Application Note

Microbiology

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Image Analysis of *Herpesvirales* Specificity Challenge using Direct Fluorescence Antibody Assay (DFA)

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Abstract

Herpes Simplex virus 1 and 2 (HSV1/2) and Varicella-Zoster virus (VZV) fluorescently tagged monoclonal antibodies (mAbs) were challenged against known Cytomegalovirus (CMV)-infected fibroblast cells to evaluate performance of an automated digital widefield inverted microscopy system. Image analysis of the direct fluorescent antibody assay (DFA) demonstrated no cross-reactivity and 100% specificity of HSV1/2 and VZV mAbs against CMV antigen. The most important parameters for differentiating positive from negative viral staining were correct filter sets, and focal, exposure, and background flattening settings.

Introduction

Direct immunofluorescence method, or DFA, is one approach used to identify distinct proteins on the surface of viruses. Compared to indirect immunofluorescence (IFA) methods, where two steps are required to bind fluorescent probe to an antigen-antibody complex, in DFA the fluorescent label is conjugated directly to the primary antibody, requiring only one binding step. After conjugate addition, nonbinding antibody is washed away, and the presence or absence of a target antigen-antibody complex is visualized by fluorescence microscopy (Figure 1). The simplicity of DFA makes it a useful tool to confirm culture of viral isolates, screen samples as part of preliminary disease diagnosis, and to monitor therapeutic efficacy among other applications. This application note describes the use of automated microscopy in a DFA assay workflow compared to non-microscopy DFA assays. Although DFA can be used in IVD workflows, the methods compared in this app note do not present a protocol for an IVD workflow

While DFA facilitates qualitative presence/absence testing, it also allows visual characterization and quantification of viral inclusions, including intracellular localization and effects on cell morphology. Moreover, assay artifacts or contaminants that may interfere with results interpretation can be characterized and excluded. In this case study, a digital widefield microscope that automates image capture, processing, and analysis is applied to DFA. Automating microscopy tends to simplify workflows, especially with multiple specimens, and can eliminate user variability in manual microscopy.

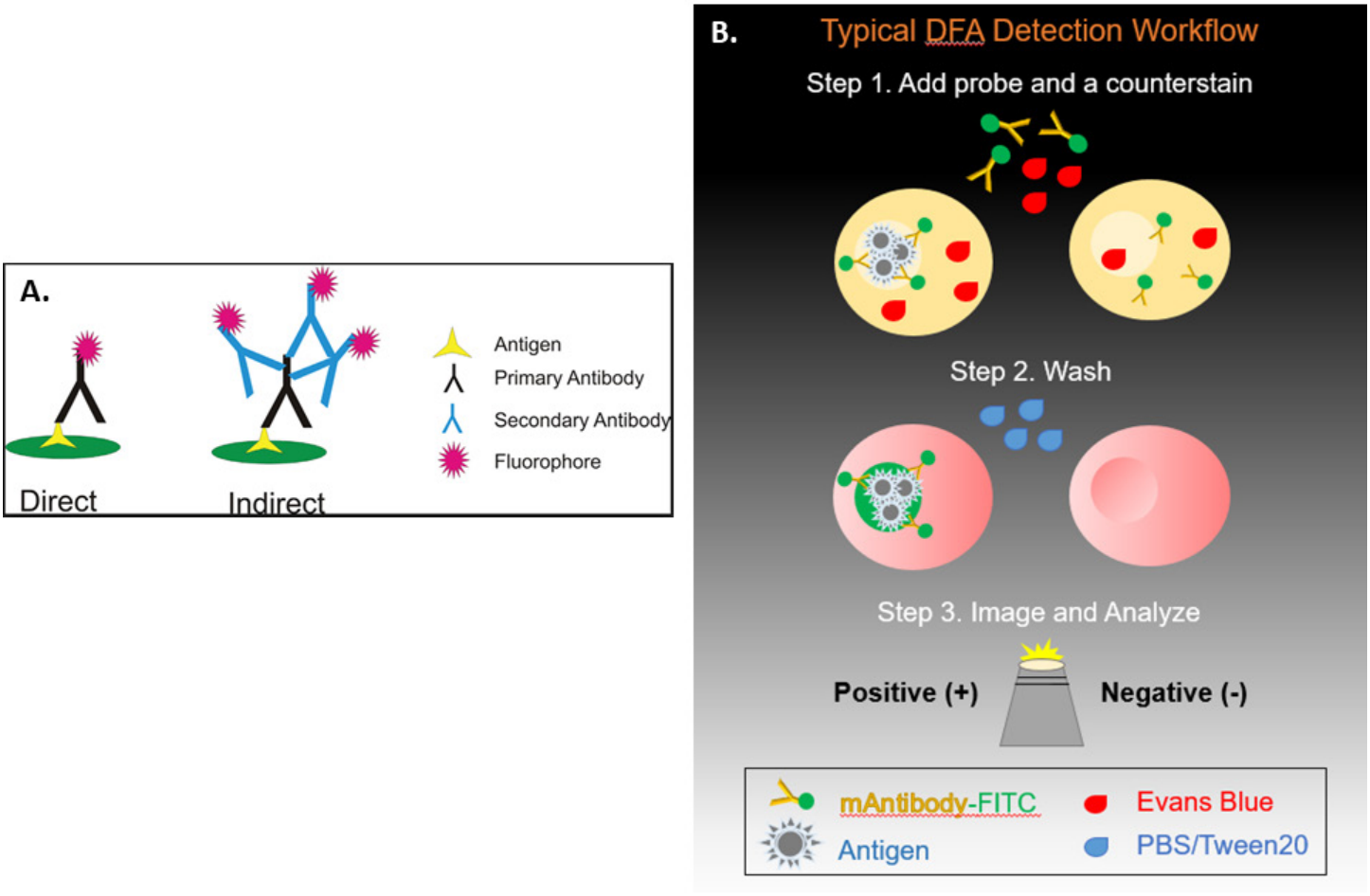


Figure 1. (A) In comparison to indirect immunofluorescence, DFA requires only one antibody binding step. (B) A typical DFA workflow involves adding fluorescent probe tagged primary antibodies specific to viral antigen to sample cells along with a counterstain. After washing, only infected cells retain the antibody. The sample is then imaged to assess for positive or negative results. If a control sample known to be positive for a defined viral antigen is treated with a nonspecific antibody the result should be negative.

As a microscopy method, DFA relies significantly on the "performance of the fluorescence microscope [that] is of critical importance in achieving satisfactory test results… objectives, bulb intensity and wattage, and filters may affect results" (Millipore 2012). DFA universally specifies the use of controls to verify functioning of reagents, culture methodology, microscope settings, and user proficiency in staining technique. When corroborating performance of a new detection and analysis instrument, a specificity challenge is one way to compare test results to expected outcomes (Table 1). In the work done here, a common IVD application was used as a model to demonstrate image analysis of direct immunofluorescence. Combined monoclonal antibodies to HSV 1 and 2 and VZV antigen were challenged to known CMV antigen-positive and -negative control cell monolayers to evaluate configuration and analysis parameters required for an automated digital widefield inverted microscopy and image analysis system to perform DFA applications.

Table 1. A common way to determine agreement between an established method and a test method is to compare results using some format of a 'truth' matrix. Diagnostic tests are often subject to accuracy testing using this approach. Specificity and sensitivity are accuracy indicators that refer to the ability of a test method to correctly identify true positives and true negatives. Using this table, percent specificity is calculated as [d/(b + d)] × 100, and percent sensitivity as [a/(a+c)] × 100. A target with 100% specificity and 100% sensitivity would indicate that interpretation of negative and positive samples respectively was correlative in all cases compared to an established method.

	Control Result	
Challenge Result	(Pos) +	(Neg (–)
Pos (+)	True pos (a)	False pos (b)
Neg (–)	False neg (c)	True neg (d)

DFA assay and challenge test principle

The ability of the automated digital microscope used in this evaluation to clearly distinguish positive and negative controls, correctly identify cross-reactivity, and exclude negative results within an acceptable %specificity range was guided by specifications for detecting and identifying viral staining patterns included in the assay user manuals (see "Materials and methods").

As defined within the user manuals, using a FITC filter set, positive staining for immediate early antigen IE1 and IE2 CMV AD169-derived protein is represented by the presence of at least 2 or more intact cells exhibiting bright green fluorescence in the nucleus, and an HSV-1 and HSV-2 155 kD major capsid protein positive reaction is indicated by at least 2 or more intact cells exhibiting bright green fluorescence in the cytoplasm and/or cell membrane. A VZV immediate early antigen glycoprotein gp I positive reaction is indicated by at least 2 or more intact cells exhibiting pink fluorescence in the nucleus, cytoplasm, and/or cell membrane using a TRITC filter set. Additional defining morphology characteristics are described, as shown by Figure 8. The expected cross-reactivity of HSV/VZV antibodies with CMV antigen is a negative result (<2 positive cells). Using the HSV/VZV DFA conjugate, negative results were correctly interpreted with an average relative specificity of 97% against predicate methods. The lowest % specificity was 85.7% with a 95% confidence interval of 79.2% to 92.2%.

Materials and methods

Challenge assay

The test procedures for the Millipore Light Diagnostics CMV DFA kit (part number 3245), CMV control slides (part number 5027-5), Simulfluor HSV/VZV DFA kit (part number 3295), HSV control slides (part number 5093-5), and Varicella-Zoster control slides (part number 5088-5) were followed according to the kit insert instructions (Figure 1). CMV assays were also run with the exception that SimulFluor HSV/VZV Reagent (part number 5235) was used in place of CMV DFA Reagent (part number 5090). The assay kits and slide accessories both include prepared positive and negative controls (Figure 2). As a means of reducing experimental variables, the challenge assay was designed to optimize, then apply automated image capture, processing, and analysis solutions to the Test Procedure, Interpretation of Results, and Limitations section of the user manuals by comparing results for control wells treated with specific antibody versus controls challenged with nonspecific antibodies.

Figure 2. Prepared positive and negative control slides.

Imaging assay

Slides were imaged cover slip down using a slide adapter (part number 1220548) on an Agilent BioTek Lionheart FX automated microscope controlled by Agilent BioTek Gen5 v3.04 microplate reader and imager software using GFP (part number 1225101 Ex: 469 Em: 525 LED cube 465 part number 1225001); Cy5 (part number 1225105 Ex: 628 Em: 685 LED cube 623 part number 1225005); and RFP (part number 1225103 Ex: 531 Em: 593 LED cube 523 part number 1225003) filter sets, and a 20x objective (part number 1320517) with a correction collar setting of 0.17. Settings for exposure (gain, integration, and LED intensity) were done on respective positive control wells and used for all images of the same assay (Figure 4). Focal height of the cell monolayer was done using Cy5 to detect the Evans blue counterstain, and was determined both manually and using a laser autofocus feature in the software to accommodate for differences between slide preparations, cell types, and mounting media application. A 5 × 4 montage of images was automatically taken for each sample to maximize statistical data point acquisition. Signal and size thresholds used to mask assay response and define subpopulations were uniformly applied to all images of the same analyte. The principle of the imaging assay is shown in Figure 3. A detail of image analysis settings is shown in Table 2.

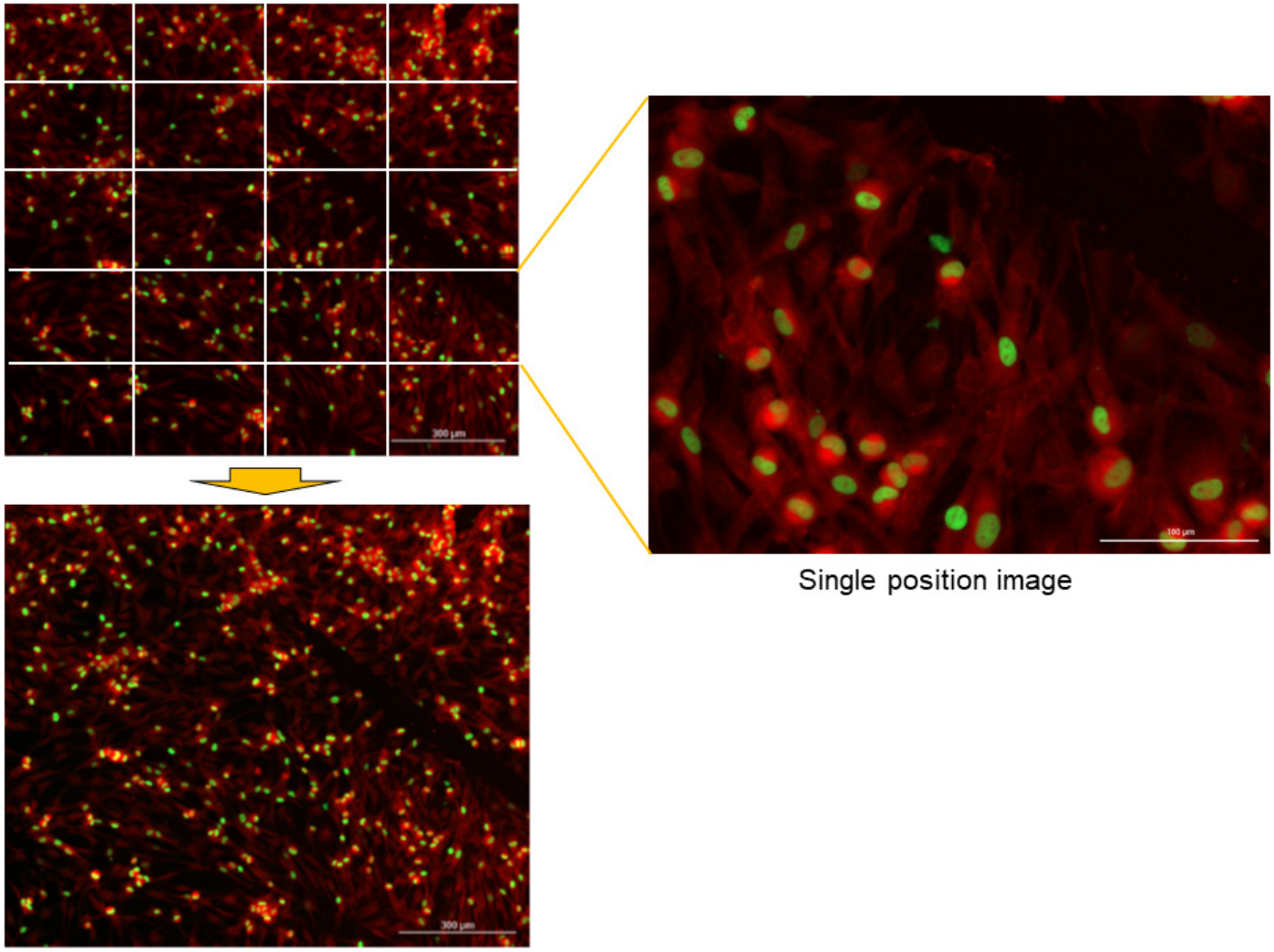


Figure 3. Imaging assay principle as shown for CMV. Multiple images captured with a 20x objective of CMV (green) in fibroblast cells (red) acquired with GFP and Cy5 filter sets respectively using a 5 × 4 montage whereby multiple individual images can be stitched into one single larger image.

Table 2. Configuration and settings used for image analysis.

		CMV		HSV1		HSV2		VZV		
Capture	**Filter Set**	**GFP**	**Cy5**	**GFP**	**Cy5**	**GFP**	**Cy5**	**RFP**	**GFP**	**Cy5**
	Detects	CMV (FITC)	Evans Blue	HSV1 (FITC)	Evans Blue	HSV2 (FITC)	Evans Blue	VZV Confirm (TRITC)	VZV Screen	Evans Blue
	Montage									
	Size		5 × 4		5 × 4		5 × 4			5 × 4
	Overlap		Default		Default		Default			Default
Process	**Background Flattening**	**Auto**[1]	**Auto**	**None**	**None**	**None**	**None**	**Auto**[1]	**Auto**[1]	**Auto**[1]
	Stitching		Linear		Linear		Linear			Linear
	Downsize (%)[2]		29.16		29.16		29.16			29.16
Analyze	**1º Mask**		**None**		**None**		**None**		**None**	**None**
	Signal Thresh	5950		5010		5010		4701		
	Size (µm)	10 to 30		10 to 00		10 to 100		6.5 to 50		
	2º Mask		**Expand 1º 7 µm**	**None**						
	2º Mask	5 µm from 1º w/ 15 µm radius		None						
	Subpopulation	**IntactVLocalztn**[3]		**None**						
	Mean_2_GFP <1263 AND			None						
	Mean_2_Cy5 >4139									

[1] Auto preprocessing was done with an index of 1 smoothing.

[2] Deactivating the downsize option did not affect cell counts, but did effect thresholding due to differences in resolution. For optimal results it is recommended to use Agilent BioTek Gen5 v3.05 with no reduced image sizing.

[3] Final positive counts include only objects defined as localized viral inclusions within intact cells (to exclude objects outside the defined region of infectivity within a single cell). GFP threshold was calculated as –1 STDev from the mean of all objects 5 µm from the nucleus with a radius of 15 µm for the CMV negative control. The Cy5 threshold was calculated from the CMV positive control and defined as the mean –2 STDev within a 7 µm radius of a positive object defined by the primary mask.

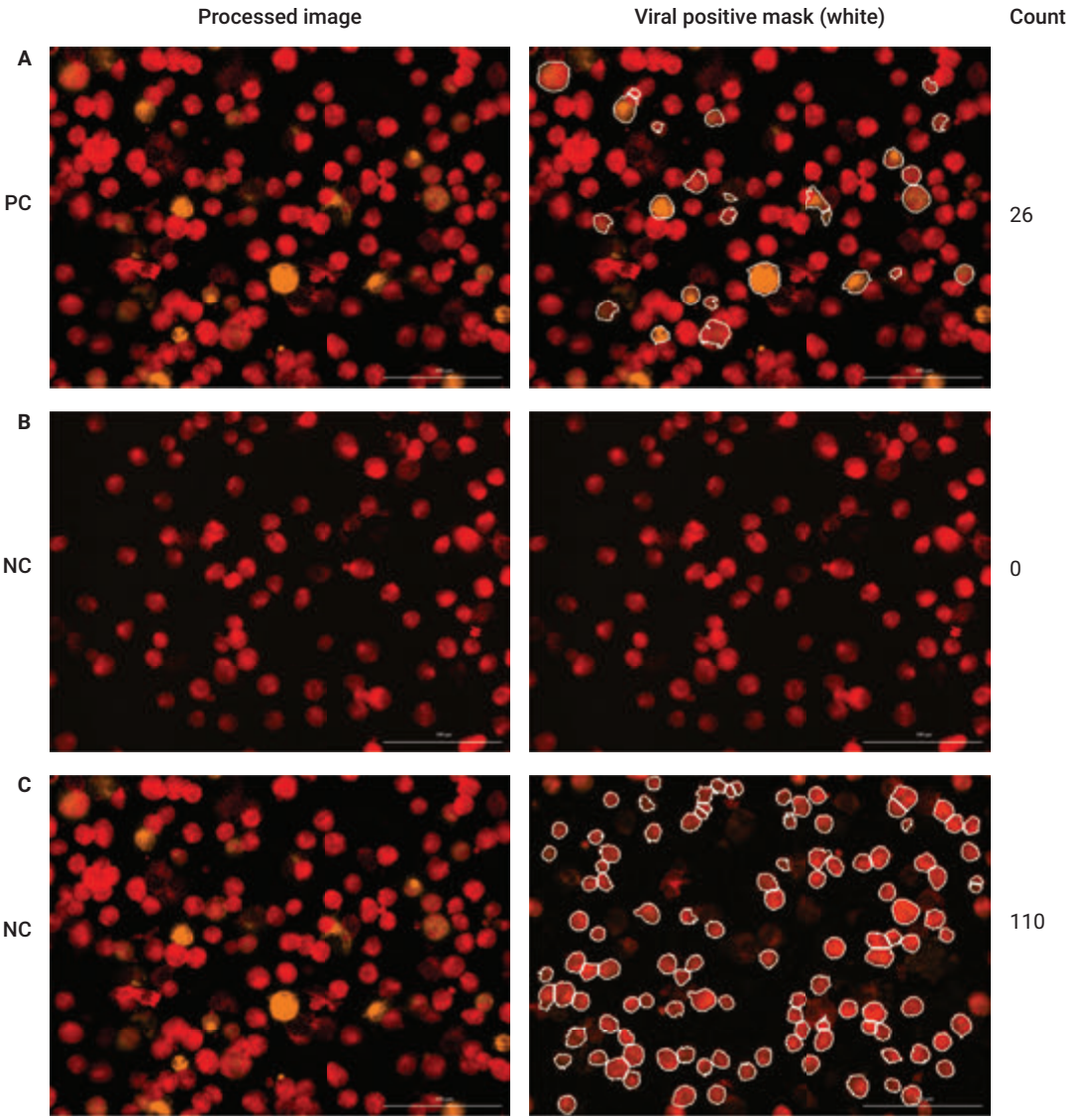


Figure 4. Incorrect image capture settings can lead to misinterpretation of results as shown for VZV DFA (A). Using the positive control (PC), focal height and optimal exposure settings are shown for VZV (RFP filter set, orange) against Evans blue counterstain (Cy5 filter set, bright red). Following application of default background correction settings, primary mask definitions on RFP result in a positive staining count of 26 objects (edge objects are excluded). (B) Focal height is determined for the negative control (NC) cell monolayer using PC exposure settings for Cy5. RFP PC exposure settings are applied at the same focal height. Using the same PC background correction and primary mask criteria, no objects are detected. (C) Autoexposure and autofocus are applied to RFP on the NC (different image area) using the Cy5 focal height as a baseline. An increase in integration time (shutter speed) of 2,924 ms from the PC exposure settings, and a focal height 10 µm away from the cell monolayer resulted in a false positive count of 110 for the NC. An aggressive decrease in the RFP rolling ball settings for background correction from 68 µm to 10 µm, and a 1.5x increase in masking signal threshold was required to eliminate false object detection for the NC (data not shown). The signal threshold increase resulted in a positive count of 20 versus 26 for the PC.

Results and discussion

Parameters required for an automated microscopy and image analysis system to interpret DFA for CMV, HSV1/2, and VZV were evaluated using a modified, abbreviated specificity challenge. Achieving valid results for DFA requires careful consideration of microscope components and imaging settings, as described below:

Correct filter sets

Evans blue has been used in DFA as a counterstain to FITC and TRITC labeled probes for decades primarily to suppress autofluorescence. There is inconsistency in reported excitation peaks for fluorescence detection of Evans blue including 470 nm, 540 nm, and 630 nm, most likely due to differences in substrate. Excitation at 470 nm and 540 nm are in the same range as FITC and TRITC probes. Although there is a difference in emission peaks between FITC and TRITC filter sets with that for Evans blue, a detectable but dull staining of Evans blue in those wavelength ranges is emitted – dull green when detected by GFP (FITC, Figures 5 and 8), and dull red when detected by RFP (TRITC). The sensitivity of the Agilent BioTek Lionheart FX automated microscope can compensate for dull staining depending on how image acquisition settings are defined. The spectral overlap of Evans blue with the mAbs probes used here was found to have the potential of leading to incorrect exposure settings and difficulty in distinguishing positive viral staining from signal background, particularly when imaging the VZV assay that defines positive staining patterns for both FITC and TRITC filter sets (GFP and RFP filter sets respectively). A spectral scan was therefore performed on Evans blue that demonstrated a distinct excitation peak of 630 nm and emission peak of 680 nm (data not shown). This spectral detection distance from both the FITC and TRITC labeled mAbs has the advantage of eliminating signal overlap and incorrect exposure settings that could contribute to misinterpretation of results. A Cy5 filter set (Ex: 630; Em: 680) was therefore chosen to image Evans blue for all analyses. This had two advantages: (1) it increased the reliability of Evans blue detection to be used as an image analysis stain rather than just a visual contrast stain (Figure 7A), and (2) provided an anchor for determining optimal focal height of the cell monolayer, making correct exposure of viral staining more accurate. For confirmation of VZV, only RFP (TRITC) is required. Although GFP is shown as a contrast to RFP in Figures 5 and 8, Cy5 is still a better choice for imaging VZV counterstaining (Figure 4). Rather than a pink appearance when detected with RFP, VZV-positive cells appear bright orange regardless of contrast filter. This is due to the default colorization of the RFP filter set in the Agilent BioTek Gen5 image analysis software. Filter channels can be recolorized without effecting analysis results. For example, RFP and Cy5 both have high red color properties. Colorization of the RFP channel can be used to increase contrast with the Evans blue counterstain (Figure 9). Based on the work performed here, IVD immunoassays that use Evans blue as a counterstain should image the dye using a filter set defined for the Ex: 630 nm; Em: 680 nm spectral region.

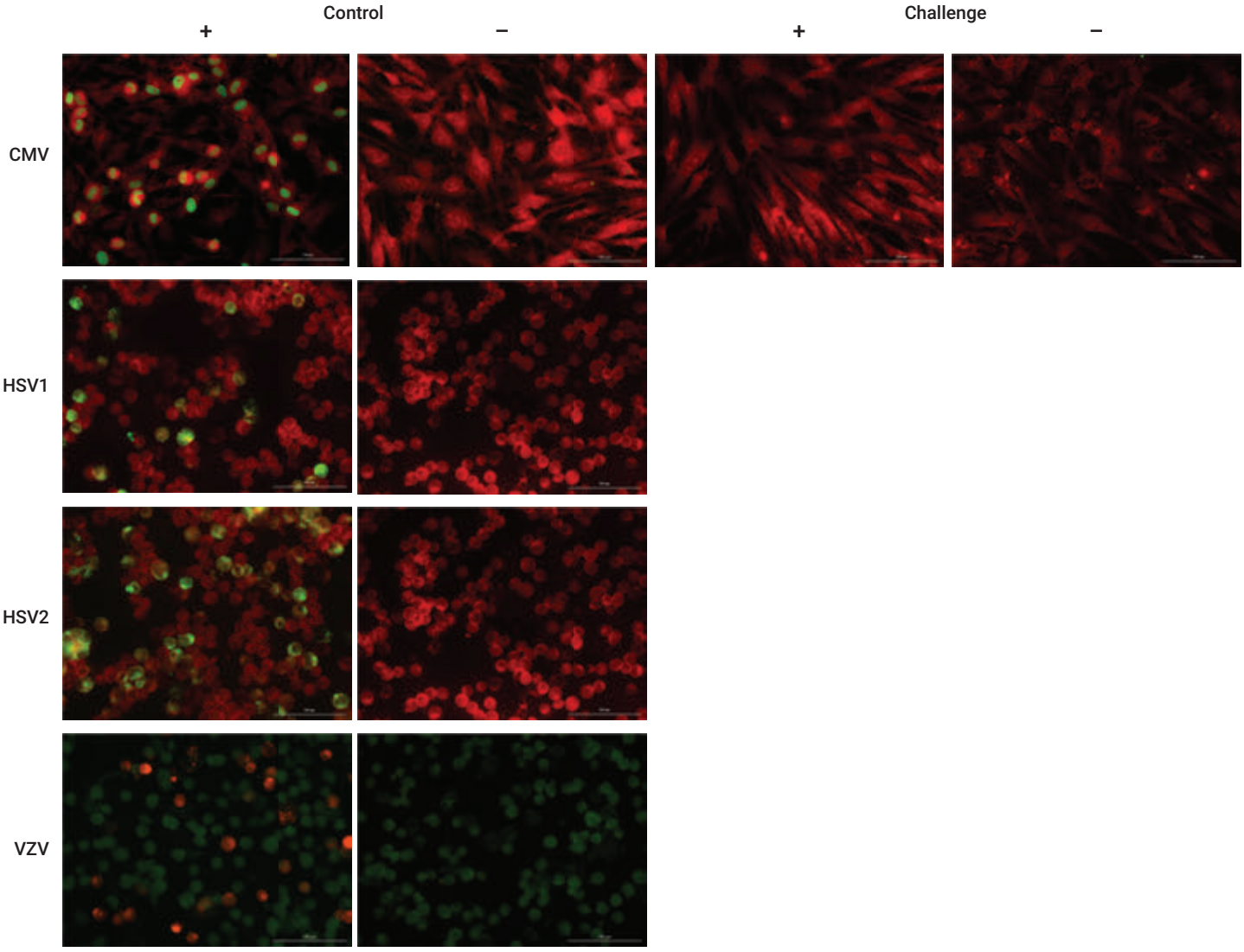


Figure 5. Image table showing representative positive and negative results. Note, VZV staining is confirmed using an RFP filter set (TRITC spectral range) where infected cells exhibit bright red-orange fluorescence due to the default color properties for the image channel in Agilent BioTek Gen5 software. Evans blue detection appears as a dull green background when imaged with GFP. Because Evans blue has a spectral overlap range with both GFP and RFP, it is recommended to use Cy5 for Evans blue detection when FITC or TRITC probes are used to identify viral staining as shown above for CMV, HSV1, HSV2, and for VZV in Figure 4.

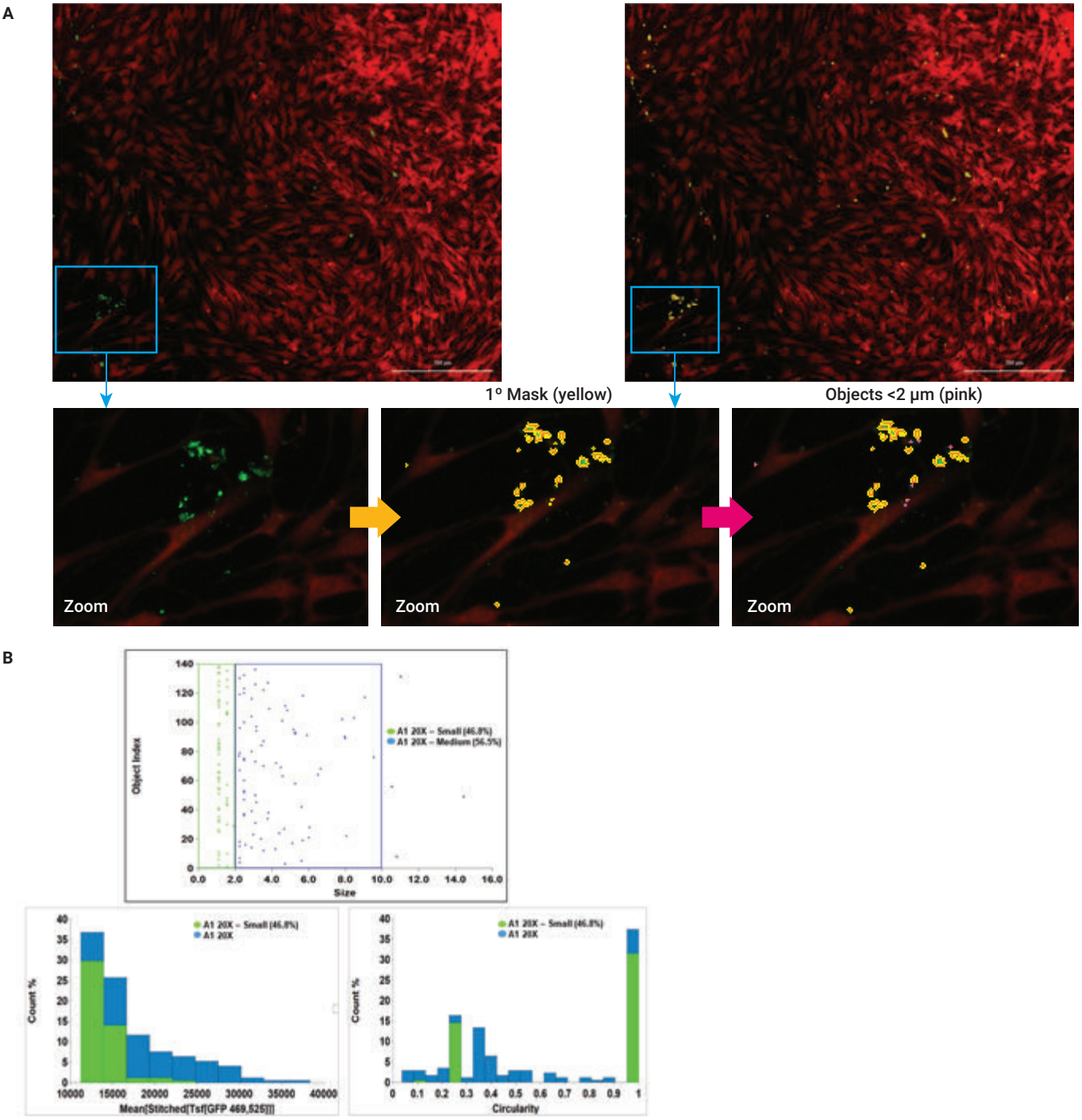
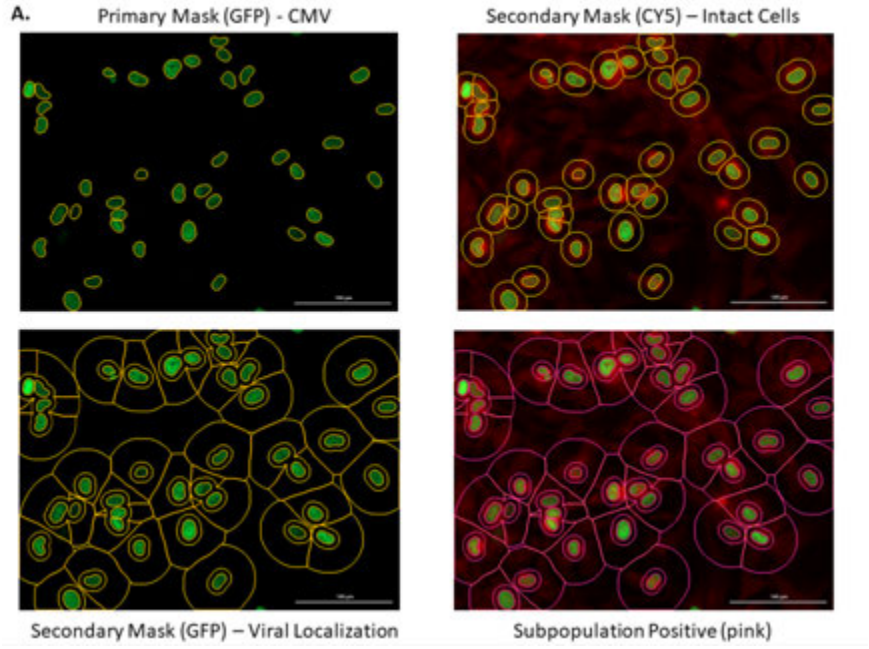
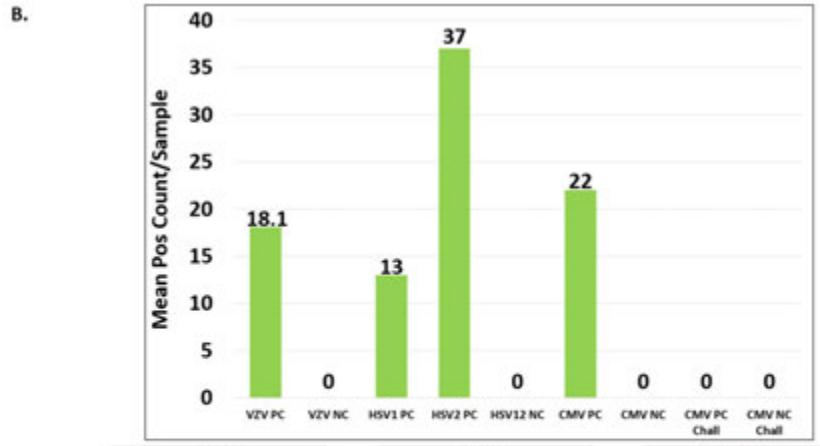


Figure 6. Characterizing nonspecific staining in negative controls (CMV shown). The user manual defines expected assay artifacts to include cell fragments due to trapping of the conjugate within the debris, Protein A produced by some bacteria that binds to the Fc portion of the mAb reagent, and potential dull staining of nonspecific viral isolates. (A) Size, morphology, and cellular localization of these artifacts can be defined using primary and secondary masking, and subpopulation analysis. (B) 46.8% of masked objects were identified as small (<2 µm) with uniform bright green staining and two distinct spherical patterns, one highly circular (≥0.9), and the other demonstrating uniform lower circularity (0.25). This profile would suggest bacterial contamination and/or nonspecific viral isolate staining respectively, with "Medium" (≥2 and ≤10 µm) and larger objects likely representing conjugate trapping. Histograms and scatterplots allow a means for defining threshold ranges that can be applied to differentiate or exclude these objects from positive viral staining. Only one object was within the size threshold for positive staining, and none of the objects met the subpopulation criteria of viral localization in intact cells (Table 2) and were therefore interpreted as negative (Figure 7).





		HSV1					HSV2					VZV			
		+	-	+|-	Total		+	-	+|-	Total		+	-	+|-	Total
CMV Chall	+	0	0	0	0	+	0	0	0	0	+	0	0	0	0
	-	20	20	0	40	-	20	20	0	40	-	20	20	0	40
	+|-	0	0	0	0	+|-	0	0	0	0	+|-	0	0	0	0
	Total	20	20	0	40	Total	20	20	0	40	Total	20	20	0	40
		HSV1 %Specificity Against CMV Antigen 40/40 * 100 = 100%					HSV2 %Specificity Against CMV Antigen 40/40 * 100 =100%					VZV %Specificity Against CMV Antigen 40/40 * 100 = 100%			

Figure 7. Cross reactivity and relative % specificity of HSV1/2 and VZV mAbs to CMV Antigen. (A) Positive cell counts are confirmed by the total count of the primary mask that are intact cell and viral localization positive as defined by two secondary masks and subpopulation criteria as shown for the PC (Table 2). A mean final count is shown for the total acquired image samples [n = 20]. CMV antigen-positive challenge monolayers were cross-reactive negative to HSV1/2_VZV mAbs as determined by the presence of <2 positive counts per image for 20 images. (B) Relative %specificity was determined by comparing CMV confirmed positive and negative controls treated with HVS1/2_VZV mAbs against HVS1/2 and VZV positive and negative controls treated with HSV1/2_VZV mAbs. For a sampling size of 20, both positive and negative CMV control cell monolayers were negative with 100% specificity when challenged with HSV1/2_VZV mAbs. Individual negative results were defined as <2 positive counts per image, and there were no positive counts for any image. Due to the modified nature of this challenge (performed on known control antigens rather than with known positive or negative clinical samples), %specificity is considered a relative measure of the ability of the imaging system to correctly exclude negative results.

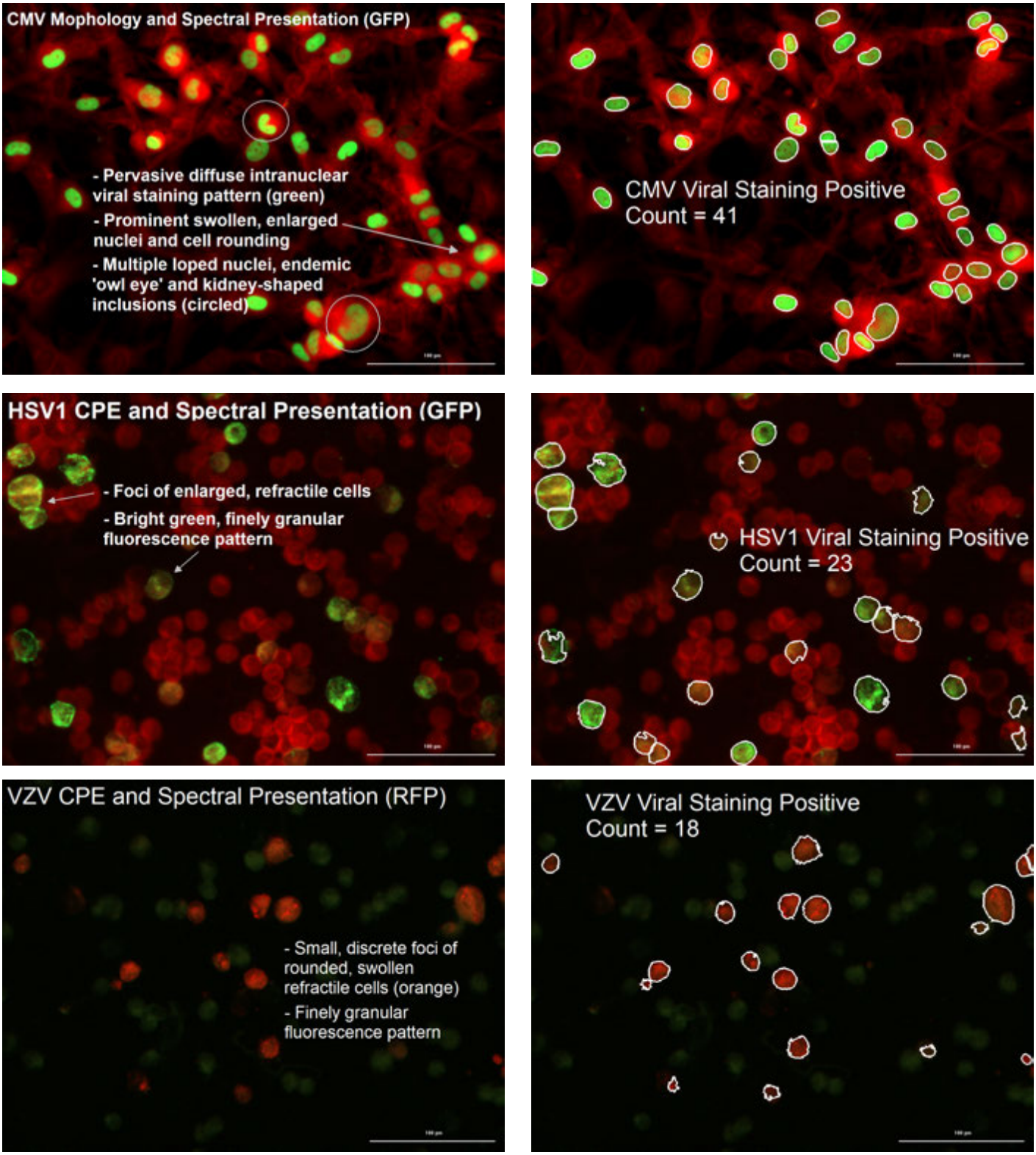


Figure 8. Microscopy allows visual confirmation of viral presentation to supplement quantitative results reporting.

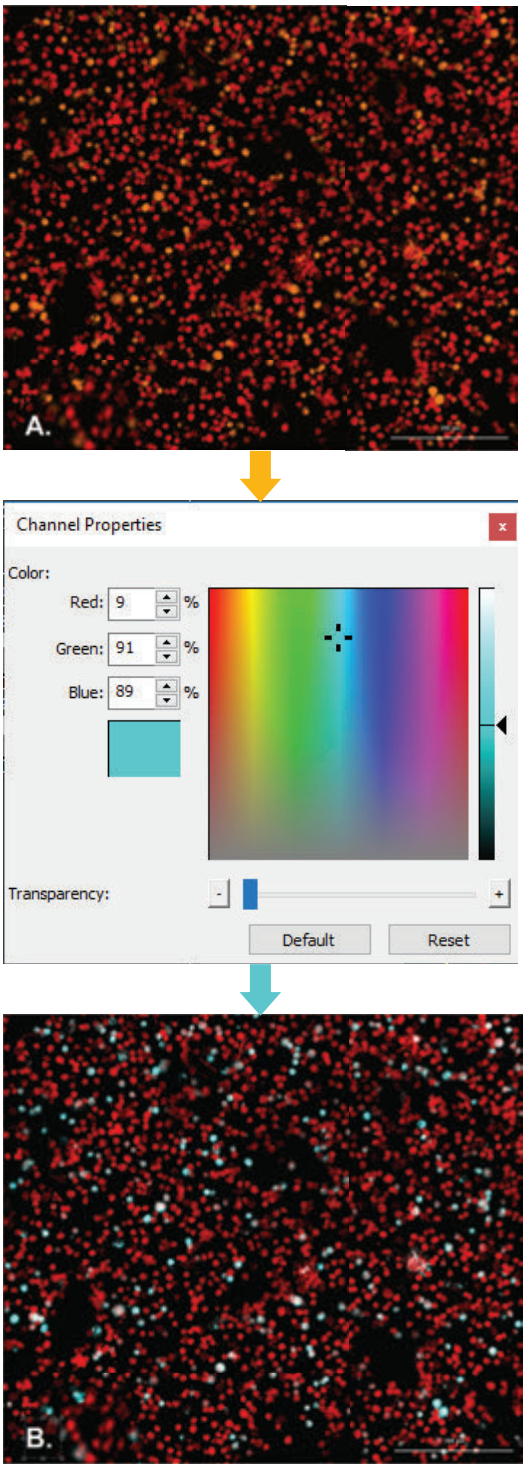


Figure 9. Recolorizing image channels can improve visual contrast. (A) Positive staining montage of VZV. Detecting VZV using the RFP filter set appears as orange against the brighter red Evans blue counterstain detected using a Cy5 filter set. (B) Editing the RFP channel color properties to turquoise increases visual contrast of positive staining. Colorization does not affect values used for image analysis. Other brightness and contrast settings can also be used to enhance image quality without effecting results.

Optimal image capture and background correction settings

As shown in Figure 4, correct focal height, exposure, and background correction proved essential for successful imaging of these assays. These settings should be optimized for each individual assay and microscopy system independently. To reduce or eliminate indeterminate findings between positive and negative staining the following best practice was used:

1. The optimal focal height and exposure for the PC (antigen-positive) cell monolayer was determined using Cy5 detection of Evans blue.
2. Using that focal height, optimal exposure settings were defined for viral staining (GFP or RFP).
3. Move to the NC (antigen-negative).
4. Using the same PC exposure settings for Evans blue, determine optimal focal height for the cell monolayer.
5. Using that focal height, capture GFP and/or RFP using the PC exposure settings.

This sequence of steps should provide a baseline of positive and negative results that can be applied to sample imaging. Optimization of these settings can be done in Manual Mode, and then transferred to an experiment file for repeated automated imaging. Tools available within the Gen5 software can assist with determining optimal exposure settings, such as a dynamic range graph, but the temptation to auto expose on each image independently can result in indeterminate results (Figure 4). Autoexposure can be useful, however, when determining initial optimal exposure settings. Likewise, the autofocus feature is not recommended within a Gen5 automated imaging step. Focal height may differ between sample preparations, or between multiple wells on the same slide. Instead, by training a laser autofocus accessory to the cell monolayer (Evans blue), the software will automatically search for the optimal focal height of the cell monolayer. Small offsets in viral staining focal heights may be seen when calibrating the laser autofocus for the PC, however these should not differ by more than a few µm from the monolayer to avoid a change in light level that can produce indeterminate results on negative wells from nonspecific Evans blue emission. A manual mode session can be used to acquire images or settings independent of, or in addition to, automated image capture. When in manual mode, the software will save the last focus and exposure settings between images within a session. Following image capture, background correction (preprocessing or rolling ball settings) can be defined for each detection channel. Analysis values are dependent on the result of these settings. Therefore,

as with image acquisition parameters, settings for each detection channel should be uniformly applied between images of a session. If auto-preprocessing does not eliminate enough signal interference, rolling ball diameter can be adjusted in 1 µm increments. A general rule for mammalian cell monolayers is that the rolling ball should be defined at about 3x the size of the object(s) of interest. Although viral inclusions can distort nucleus and cell size, a rolling ball of 68 to 70 µm was found sufficient for these assays. Due to the morphology of the viral staining patterns, a smoothing feature in concert with adjustments to the rolling ball diameter can be applied that result in distinct, concentrated viral staining for masking while also excluding signal interference from nonspecific staining. These parameters can be optimized using the assay PC. As augmentation to the work done here, it is highly recommended that inter- and/or intra-assay reproducibility testing on multiple assay lots by any intended user be undertaken for each instrument to build a machine learning library of baseline positive and negative data of optimal parameter settings and confirm repeatability when implementing experiment level automated image acquisition.

Identifying and differentiating nonspecific staining

The user manuals for the assays performed here recommend viewing NCs for the presence of assay artifacts. Nonspecific staining that is still detectable within the exposure and focal settings defined for positive viral staining can be characterized using image analysis tools. Results from this analysis can be used to inform on viral object size and signal cutoffs to define positive staining and differentiate it from assay artifacts. For example, size thresholds for CMV viral inclusions defined ≥10 µm will exclude 97% of nonspecific artifacts defined by the image analysis parameters shown in Figure 6. Additional analysis, such as shown in Figure 7, can be defined to increase exclusion of remaining artifacts based on signal localization within the cell for example.

Scanning for, counting, and characterizing positive staining

Finding regions of interest within the positive and negative control cell monolayers is made easier by having a defined geometry on the slide that contains the cell staining. In Gen5 this geometry can be programmed into a plate type definition so that the software always knows where to go to find the center of defined vessel areas. From that point, Cy5 exposure and focal height, and GFP or RFP exposure, can be optimized for the cell monolayer. Using a lower magnification objective (e.g. 4x) and brightfield imaging, edges of the image area can be detected. The slide can be scanned from within the image area using a joystick or mouse to move around the vessel. By switching to the filter set defined for detecting the cell monolayer, and using autoexposure and autofocus, regions of interest (ROI) can be precisely determined before moving to a higher magnification objective to define final focal and exposure settings. Applied montage settings from a starting ROI using the optimized capture parameters can then be defined to cover an area up to as large as the entire dimension of a standard coverslip. A 5 × 4 montage size is shown by Figure 3. The larger the montage, the larger the statistical pool for interpreting positive or negative results. Alternatively, a higher magnification (e.g. 40x) can be used for imaging. In that case, a larger montage would then be required to cover the same image area as that obtained for a 20x objective. To make automation easier, it is recommended that if samples are imaged using a slide and coverslip that they be mounted in the same region from sample to sample, either in the center of a microscopy slide, or offset to an area on the slide that match those of the assay control locations. This will allow a reference location that can be defined in a plate type definition so that subsequent assay ROI scanning begins at the same starting point for each new sample.

Final viral staining counts were used to determine cross-reactivity and specificity results for reactivity of HSV1/2 and VZV mAbs to CMV antigen-positive and -negative cells, as described in Figure 7. Definition of cell counting analysis parameters for identifying viral staining was influenced by: (1) granular staining patterns of viral inclusions causing variable threshold values across the entire infected region of the cell; (2) incongruent size and shape of viral inclusion within a cell; and (3) excluding nonspecific object staining. Viral objects were recognized using a primary mask favoring relatively larger objects with lower fluorescence signal thresholds instead of thresholds that masked multiple bright, discrete viral areas within a single cell. This resulted in both detecting more whole areas of viral inclusion, and in the exclusion of nonspecific staining objects. Higher signal thresholds may be required to eliminate background interference that could produce false positives. This may result in fewer, but still specific, true positive counts (Figure 4). Secondary masking and subpopulation analysis can also be employed to exclude nonspecific staining, or confirm viral localization, if desired (Figure 7A). Following characterization of positive viral staining using the PC, the same analysis settings should be applied to all sample images acquired for the same assay. A process of optimization and reverification of threshold and subpopulation values may be required to fine tune the masking, but the same values for the controls should be consistent, and reference ranges can ultimately be defined and used to validate functioning of reagents, culture methodology, microscope settings, and user proficiency in staining technique.

Reporting results

Gen5 automatically calculates a selected number of default image data metrics for each image channel. These metrics can be used to define additional parameters that can be reported, such as viral positive object counts shown in Figures 7B and 8. Tools in Gen5 are available that allow custom reporting of image data, such as the annotation feature shown in Figure 8. From within Gen5, images and image data can be saved and/or printed in .pdf or other formats, and easily exported to Excel, making results transportable between multiple platforms.

During the course of this evaluation benefits and limitations to DFA were encountered. Potential enhancements to DFA are topics for future discussion.

Conclusion

Optimization of parameters required for the Agilent BioTek Lionheart FX automated microscope and the Agilent BioTek Gen5 Image Prime software to interpret DFA for CMV, HSV1/2, and VZV was evaluated using a modified, abbreviated specificity challenge. The results indicate that using the optimized parameter settings shown in Table 2, a relative specificity of 100% for HSV1, HSV2 and VZV mAbs against CMV antigen was achieved. In addition to correct filter sets, exposure, and focal height settings, image analysis tools such as subpopulation analysis to identify and exclude non-specific staining, and isolate and count positive viral staining, further enabled this performance as described in the Discussion section. Ancillary features such as scanning for regions of interest, montage and stitching during image capture, and annotation and reporting of imaged results, were found useful to increase targets areas for analysis and annotate and report on viral morphology characteristics respectively.

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3. Millipore Light Diagnostics CMV DFA Kit User Manual, July **2012** Revision A; 3245MAN

Acknowledgements

Figure 1A is courtesy of Held, P., July 28, 2015, Sample Preparation for Fluorescence Microscopy: An Introduction – Concepts and Tips for Better Fixed Sample Imaging Results (Figure 4), BioTek Instruments, Inc.

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