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Automated high-throughput method for assessing pathogenic infectious dose (TCID$_{50}$) using Celigo imaging cytometer

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1. ABSTRACT

Qualitative assessment of pathogenic infections, as well as the protective effects of candidate therapeutics, often relies on the observation of specific pathological changes within the host cell. These phenotypic changes, such as cell rounding, swelling/shrinking, granularity, etc., are known as a Cytopathic Effect (CPE) and can be visualized via light microscopy. As the magnitude and localization of the CPE may vary considerably, careful examination of replicate samples at various titers is required for reliable, qualitative results. This subjective approach which is specific to the infectious agent as well as the host cell is tedious, time consuming and low throughput, requiring manual well-by-well examination by highly skilled personnel. In this study, the Celigo imaging cytometer has been used to rapidly assessment of viral infectivity in a range of plate formats. The infectivity level was assessed by characterizing the cell count and cell morphology when induced with different MOI levels. In addition, the viral infectivity at 0, 6, 12.5, 25, and 50 ng of Lentivirus concentration was assessed by using a GFP fluorescence reporter to measure % GFP expression in the cell population. The total number of cells were counted using the Hoechst stain, and GFP of each was assessed to determine the % GFP in the population. The Celigo imaging cytometer uses the f-theta optics, which can generate uniform whole well bright-field and fluorescent images. Image-based analysis can rapidly and objectively quantify output of CPE based on characteristic changes to the host cell monolayer. By directly counting cells in whole well on microplates, the viral infectivity can be quickly quantified for both suspension and adherent cells without trypsinization, which can reduce assay time and improve research efficiency.

2. CELIGO IMAGING CYTOMETRY FOR ASSESSING PATHOGENIC INFECTIOUS DOSAGE
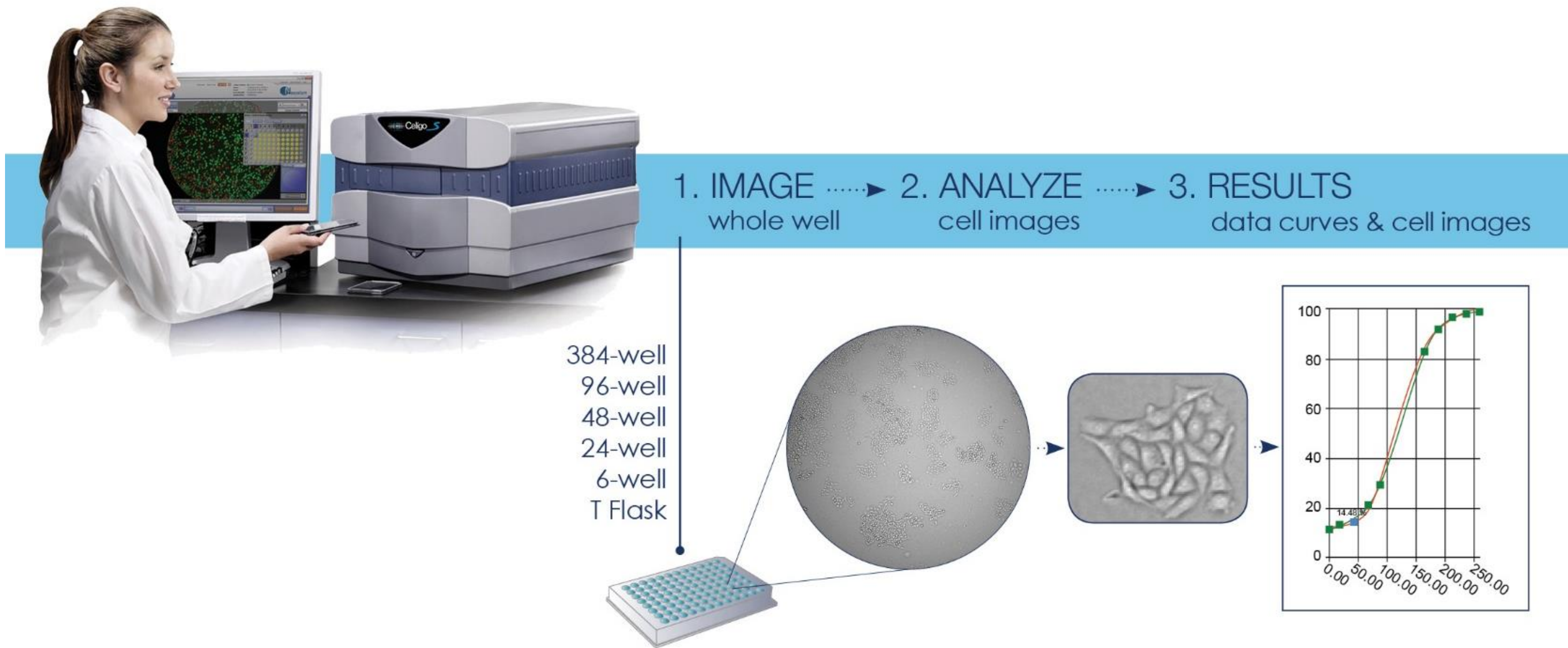
1. Celigo Imaging Cytometer is a plate-based cytometer that can scan the entire well of standard microplates and captures bright-field and fluorescent images

2. The captured images are analyzed with the Celigo software to measure size, morphology, cell count, confluence, and fluorescent intensity

3. The measured parameters are used to generate cell proliferation kinetic data, GFP/RFP expression, tumor spheroid size change, DNA cell cycle analysis, apoptosis, and ADCC cytotoxicity results

## 3. TCID$_{50}$ ASSAY PROTOCOL
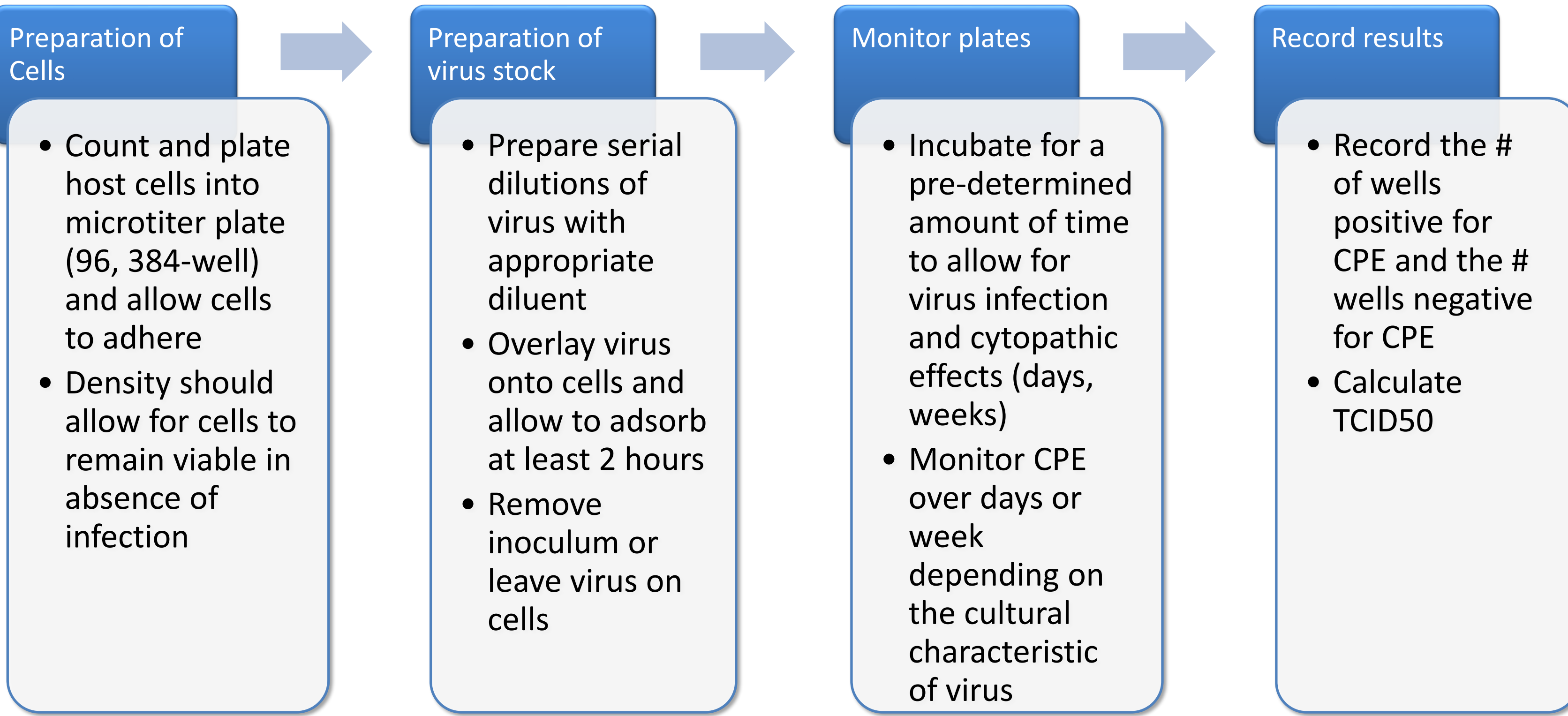
Preparation of Cells
- Count and plate host cells into microtiter plate (96, 384-well) and allow cells to adhere
- Density should allow for cells to remain viable in absence of infection

Preparation of virus stock
- Prepare serial dilutions of virus with appropriate diluent
- Overlay virus onto cells and allow to adsorb at least 2 hours
- Remove inoculum or leave virus on cells

Monitor plates
- Incubate for a pre-determined amount of time to allow for virus infection and cytopathic effects (days, weeks)
- Monitor CPE over days or week depending on the cultural characteristic of virus

Record results
- Record the # of wells positive for CPE and the # wells negative for CPE
- Calculate TCID50

4. TRADITIONAL VS CELIGO ASSAY PROTOCOL

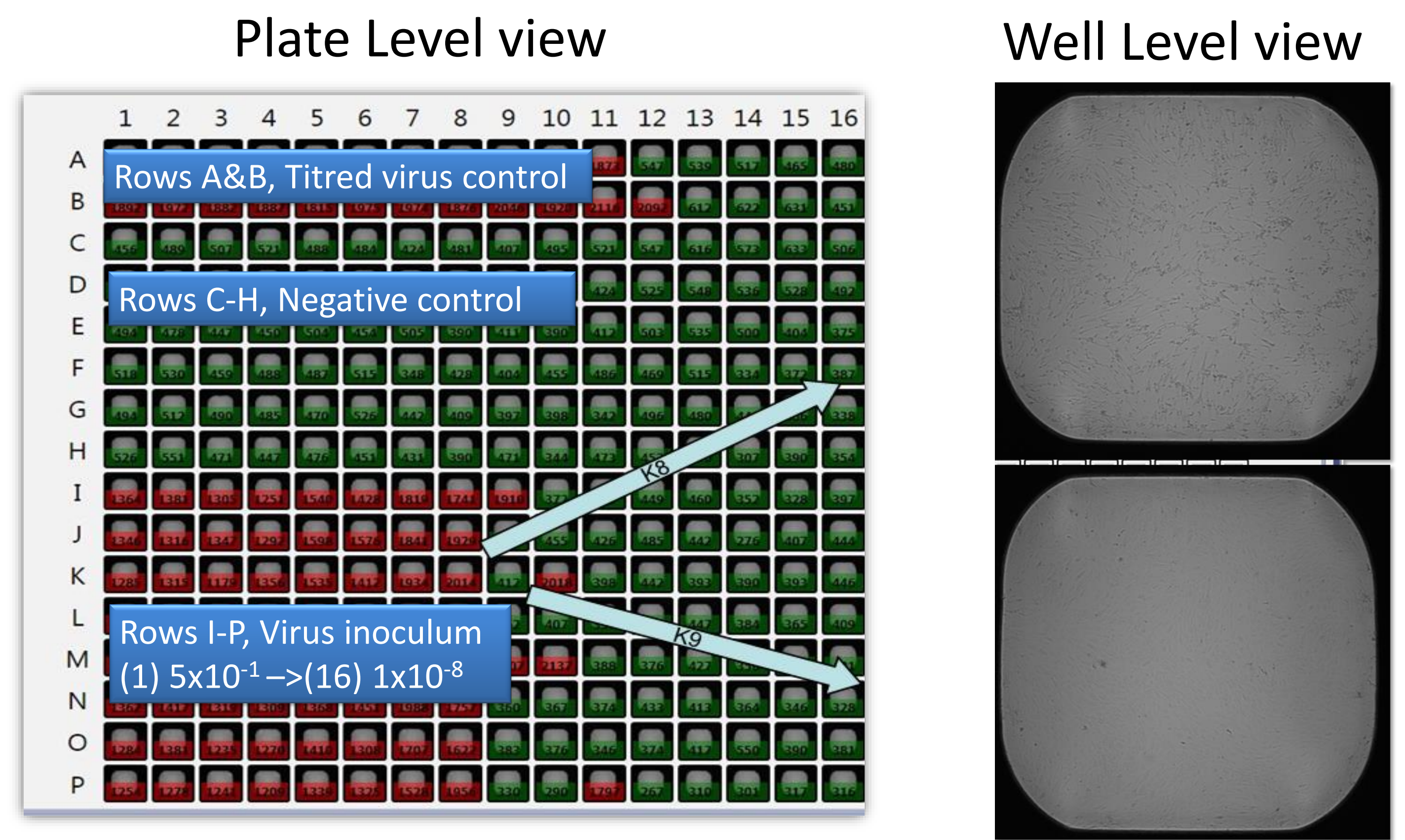Uninfected Control — Moderate MOI — High MOI
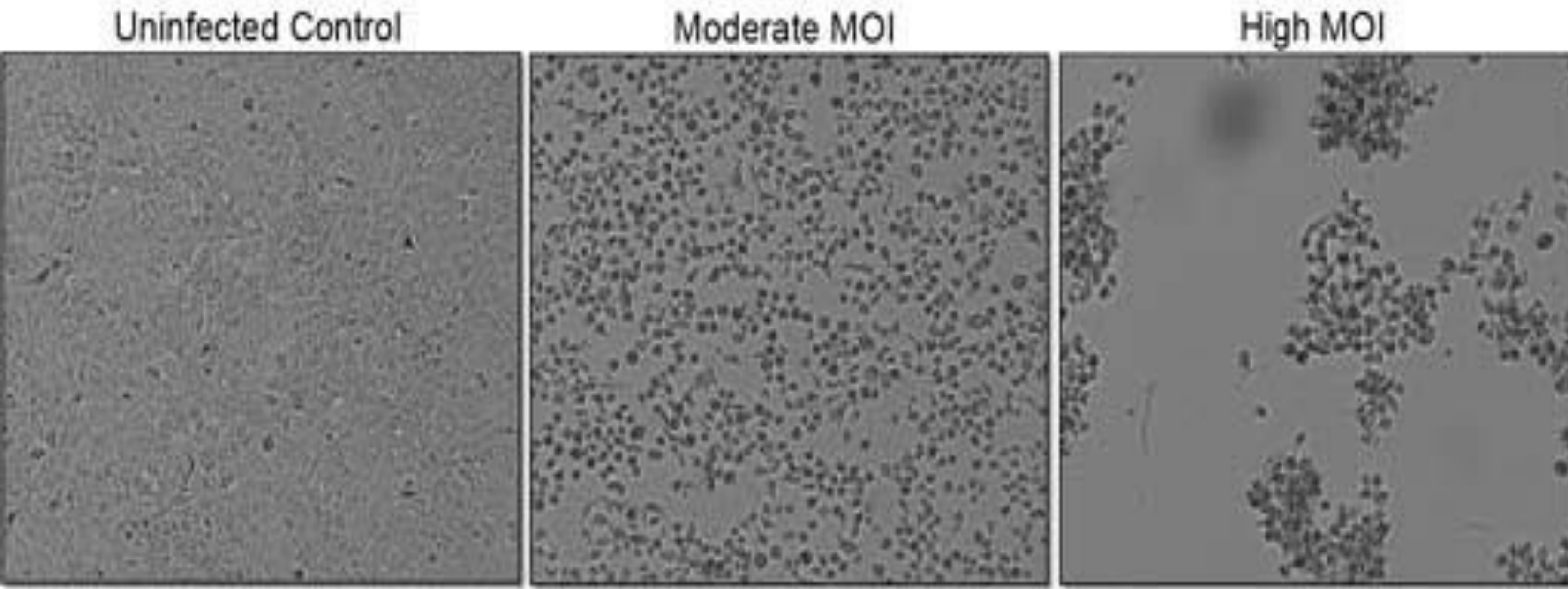
- BVDV (Bovine Viral Diarrhea Virus) Infection of BK6 (Bovine kidney) cells
- 96-well plates were scanned on the Celigo using the Cell Counting Application
- A dark focus was selected to better separate the healthy monolayer from the infected cells

5. BRIGHT FIELD IMAGING


(a) Direct Cell Counting   (b) Confluence
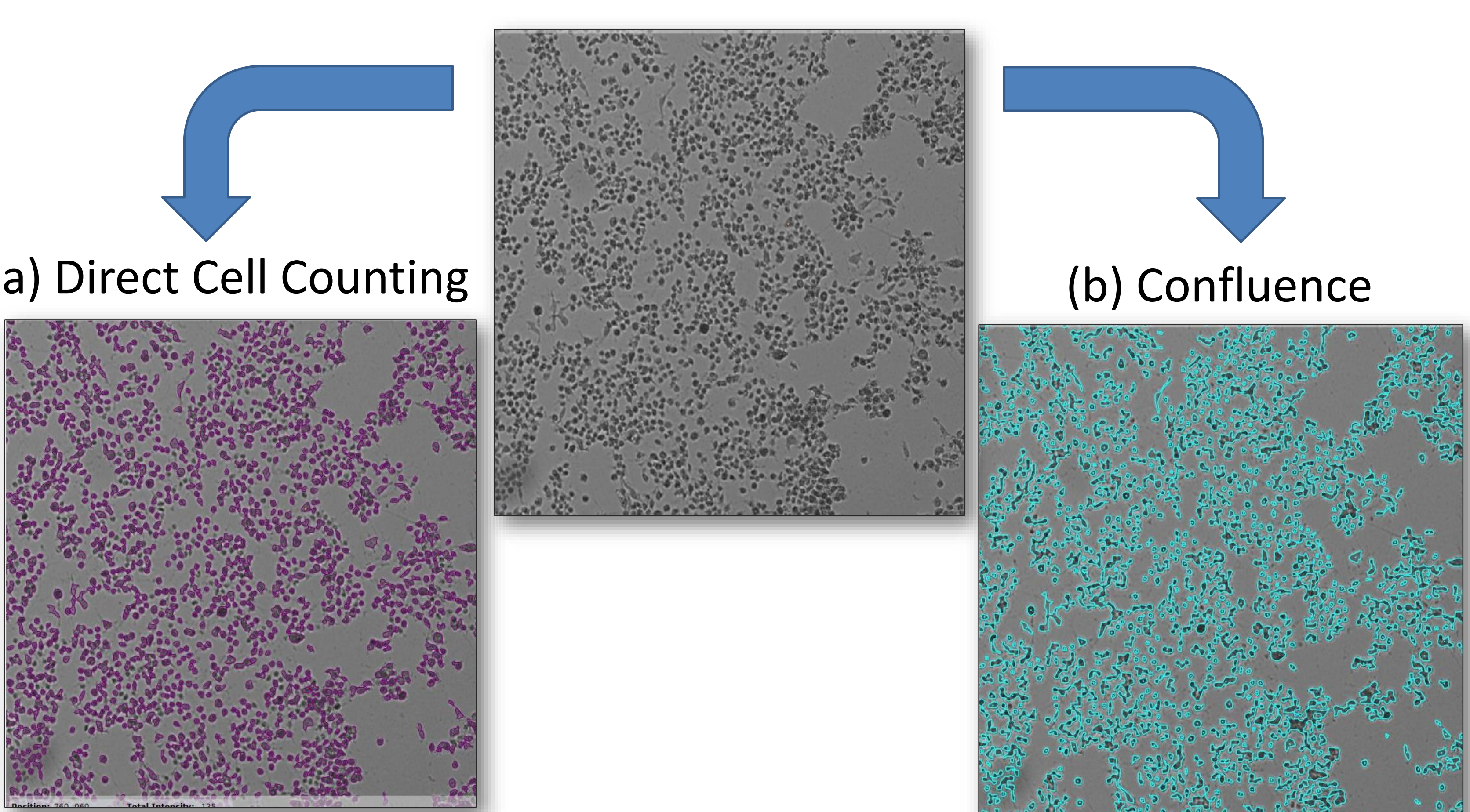
- Each cell/virus pair will have distinct morphological changes
- Analysis setting can be created and saved for each pair
- CPE was scored + based on the appearance of dark, rounded cells
- In this example, the reduction in confluence (a) and the increase in sick/dying cells (b) scored equally well

6. DETERMINING BEST ANALYSIS METHOD TO SCORE CPE

Plate Level view   Well Level view

Rows A&B, Titred virus control
Rows C-H, Negative control
Rows I-P, Virus inoculum (1) 5x10^{-1} —>(16) 1x10^{-8}

- Bovine Viral Diarrhea (BVD) infection of Bovine Turbinate cells (BT2)
- Threshold function indicates red wells (infected) vs. green wells (uninfected)
- Breakpoint of infection is clearly visible at plate level
- Well level data is exported as CSV
- Celigo results correlated 100% with assessment via light microscope

7. MOI BY GFP-INDICATOR VIRUS
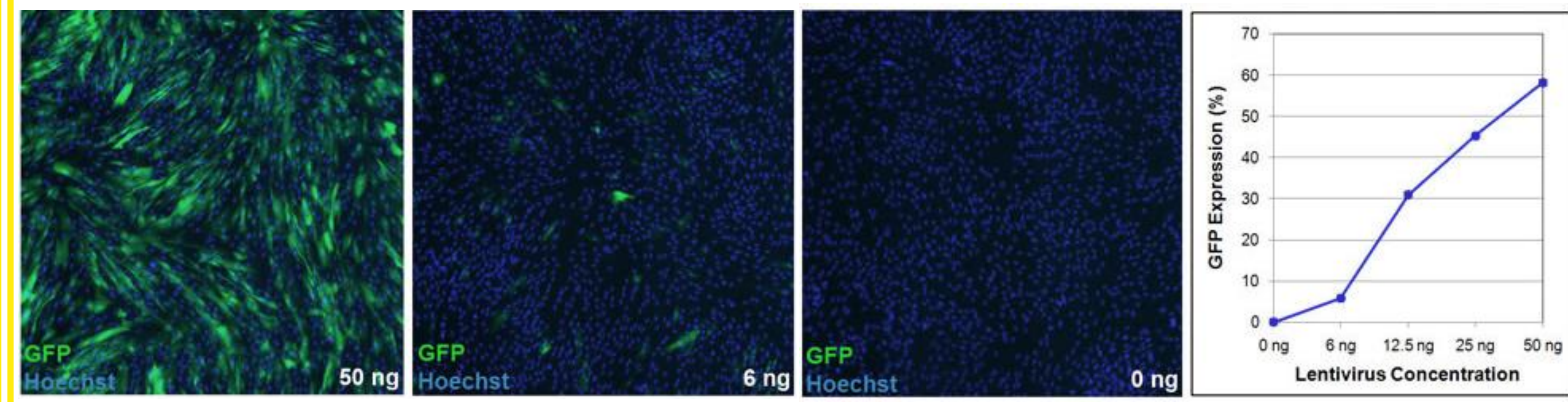
- Known concentrations of viral stock (ng/ml) are used to infect primary fibroblasts
- Infectious titre is calculated by measuring the % of infected cells/dose
- Viability and overall cell health can also be determined by staining with propidium iodide for sick/dying cells (not shown)
- The Celigo can scan and report up to 4 separate channels at one time (Brightfield, Red, Green, Blue)

8. FLUORESCENT TITERS FROM IFA

$$X = \frac{AVG\ of\ Virus\ Control - AVG\ Cell\ Control}{2} + AVG\ Cell\ Control$$
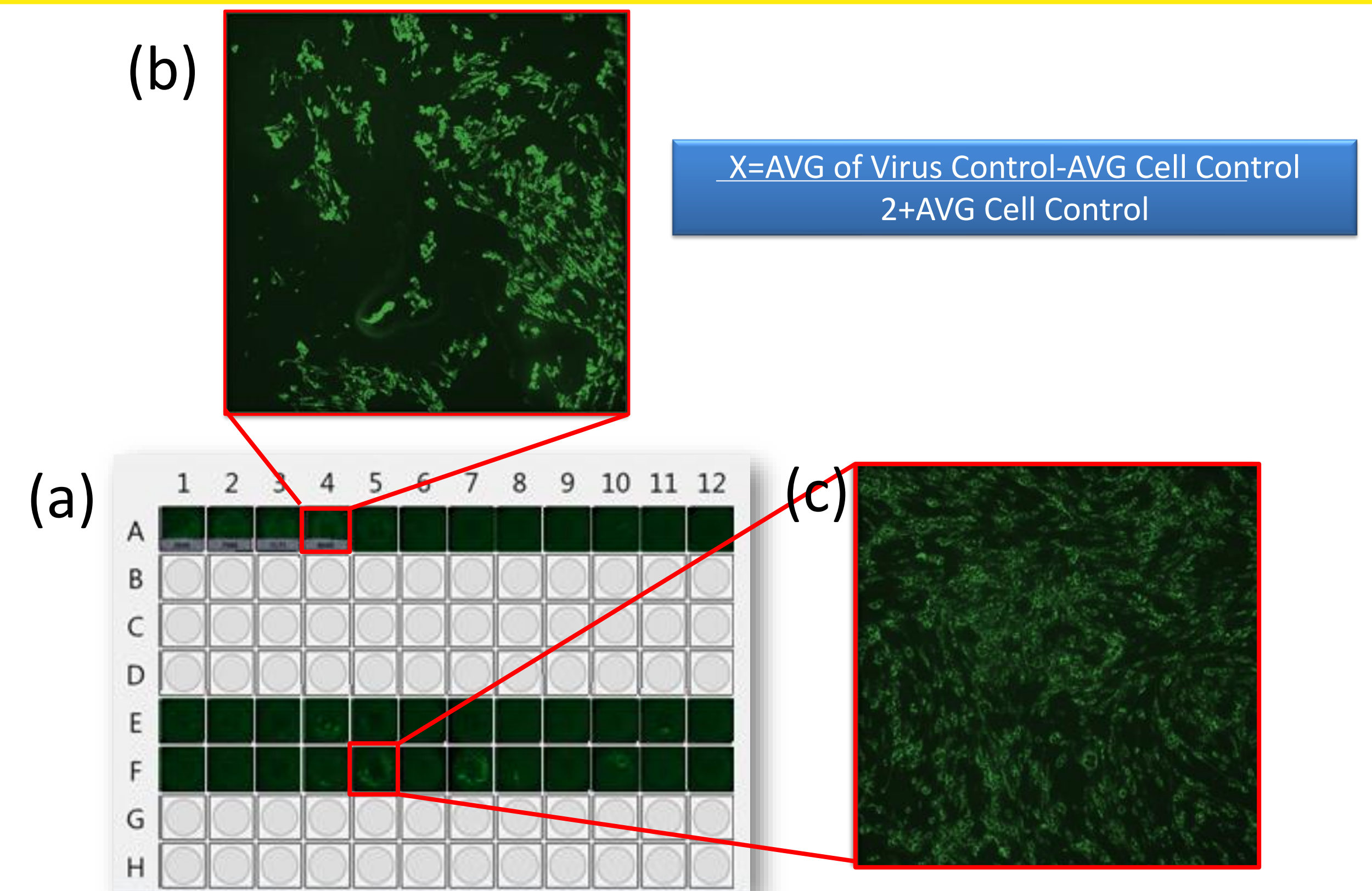
- Celigo allows for multiple analyses/plate to be carried out in one experiment. (a) Result thumbnails following scan are displayed. Zoomed images of infection by BVD1 (b) and BVD2 (c) demonstrate the varied morphologies of infected cells as well as the localization of the viral antigen
- Antibodies directed against viral antigen are often used as indicators of infection. Unlike TCID50 where a single event of infection is scored as a positive, these assays measure % infection. Intensity of FL signal may also be used to indicate strength of infection.

9. CALCULATION OF TCID$_{50}$

Definition: 50% tissue culture infectious dose of a virus

Spearman-Karber formula:
$$M = xk + d\ [0.5 - (1/n)\ (r)]$$

xk = dose of the highest dilution.
r = sum of the number of "-" responses.
d = spacing between dilutions.
n = wells per dilution.

Reed and Muench formula:
% infection immediately above 50% - 50%
% infected immediately above 50% - %infected immediately below 50%

- Determination of
  - TCID$_{50}$
  - MOI
  - neutralization assays
- They are easily calculated by exporting the well-level data as CSV into a number of online calculators

10. SUMMARY AND CONCLUSION

- Settings for each cell type and virus can be saved and re-used to assure objective results
- Additional information such as cell health, cell cycle status can be determined from the same plates using Nexcelom designed applications.
- Time course studies can be carried out by scanning plates over several days/weeks allowing for additional information than may be generated by carrying out end-point assays alone.
- User-friendly and intuitive software allows even those with little imaging experience to generate valuable data.