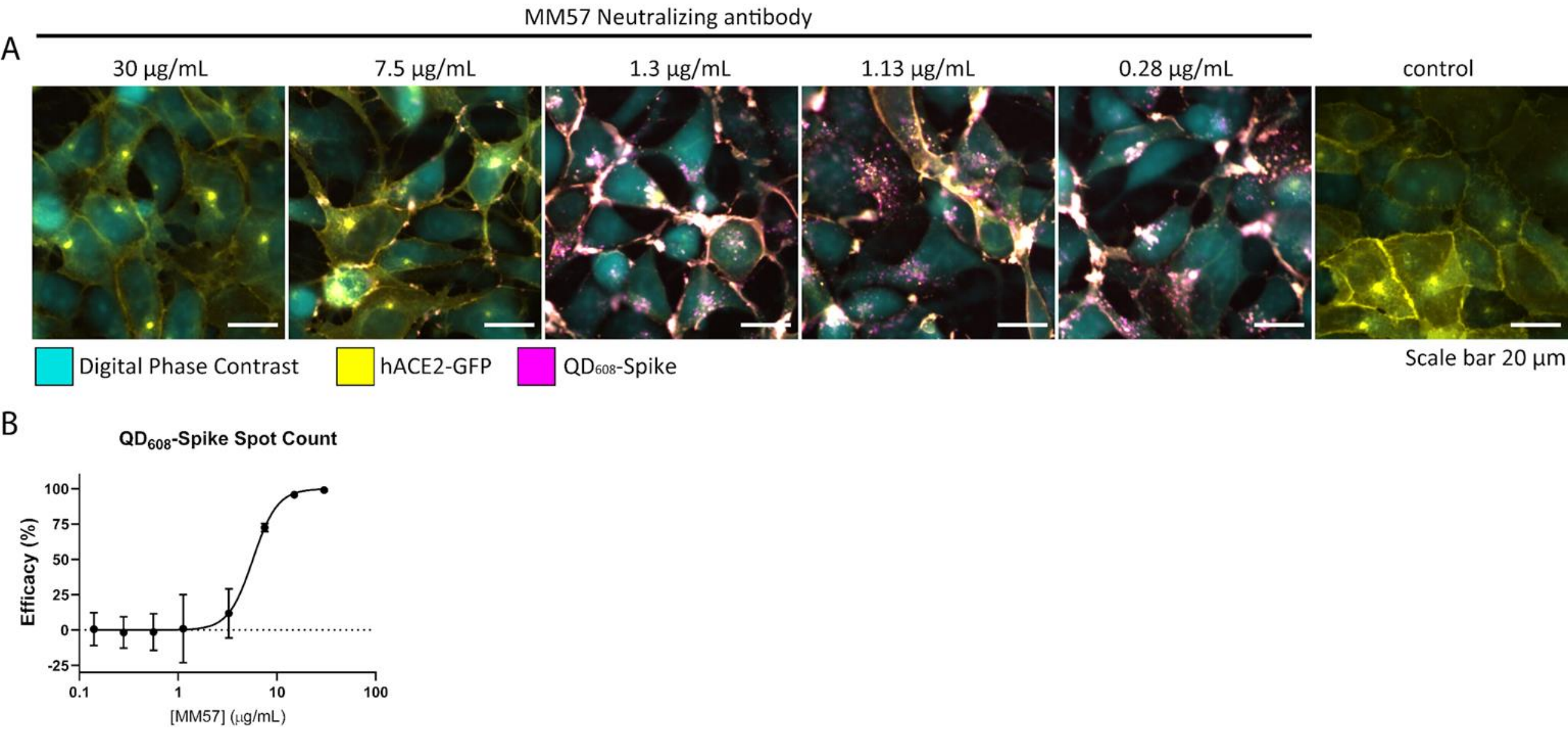


Table of materials

Click here to access/download

Table of Materials

Table of Materials_final.xlsx

Editorial Changes
Changes to be made by the Author(s):
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

Authors' response: We thank the editor for this reminder and have proofread the manuscript.

2. Please begin the protocol numbering from the section heading. For example, 1. Cell culturing and seeding, followed by 1.1 Prepare cell culture…, 1.1.1 For 500 mL of media…, and so on.

Authors' response: We have adhered to the protocol numbering format as indicated above.

3. 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 OptiMEM, DRAQ5, Harmony Perkin Elmer Phenix, Greiner µClear, HCS Cell Mask Deep Red, Columbus, GraphPad, Prism, etc.

Authors' response: We have removed all commercial names and replaced them with OptMEM (imaging media), DRAQ5 (deep red nuclear dye), Perkin Elmer Phenix (imaging platform), Greiner uClear (clear bottom imaging plate), HCS Cell Mask Deep Red (deep red cell stain), Columbus (image analysis software), Graphpad Prism (graphing software), etc. Please note that one reviewer asked us to provide background information on the Columbus imaging software, but without using commercial product names we can only describe it in a limited way.

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

Authors' response: We have revised the text accordingly to remove personal pronouns.

5. The protocol steps should be described in the imperative tense as if telling someone how to do the technique (e.g., “Do this,” “Ensure that,” etc.). Any text that cannot be written in the imperative tense may be added as a “Note.” However, notes should be concise and used sparingly. Please include all safety procedures and use of hoods, etc.

Authors’ response: We have revised the manuscript to use the imperative tense and included safety procedures and use of hoods, etc.

6. Please add more details to your protocol steps. Please ensure you answer the “how” question, i.e., how is the step performed?

Authors’ response: We have added more detail to the protocol steps where possible to explain how the step is performed.

Line 80: How were the cells counted?

Authors' response: We have added a description of how cells were counted.

Line 92-102: In this protocol, how many QD-Spike variants were used? Were the experiments performed in triplicates? What was the final volume added to each well?

Authors' response: In this protocol, we only demonstrate use of the Washington WA-1 strain of SARS-CoV-2 Spike. The experiments were performed in triplicate and the final volume of QD-Spike added to each well was 50 µL.

Line 104: Was 100 µL of media removed?

Authors' response: 100 uL of spent media was removed prior to the addition of QD-Spike in Optimem Reduced Serum media containing 0.1% BSA. We have amended this section to be more clear about the handling of the media.

Line 106: The QD-Spike solution has variants as well as BSA? Is the solution added corresponding to the plate set up with all the dilutions?

Authors' response: The 0.1% BSA is added to the media used to dilute the QD-Spike solution. Only one variant was used in this protocol. We have clarified this point in the text.

Line 112: what is the volume of the solution aspirated?

Authors' response: The volume of solution aspirated is 50 µL .

Line 118: Was the fluorescent probe added earlier as well? if yes, at what step and what was the volume added?

Authors' response: The fluorescent probe was added to the cells only at Step 2.4 with a volume of 50 uL. We have amended the text for this point.

Line 154,186: What is used as the positive control?

Authors' response: The positive control in the case where we are assessing the internalization of QD-Spike is the highest concentration of QD-Spike. If we are assessing the efficacy of a neutralizing antibody, the positive control are the wells containing media-only.

7. Please ensure that the highlighted part of the step includes at least one action that is written in the imperative tense.

Authors' response: We have amended the highlighted portion to ensure that each step has one action written in the imperative tense.

Reviewers' comments:

Reviewer #1:

The manuscript describes the procedure to image ACE2 internalization upon interaction with SARS-CoV2 spike trimer-decorated quantum dot. The procedures from cell culture to imaging and data collection are well described. . As the authors mentioned in the Introduction, SARS-CoV-2 spike interacts with ACE2 which are on the cellular surface and undergoes cleavage by TMPRSS2, which may lead to endocytosis of the virus or conformational change of spike followed by membrane fusion. Whereas QD-Spike makes it possible track endocytosis, this protocol seems to be difficult to assess the membrane fusion. In addition, it is hard to examine the effect of cleavage of S1-S2 by TMPRSS2 on imaging. Thus, although the imaging of endocytosis using QD-RBD is a fine approach, the model system used here (SARS-CoV-2 spike) is unlikely to be a suitable example. The protocol just relied on the use of the QDs which have been produced by Naval Research Laboratory Optical Nanomaterials Section. Thus, researchers seems to be difficult to access the protocol.

Authors' response: We thank the reviewer for their effort in reviewing our submission. The reviewer's point regarding membrane fusion is well taken. Our model enables the study of SARS-CoV-2 spike and hACE2 endocytosis rather than the activity of TMPRSS2 and membrane fusion. Because of the nature of our QD model and the C-terminal His-tag on the commercially available spike trimer, any TMPRSS2 cleavage of spike S1 and S2 domains would leave the quantum dot attached to the S2 only, which may prevent internalization given that the RBD is found in S1. Therefore, if the sequence of events were to be precise at the cell surface where hACE2 is bound and then TMPRSS2 cleaves spike, we would expect a negative signal with no internalization. Our model is best suited for cells where endocytosis is the main route of entry.

Specific comments:

1. Although the authors recommended the use of QD-Spike concentration over 10 nM, there is only single data that suit this condition in Figure 1 (20 nM). It would be more helpful if more images in the recommended concentration range are provided.

Authors' Response: We thank the reviewer for the suggestion but we did not use any concentration between 20 and 6.66 nM for this study. We have shown the representative images for the work.

2. The legend of Y-axis in Figure 1B should change to QD608-Spike, not QD608-RBD.

Authors' Response: We thank the reviewer for catching this error and have made the correction.

Reviewer #2:

Manuscript Summary:

The method by Tran et al. is a useful guide and will be helpful to researchers who wish to perform high-content screening of endocytosis. I have included some comments below that I hope the authors will find useful in fine-tuning their manuscript prior to publication.

Major Concerns:

1. The introduction and/or conclusion may benefit from some wider context since this method is applicable to high-content screening of endocytosis of any protein-QD complex.

Authors' response: We thank the reviewer for this comment and have added general comments regarding high-content screening to the introduction since it is the type of analysis used in the study.

2. As mentioned above, some of the steps (e.g., 19.4.2, 27.2, 30.1-30.2, 37.2) are a little too generalised which makes the method difficult to follow. For example, the recommended products include ACE2-GFP so there is no need to mention 'if' at step 19.4.2; similarly, if one follows the recommended procedure are the 'if' conditions at step 27.1 necessary? I believe the method could be easier to follow if the authors can be more definite about what needs to be done in this particular experiment. Alterations to the protocol for other proteins could be discussed separately.

Authors' response: We agree with the reviewer's comments and have made the protocol more specific.

3. Some of the QD signal in Figure 1a does not appear spot-like - for example in the 20 nM condition there are some very large and intense signals in the bottom right corner. Are these really quantifiable with respect to endocytosis 'spots' and what implications does this have for analysis?

Authors' response: We appreciate the reviewer's keen eye and agree that not all of the fluorescent objects are spotlike. The biology of Spike:ACE2 complex and the context of our in vitro experiment produce what is shown in the figure. The image analysis software will identify objects that fit within the parameters of the method used for spot identification. This selection is dependent on the preference of the researcher and is applied to all samples tested.

4. Where is the quantification performed in the graph in Figure 1b - the caption only mentions spot counts but is this in the nucleus or cytoplasm? Similarly, step 30 is a little confusing to me. Is the ROI population defined as nuclei for spot counts? Would one expect to see puncta in the nucleus, which are characteristic of endolysosomal entrapment (e.g., Bioconjug Chem 28, 2932 (2017))? This point could be expanded upon in the discussion.

Authors' response: We thank the reviewer for bringing up this point. The quantification of spots is performed in the whole cell. The terminology used for image segmentation in the Columbus analyzer software sets the name of the population of objects segmented using the nucleus building block as "Nuclei". They do not necessarily have to be nuclei. We have discussed and clarified this point.

5. Are all membrane interactions corrected for in this method by including BSA in the media? Is there a way to exclude any QDs localised at the membrane to avoid false positives, for example, using QDs conjugated to a mutant spike protein as a control (see also point 5)?

Authors' response: This is a great point and worthy of discussion. Our protocol did not account for membrane interactions, but this could be done by adding an additional membrane stain that is independent of ACE2-GFP trafficking such as Cell Mask Green Membrane stain. 0.1% BSA is included in the assay media (OMEM + 0.1% BSA), and the cells are grown in 10% FBS as well. Mutant spike proteins could be used, but we were limited to commercially available spike proteins. In this case, a SARS-CoV-2 mutant non-ACE2 binding Spike protein was not available. Our protocol demonstrated QD-Spike cell-based assays using the Washington WA-1 reference strain.

6. At line 255, the authors state that strong colocalization of ACE2-GFP and QDs should be observed. Should spots be filtered to only include GFP+/QD+ spots in the analysis?

Authors' response: We cannot be certain of the duration of an individual ACE2:spike interaction after binding and internalization into the cell. To maximize the assay window, all objects can be counted. We have previously demonstrated in our initial publication that QDs conjugated to SARS-CoV-2 Spike do not engage with cells to any appreciable extent as measured by high content microscopy. Similarly, unconjugated QDs do not bind or internalize into cells regardless of ACE2 expression.

7. At steps 19.5 and 26, what is meant by a positive control well? What are the conditions? Both positive and negative controls to include in the experiment should be explained at the "Treatment of cells with QDs" stage.

Authors' response: We apologies for the lack of clarity in the protocol regarding positive controls. We have amended these steps and clarified the proper well to use for setting the proper settings. The positive control in the case where we are evaluating the internalization of QD-Spike is the highest concentration of QD-Spike. When we evaluate the efficacy of a neutralizing antibody, the positive control are the wells containing media-only.

8. Only spot counts are shown in the figures in this manuscript. Since spots may vary in size and intensity, is it possible to add some other more quantitative measure of total uptake, such as integrated intensity?

Authors' response: Indeed, more quantitative measurements can be made including spot area multiplied by spot intensity to produce integrated intensity.

9. At step 19.4.1, what should the reader look for in an image for good segmentation?

Authors' response: We thank the reviewer for the question and have amended step 19.4.1 to better guide the reader in image analysis. Good segmentation includes accurately defining the region of interest for the nucleus, cell, and spots. This means only the pixels that are positive for the marker

should be captured within an individual ROI. Background signal should be excluded. If background is being captured in the ROI, the sensitivity of the method could be decreased or the threshold of intensity could be increased to increase the stringency of the segmentation algorithm.

10. In the discussion, protein-QD aggregates are mentioned (line 262). How are solutions cleared of such aggregates and how is the actual concentration of protein quantified?

Authors' response: Once aggregates form the pH may be adjusted to 8.0. The concentration of the protein is known before mixing with the QDs and can be calculated based on the total amount of protein added to the solution and the total volume.

11. At step 28.1, it is claimed the digital phase contrast (DPC) image can be used to identify nuclear regions of interest. However, in most of the images in Fig. 1, the DPC channel does not appear to show cells clearly, let alone the nucleus. Could this negatively affect the analysis if live imaging is used?

Authors' response: The reviewer's astute observation reveals that DPC is an approximate indicator of cell morphology not specifically the nucleus. The area of interest overlaps with the DPC signal and is sufficient for detecting internalized QDs. The total number of cells would be underestimated if DPC does not capture cell bodies in enough fields to offset the average.

Minor Concerns:
1. The protocol includes some rather general comments regarding analysis. For example, point 27.2 lists the available options. What are appropriate settings to use in this case?

Authors' response: We have removed this since it does not apply to this experiment.

2. Some introduction to the Columbus software (what it does, the company that makes it, the version used, alternatives etc.) could be helpful to guide the reader.

Authors' response: We thank the reviewer for pointing out a lack of software introduction and have amended the text to incorporate Columbus more specifically. Columbus, v2.9.1 is a cloud-based high content imaging analysis software that can accommodate a large variety of instrument data outputs including the Perkin Elmer Opera Phenix high content imaging platform. Columbus is also made by Perkin Elmer. The alternatives to Columbus include but are not limited to CellProfiler from the Broad Institute, and ImageJ from the NIH.

Please note, the editor has requested that we remove all commercial language from our manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials.

Reviewer #3:
Manuscript Summary:
This protocol provide methods for looking to utilize quantum dots to study protein-protein interactions

and trafficking in the context of cellular physiology.

Major Concerns:
there is no introduction of how the necessary materials are prepared and quality verified, for example the QD-spike?

Authors' response: We thank the author for this comment. The manuscript describing the generation and characterization has been published previously, referenced below. In this protocol, we are focused on the execution of the assay using ready-made QD-spike.

Quantum Dot-Conjugated SARS-CoV-2 Spike Pseudo-Virions Enable Tracking of Angiotensin Converting Enzyme 2 Binding and Endocytosis

Kirill Gorshkov, Kimihiro Susumu, Jiji Chen, Miao Xu, Manisha Pradhan, Wei Zhu, Xin Hu, Joyce C. Breger, Mason Wolak, and Eunkeu Oh

ACS Nano 2020 14 (9), 12234-12247

DOI: 10.1021/acsnano.0c05975

How to make sure the specific labeling and activity of the QD-spike?

Authors' response: We appreciate the Authors' concern. During the labeling of QDs with spike, specific concentrations of QD and Spike are mixed so that we achieve a specific ratio of molecules. Only QD and spike are mixed together, and both solutions are highly purified. To assess the labeling of QDs, an acrylamide gel can be cast and loaded with QD alone as well as QD-conjugated to spike that results in heavier mW bands. We have added this point into the manuscript.

some of the text is highlighted by yellow background, why is that?

Authors' response: We highlighted a portion of the text for videography as a courtesy to the editorial staff.

High-throughput confocal imaging of quantum dot-conjugated SARS-CoV-2 Spike trimers to track binding and endocytosis in ACE2-GFP HEK293T cells

List of Changes

1) The numbering of the steps has been revised according to the editor's comments.
2) We have removed commercial names from the manuscript and amended the Table of Materials
3) We have revised the text to remove personal pronouns.
4) We have revised the manuscript to use the imperative tense and included safety procedures and use of hoods and personal protective equipment.
5) We have added more detail to the protocol steps where possible to explain how the step is performed.
6) We have added a description of how cells were counted.
7) We clarified that only one strain of SARS-CoV-2 Spike was utilized in this protocol.
8) We clarified volumes and procedures for liquid handling in the text.
9) We clarified that 0.1% BSA in Optimem I reduced serum media was used with the QD-Spike fluorescent probe.
10) We amended the text to specify what wells to use for normalizing the data.
11) We highlighted the portion of the text to be used for video recording that includes imperative tense sentences.
12) We have amended the Y-axis on Figure 1B to read QD608-Spike
13) We have added general comments regarding high-content imaging and analysis to the introduction since it is the type of analysis used in the study.
14) We have made the protocol more specific and less ambiguous based on the reviewer comments particularly for the previous sections of 19.4.2, 27.2, 30.1-30.2, and 37.2. The sections have been renumbered based on editor's suggestions.
15) We have discussed the naming scheme in the image analysis software in regards to the "Nuclei" population identifier.
16) We clarified that the appropriate image segmentation refers to the object of interest and the accuracy of the generated ROI for each particular object (nuclei, cell body, spots).
17) We removed some sections of the protocol that were not relevant to the immediate experiment, while balancing the presentation of available options to the reader.
18) We highlighted a portion of the text for videography as a courtesy to the editorial staff.

Dear editor,

We thank you for the opportunity to revise our manuscript titled “High-throughput confocal imaging of quantum dot-conjugated SARS-CoV-2 Spike trimers to track binding and endocytosis in ACE2-GFP HEK293T cells” after editorial and reviewer comments. We appreciate the feedback and specific suggestions to make our protocol clear and accessible to the readers. We have answered all of the comments and revised the manuscript according to the recommendations. We hope you agree that this manuscript is now suitable for publication in JoVe for the special issue.

Sincerely,

Kirill Gorshkov

Ph: 713-444-5317
Email: kgorshkov89@gmail.com