Stain-free, rapid, and quantitative viral plaque assay using deep learning and holography

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Abstract

Plaque assay is the gold standard method for quantifying the concentration of replication-competent lytic virions. Expediting and automating viral plaque assays will significantly benefit clinical diagnosis, vaccine development, and the production of recombinant proteins or antiviral agents. Here, we present a rapid and stain-free quantitative viral plaque assay using lensfree holographic imaging and deep learning. This cost-effective, compact, and automated device significantly reduces the incubation time needed for traditional plaque assays while preserving their advantages over other virus quantification methods. This device captures ~0.32 Giga-pixel/hour phase information of the objects per test well, covering an area of ~30 × 30 mm², in a label-free manner, eliminating staining entirely. We demonstrated the success of this computational method using Vero E6 cells and vesicular stomatitis virus. Using a neural network, this stain-free device automatically detected the first cell lysing events due to the viral replication as early as 5 hours after the incubation, and achieved >90% detection rate for the plaque-forming units (PFUs) with 100% specificity in <20 hours, providing major time savings compared to the traditional plaque assays that take ≥48 hours. This data-driven plaque assay also offers the capability of quantifying the infected area of the cell monolayer, performing automated counting and quantification of PFUs and virus-infected areas over a 10-fold larger dynamic range of virus concentration than standard viral plaque assays. This compact, low-cost, automated PFU quantification device can be broadly used in virology research, vaccine development, and clinical applications.
Introduction

Viral infections pose significant global health challenges by affecting millions of people worldwide through infectious diseases, such as influenza, human immunodeficiency virus (HIV), human papillomavirus (HPV), and others\(^1\). The US Centers for Disease Control and Prevention (CDC) estimates that, since 2010, the influenza virus has resulted in 16-53 million illnesses, 0.2-1 million hospitalizations, and 16,700-66,000 deaths in the United States alone\(^2,3\). Furthermore, the ongoing COVID-19 pandemic has already caused >500 million infections and >6 million deaths worldwide, bringing a huge burden on public health and socioeconomic development\(^4\). To cope with these global health challenges, developing an accurate and low-cost virus quantification technique is crucial to clinical diagnosis\(^5\), vaccine development\(^6\), and the production of recombinant proteins\(^7\) or antiviral agents\(^8,9\).

Plaque assay was developed as the first method for quantifying virus concentrations in 1952 and was advanced by Renato Dulbecco, where the number of plaque-forming units (PFUs) was manually determined in a given sample containing replication-competent lytic virions\(^10,11\). These samples are serially diluted, and aliquots of each dilution are added to a dish of cultured cells\(^10\). As the virus infects adjacent cells and spreads, a plaque will gradually form, which can be visually inspected by an expert. Due to its unique capability of providing the infectivity of the viral samples in a cost-effective way, the plaque assay remains to be the gold standard method for quantifying virus concentrations despite the presence of other methods\(^12–19\) such as the immunofluorescence focal forming assays (FFA)\(^14\), polymerase chain reaction (PCR)\(^16\), and enzyme-linked immunoassay (ELISA) based assays\(^19,20\). However, plaque assays usually need an incubation period of 2-14 days (depending on the type of virus and culture conditions)\(^21\) to let the plaques expand to visible sizes, and are subject to human errors during the manual plaque counting process\(^22\). To improve the traditional plaque assays, numerous methods have been developed\(^23\). While these earlier systems have unique capabilities to image cell cultures in well plates, they require either fluorescence markers\(^22\) or special culture plates with gold microelectrodes\(^25\). In addition, human counting errors still remain to be a problem for these methods. Hence, an accurate, quantitative, automated, rapid, and cost-effective plaque assay is urgently needed in virology research and related clinical applications.

Some of the recent developments in quantitative phase imaging (QPI), holography, and deep learning provide an opportunity to address this need. QPI is a preeminent imaging technique that enables the visualization and quantification of transparent biological specimens in a non-invasive and label-free manner\(^25,26\). Furthermore, the image quality of QPI systems can be enhanced using neural networks by improving e.g., phase retrieval\(^27\), noise reduction\(^28\), auto-focusing\(^29,30\), and spatial resolution\(^31\). In addition, numerous deep learning-based microorganism detection and identification methods have been successfully demonstrated using QPI\(^32–41\).

Here, we report a cost-effective and compact label-free live plaque assay that can automatically provide significantly faster quantitative PFU readout than traditional viral plaque assays without the need for staining. A compact lensfree holographic imaging prototype was built (Fig. 1 and Supplementary Video 1) to image the spatio-temporal features of the target PFUs during their incubation; the total cost of the parts of this entire imaging system is < $880, excluding a standard laptop computer. This lensfree holographic imaging system rapidly scans the entire area of a 6-well plate every hour (at a throughput of ~0.32 Giga-pixels per scan of a test well), and the reconstructed phase images of the sample are used for PFU detection based on the spatio-temporal changes observed within the wells. A neural network-based classifier was trained and used to convert the reconstructed phase images to PFU probability maps, which were then used to reveal the locations and sizes of the PFUs within the well plate. To prove the efficacy of our system, early detection of vesicular stomatitis virus (VSV) was performed on Vero E6 cell plates. Our stain-free device could automatically detect the first cell-lysis event due to the virus replication as early as 5 hours after the incubation and achieve >90% PFU detection rate in <20 hours, providing major time savings compared to the traditional plaque assays that take ≥48 hours. A quantitative relationship was also developed between the incubated virus concentration and the virus-infected area on the cell monolayer. Without any extra sample preparation steps, this deep learning-
enabled label-free PFU imaging and quantification device can be used with various plaque assays in virology and might help to expedite vaccine and drug development research.

Figure 1: Stain-free, rapid and quantitative viral plaque assay using deep learning and lensless holography. (a) Photograph of the stain-free PFU imaging system that captures the phase images of the plaque assay at a throughput of ~0.32 Giga-pixels per scan of each test well. The processing of each test well using the PFU classifier network takes ~7.5 min/well, automatically converting the holographic phase images of the well into a PFU probability map (see Fig. 2). (b) Detailed illustration of the system components. (c) A 6-well plate sample with ventilation holes on the cover and parafilm sealed from the side. Also see Supplementary Video 1.

Results

To demonstrate the efficacy of the presented device, we prepared 14 plaque assays using the Vero E6 cells and VSV. The sample preparation steps are summarized in Fig. 2a (also see the Methods section); for each well-plate, 5 wells were infected by the VSV and 1 well was left for negative control. After each sample was prepared, it was first placed into our imaging set-up for 20 hours of incubation, performing time-lapse imaging to capture the spatio-temporal information of the sample. Then, the same sample was left in the incubator for an additional 28 hours to let the PFUs grow to their optimal size for the traditional plaque assay (this is only used for comparison purposes). Finally, each sample was stained using crystal violet solution to serve as the ground truth to compare against our label-free method.

To train and test our network-based PFU classifier, 54 wells were used for training and 30 wells were used for testing. During the training phase, a machine learning-based coarse PFU localization algorithm was developed to both accelerate the training dataset generation and delineate the potential false positives (see the Method sections for details). After this PFU localization algorithm screened each sample, the resulting PFU candidates were further examined manually for confirmation using a custom-
developed Graphical User Interface (Supplementary Figure 1); this manual examination was only used during the training phase to correctly and efficiently prepare the training data. The negative training dataset was populated purely from the negative control well of each well plate. In total, 357 true positive PFU holographic videos and 1169 negative holographic videos were collected for training the PFU decision neural network. This dataset was further augmented to create a total of 2594 positive and 3028 negative holographic videos (see the Method sections), where each frame had 480×480 pixels, and the time interval between two consecutive holographic frames was 1 hour.

Figure 2: Schematics of the workflow of the label-free viral plaque assay and its comparison to the standard PFU assay. (a) Plaque assay sample preparation workflow. The traditional plaque assay at the last step in (a) is only performed for comparison purposes and is not needed for the operation of the presented PFU detection device. (b-f) Detailed image and data processing steps for the live viral plaque assay.

After the neural network-based PFU classifier was trained, it was blindly tested on all the 30 test wells in a scanning manner (shown in Fig. 2b) without the need for the PFU localization algorithm, which was only used for the training data generation. For each test well, we have ~18000×18000 effective pixels (representing a 30x30 mm² active area after discarding the edges); the digital processing of each test well using the PFU classifier network takes ~7.5 min, which automatically converts the holographic phase images of the well into a PFU probability map (Fig. 2d). Each pixel of the well on this map indicates the statistical probability of the local area (0.8×0.8 mm²) centered at this pixel having a PFU. Using a probability threshold of 0.5 (50%), the final PFU detection and quantification result was obtained across the entire test well area (see e.g., Fig. 2 e-f).

Figure 3a shows examples of our device's output after 15 hours of incubation. Three representative PFUs are also selected and shown in Fig. 3b. When a PFU is in its early stage of growth, with its size much smaller than our 0.8×0.8 mm² virtual scanning window, it appears as a square (shown by the PFU① in Fig. 3b) in the final detection result, which effectively is the 2D spatial convolution of the small scale PFU with our scanning window. As another example, PFU③ shows a cluster forming event where the two neighboring PFUs can be easily differentiated using our method as opposed to the traditional plaque assay where they physically merged into one. Fig. 3c further shows the PFU quantification achieved by our device compared to the 48-hour traditional plaque assay results. We achieved a detection rate of >90% at 20 hours of incubation without having any false positives at any time point despite using no staining.

The presented device is cost-effective, compact, and automated, and can also handle a larger virus concentration range with a more reliable PFU readout. To demonstrate this, we prepared another 5 titer test plates, where for each plate, all the 6 wells were infected by VSV, but with a 2 times dilution
difference between each well, covering a large dynamic range in virus concentration from one test well to another. As shown in Fig. 4, our method is effective even for the higher virus concentration cases; see, for example, the dilution cases of \(2^{-2}\times10^4\) and \(2^{-3}\times10^4\). In the traditional 48-hour plaque assay, only the lowest virus concentration is suitable for the PFU quantification due to significant spatial overlapping, whereas for our label-free device, we can automatically and accurately count the individual PFUs at an early stage, even for the highest virus concentration (see Fig. 4c).

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**Figure 3: Performance of the stain-free plaque assay for samples with low virus concentration.**

(a) Whole well comparison of the stain-free viral plaque assay after 15-h incubation against the traditional plaque assay after 48-h incubation and staining. (b) The growth of three featured PFUs in the positive well from (a). The reconstructed phase channel is overlaid with the mask generated using the PFU localization algorithm to reveal their locations better. (c) Average PFU detection rate using the label-free viral plaque assay. The error bars show the standard deviation across the 5 testing plates.
Furthermore, our method provides a more reliable readout; for example, in the circled region in Fig. 4 a-b, the absence of the cells was caused by some random cell viability problems that occurred during the plaque assay. In our device, these artifacts can be easily differentiated from the cell lysing events caused by the viral replication, since the spatio-temporal patterns for these two events are vastly different (assessed by the trained PFU probability network). This makes our deep learning-enabled device resilient to potential artifacts or cell viability issues randomly introduced during the sample preparation steps.

Figure 4: Performance of the stain-free viral plaque assay as a function of the virus concentration. (a-b) Whole plate comparison of the stain-free viral plaque assay after 15-h incubation against the traditional plaque assay after 48-h incubation and staining. (c) The growth of PFUs in their early stage
Due to the high virus concentration used in these 5 titer test samples, PFUs quickly clustered and were no longer suitable for manual counting, as shown in Fig. 5a. However, the quantitative readout and the PFU probability map of our presented device allowed us to obtain the area of the virus-infected regions across all the time points during the incubation period, as shown in Fig. 5b. To better illustrate this, we plot in Fig. 5c the virus dilution factor vs. the ratio of the infected cell area per test well (in %) for all the samples at 6, 8, and 10 h of incubation time. Despite the existence of some serial dilution errors, late virus wakeups, and PFU clustering events, the infected area percentage that our device measured is monotonically decreasing with the increasing dilution factor for all the incubation times. This suggests that, by calibrating the system, the virus concentration (PFU/mL) can also be estimated from the percentage of the infected cell area per well.

Furthermore, using the area percentage of the virus-infected region as a label-free quantification metric, the presented framework can provide earlier PFU readouts. To show this, we computed the infected area percentage for all the 25 positive/infected wells of the blind testing plates used to generate Fig. 3c. As shown in Fig. 6, when the infected area percentage is sufficiently large (>1%), a faster PFU
concentration readout can be provided at 12-h or 15-h. Since the size of an average PFU on the well is physically larger at 15 hours of incubation compared to 12 hours, the slope of the red calibration curve in Fig. 6b is smaller than Fig. 6a, as expected. For samples with even higher virus concentrations, the infected cell area percentage could reach >1% in ≤10 hours of incubation (shown in Fig. 5c), providing the PFU concentration readout even earlier.

Figure 6: Infected area percentage (%) measured by our stain-free device at different time points vs. the virus concentration per well (PFU/mL). The virus concentrations in y-axis were obtained from the 48-h traditional plaque assay for each test well. Different test wells of the same plate were marked with the same color/symbols. There are 25 infected test wells in each plot. The red calibration curves were obtained by quadratic polynomial fitting.

Discussion

We demonstrated a cost-effective and automated early PFU detection system using a lensfree holographic imaging system and deep learning. This deep learning-based stain-free device captures time-lapse phase images of a test well at a throughput of ~0.32 Giga-pixels per scan, which is then processed by a PFU quantification neural network in ~7.5 min to yield the PFU distribution of each test well. The high detection rate of this label-free device with 100% specificity shown in Fig. 3c is a conservative estimate since the ground truth data were obtained after 48-h of incubation. In the early stages of the incubation period, many PFUs did not even exist physically, which means that if we were to use the existing PFUs as the ground truth for our quantification at each time point, our detection rate would be even higher.

The modular design employed by the presented PFU detection platform brings the potential for further system improvements. For example, parallel imaging can be achieved by installing several image sensors on the same system without significantly increasing the cost of the device, which will further improve the 30 cm²/min effective imaging throughput of the device⁴⁵. More accurate scanning stages can also help reduce the image registration steps needed during image pre-processing. Multi-wavelength phase recovery⁴⁴ can also be implemented to improve the overall image quality of the label-free plaques. The presented deep learning-enabled PFU detection framework can be potentially adapted to other imaging modalities that can provide the spatio-temporal differences in the PFU regions for various types of viruses; similarly, the trained PFU classifier network also has the adaptability to these system changes (see the Supplementary Note 1).

All in all, we presented a stain-free, rapid, and quantitative viral plaque assay using deep learning and holography. The presented compact and cost-effective device preserves all the advantages of the traditional plaque assays while significantly reducing the required sample incubation time in a label-free manner, saving time and eliminating staining. It is also resilient to potential artifacts during the sample preparation, and can automatically quantify a larger dynamic range of virus concentrations per well. We expect this technique to be widely used in virology research, vaccine development, and related
clinical applications.

**Material and Methods**

**Safety practices:** We handled all the cell cultures and viruses during our experiments at our biosafety level 2 (BSL2) laboratory according to the environmental, health, and safety rules and regulations of the University of California, Los Angeles. All operations were carried out under strict aseptic conditions.

**Studied organisms:** We used Vero C1008 [Vero 76, clone E6, Vero E6] (ATCC® CRL-1586™) (ATCC, USA) and vesicular stomatitis virus (ATCC® VR-1238™). Vero E6 cells are African green monkey kidney cells and are epithelial cells.

**Cell propagation:** We placed the frozen stock culture immediately in the liquid nitrogen vapor, until ready for use, just after the delivery of the frozen stock culture from ATCC. ATCC formulated Eagle's Minimum Essential Medium (EMEM) (product no. 30-2003, ATCC, USA) was used as a base medium for the cell line. For the complete growth medium, the base medium was mixed with fetal bovine serum (FBS) (product no. 30-2021, ATCC, USA) with a final concentration of 10%. The FBS stock was aliquoted into 4 mL microcentrifuge tubes and stored at -20°C until use.

We used tissue culture flasks (75 cm² area, vented cap, TC treated, T-75) (product no. FB012937, Fisher Scientific, USA) for cell culturing. The base medium in a T-75 flask and FBS were brought to 37°C in the incubator (product no. 51030400, ThermoFisher Scientific, Waltham, MA, USA) and fed with 5% CO₂ before handling it for cell culturing steps. The complete growth medium was prepared. The frozen cell culture was removed from liquid nitrogen and thawed under running water. After thawing the cells, the cell suspension was added to a T-75 flask containing 8 mL of complete growth medium (i.e., EMEM + 10% FBS). The flask was incubated at 37°C and 5% CO₂ in the incubator. The adherence of the cells to the flask surface was analyzed daily under a phase-contrast microscope. The medium in the flask was renewed 2-3 times a week. The cells were sub-cultivated in a ratio of 1:4 when 95% confluency of the cells as a monolayer was reached.

**Subculturing of cells:** After the removal of the medium from the cell culture flask, the cells were exposed to 2-3 mL of 0.25% Trypsin/0.53 mM EDTA (ATCC® 30-2101™, ATCC, USA) per flask for dissociation of cell monolayers. The flasks were kept in the incubator for 5-6 minutes for rapid dissociation of cells. 8 mL of complete medium per flask was added to each of them and 2-3 mL of the mixture containing suspended cells was transferred into a new T-75 flask. 8 mL of complete medium was added to the new flask and after gentle mixing, it was incubated at 37°C and 5% CO₂ for the growth of new cells.

**Virus propagation:** After the delivery of the virus stock sample, it was stored at 4°C until use. Virus propagation requires to have Vero cells to be cultured and reach 90-95% confluency on the day of infection. Therefore, Vero cells were cultured 1-2 days before the virus propagation using a seed cell suspension of Vero cells that were subcultured more than 3 times. The virus titer for the stock solution that we had was 10⁶.⁴ TCID₅₀/mL, which was equal to ~1.75x10⁶ PFU/mL, according to its specifications. The multiplicity of infection was chosen as 0.005, which is recommended to be between 0.005 and 0.00001⁴⁵.

The growth medium in the Vero cell culture flask was removed and discarded. It was rinsed using 5 mL Dulbecco's Phosphate Buffered Saline (D-PBS), 1X (ATCC® 30-2200™) (product no. 30-2200, ATCC, USA). After incubation of the D-PBS containing flask for 3 min in the cabinet, the buffer solution was removed and discarded. 14 μL of stock virus and 6 mL of EMEM (without FBS) were added to each flask. The flasks were incubated at 37°C for 1 hour and rocked at 15 min intervals to have a uniform spread of virus inoculum. After 1 hour, 10 mL of complete medium was added to each flask and the flasks were incubated at 37°C and 5% CO₂ for 48 h to 72 h.

After the incubation, the flasks were analyzed under a phase-contrast microscope. The cells should dissociate from the surface and round cells should be observed in the mixture if the virus propagation
process is successful. The mixture was collected into a 50 mL tube (product no. 06-443-20, Fisher Scientific, USA) and the tubes were sealed using a parafilm layer. The suspension in the tube was centrifuged at ~2600 g for 10 min using a centrifuge with swing-out rotors (product no. 22500126, Fisher Scientific, USA). The supernatant containing the virus was collected from the tube and pooled in a new tube. After gentle mixing of the tube to have a uniform suspension, the suspension was aliquoted into 1 mL cryogenic vials with O-ring (product no. 5000-1012, Fisher Scientific, USA). The tubes were labeled and stored in liquid nitrogen.

**Preparation of agarose solution:** 4% Agarose (product no. MP11AGR0050, Fisher Scientific, USA) in reagent grade water (product no. 23-249-581, Fisher Scientific, USA) was prepared and well mixed. The suspension was then aliquoted into the glass bottles. The solution was sterilized at 121°C for 15 min in an autoclave and 50 mL aliquots were stored at 4°C until use.

**Preparation of agarose overlay solution:** One of the tubes containing the 50 mL of sterile agarose solution was heated up in a microwave oven for ~30 s. The solution was cooled down to 65°C in a water bath. 23.9 mL EMEM medium was mixed with 0.6 mL FBS and warmed to 50°C. 3.5 mL of agarose solution were added into the warmed medium mixture using a 10 mL serological pipette and kept at 50°C until use.

**Well plate preparation:** First, the adhered cells in the flask were resuspended using trypsin. The solution was gently mixed to have uniform cell suspension and 10 μL of the suspension was taken for cell counting using a hemacytometer chamber. The cells were counted using a phase-contrast microscope. According to the cell count, the concentration of cells was adjusted to ~6.5×10^5 cells/mL by diluting the suspension using the complete medium. ~6.5×10^5 cells were added to each well of a new 6-well plate. Then, 2 mL of complete medium was added to each well and the plate was stored at 37°C and 5% CO₂ for 24 h. Next, the cell coverage on each well was checked under the microscope. The cell coverage should reach ~95% to perform the PFU assay. The cells were infected with diluted virus suspension and the overlay solution was added to the cells. After the solidification of the overlay at room temperature, the plate was incubated for 48 h, where the first 20 hours were used for the lensfree PFU imaging device.

**Preparation of crystal violet solution:** 0.1 g of crystal violet powder (product no. C581-25, Fisher Scientific) was mixed with 40 mL reagent grade water in a 50 mL centrifuge tube. The mixture was gently mixed to dissolve the powder. 10 mL methanol (product no. A452-4, Fisher Scientific) was added to the mixture and stored at room temperature.

**Fixation and staining of cells:** These steps were only performed for comparison against our device’s PFU readings. After 48 h of incubation, the plate was removed from the incubator and the cells were fixed using 0.5 mL methanol/acetic acid solution for 30 min. After 30 min, the wells were washed with water gently to remove the agarose layer. The excess water was removed, and 1 mL of crystal violet (CV) solution was added to each well. The plate with CV was placed into the shaker incubator and mixed at 100 rpm for 30 min. After 30 min of incubation, tap water was used to remove excess stain from the plate and the waste was collected into a large beaker. The plate was left to dry in a fume hood and stored at room temperature by covering with an aluminum foil.

**Lens-free imaging set-up:** An automatic lens-free PFU imaging set-up was built to capture the in-line holograms of the samples. This set-up includes: 1) a holographic imaging system, 2) a 2D mechanical scanning platform, 3) a cooling system, 4) a controlling circuit, and 5) an automatic controlling program. Three green laser diodes (515 nm, Osram PLT5510) were used for coherent illumination, where each laser diode illuminates two wells on the same column of the 6-well sample plate (see Supplementary Video 1). The laser diodes were controlled by a driver (TLC5916, Texas Instruments, Texas, US) and mounted ~16 cm away from the sample. A CMOS image sensor (acA3800-14 μm, Basler AG, Ahrensburg, Germany, 1.67 μm pixel size, 6.4 mm × 4.6 mm FOV) was placed ~5 mm beneath the sample forming a lensfree holographic imaging system. The phase changes in the PFU regions were encoded in the acquired holograms.

The FOV of the CMOS image sensor is ~0.3 cm², hence mechanical scanning is needed for imaging
the whole area of a 6-well plate. A scanning platform was built using a pair of linear translation rails, a pair of linear bearing rods, and linear bearings. 3D printed parts were also used to aid with housing and joints. Two stepper motors (product no. 1124090, Kysan Electronics, San Jose, CA, USA), driven by two driver chips (DRV8834, Pololu Las Vegas, NV, US), were exploited to enable the CMOS sensor to perform 2D horizontal movement. This low-cost platform carries the CMOS sensor moving in a raster pattern and images a total of 420 holograms (21 horizontal, 20 vertical, with 15% overlap) in ~3 min to complete the whole sample scanning (see Supplementary Video 1). The selected CMOS sensor could heat up to >70°C during its operation, which could disturb the growth of the sample and vaporize the agarose layer, especially for regions that are near the sensor parking location between successive holographic scans. Hence, a cooling system was built using fans (QYN1225BMG-A2, Qirssyn, China). We also sealed the sides of the sample using parafilm (product no. 13-374-16, Fisher Scientific, Hampton, NH, USA) and opened 4 holes on the top cover to form a gentle ventilation system.

A microcontroller (Arduino Micro, Arduino LLC) was used to control the two stepper motor driver chips, the illumination driver chip, and a field-effect transistor-based digital switch (used to turn the CMOS sensor on/off). All these chips along with the digital switch, wires, and capacitors, were integrated on one printed circuit board (PCB), powered by a 6V-1A power adaptor connected to the wall plug.

An automatic controlling program with a graphical user interface (see Supplementary Figure 2) was developed using the C++ programming language. It can be used to adjust the image capture parameters (e.g., exposure time etc.) of the CMOS image sensor and communicate with the microcontroller to further switch the laser diodes or CMOS sensor on/off and control the movement of the mechanical scanning system.

All the components along with their unit prices are also summarized in Supplementary Table 1. The cost of the parts of this entire imaging system is < $880, excluding the laptop computer. At higher volumes of manufacturing, this cost can be significantly reduced.

**Image pre-processing:** After the image acquisition for each time interval, the raw holograms were firstly reconstructed using the angular spectrum approach based back-propagation\(^{46-50}\). The accurate sample-to-sensor distance was estimated at the central region of each well using an auto-focusing algorithm based on the Tamura-of-Gradient (ToG) metric\(^{51}\). The same sample-to-sensor distance was used for the entire well since the neural network-based method can tolerate de-focusing. Then, the phase channel of the reconstructed holograms was stitched into the whole FOV image using a correlation-based method and linear blending\(^{52}\).

Starting from the second time interval, a 2-step image registration was performed to compensate for the low accuracy of the mechanical scanning stage. A coarse whole FOV correlation-based image registration was firstly performed, then a local fine elastic image registration was followed\(^{53}\). The impact of this 2-step image registration is shown in Supplementary Video 2.

**Coarse PFU localization algorithm**

First, each current frame was stacked with the previous 3 frames (shown in Supplementary Figure 3a) and a background image (shown in Supplementary Figure 3b) was estimated through singular value decomposition\(^{53}\). By subtracting this background image, signals from the static regions were suppressed (shown in Supplementary Figure 3c). Then, by applying bilateral filtering, the PFU regions with high spatial frequency features were further enhanced (shown in Supplementary Figure 3d).

93 images patches (256 × 256 pixels) in PFU regions and 93 image patches from non-PFU regions were cropped manually from 3 experiments. Each pixel of these image patches was labelled as 1 for the PFU region and 0 for the non-PFU region. A Naïve Bayes pixel-wise classifier was trained using this dataset, where the Tamura-of-Gradient (ToG) metric\(^{51}\) was computed at 2×, 4×, 8×, 16×, and 32× down-sampling scales to serve as the manually selected features. The effect of this classifier is shown in Supplementary Figure 3e. Finally, by applying several morphological operations (such as image close, image fill, etc.), the PFU regions are coarsely located (shown in Supplementary Figure 3f).
Though this coarse PFU localization algorithm was still subject to detect false positives (shown in Supplementary Figure 3g), it could significantly simplify the effort needed for populating the network training dataset. In addition, applying this algorithm to a negative well would help delineate the potential false positives during network training (shown in Supplementary Figure 3h). Ultimately, this coarse PFU localization algorithm helped label 357 positive videos and 1169 negative videos used to train the PFU classification network. The positive videos were populated to 2594 by performing augmentation over time; the negative videos were populated to 3028 by further random selection from the negative control wells. Important to note that this PFU localization algorithm was only used for the training data generation, and was not employed in the blind testing phase as its function was to streamline the training data generation process to be more efficient.

**Network architecture and training schedule**

Our PFU classifier network was built based on the DenseNet\textsuperscript{54} structure, with 2D convolution layers replaced by the pseudo-3D building blocks\textsuperscript{55}. The detailed architecture is shown in Supplementary Figure 4 and described in Supplementary Note 2. We used ReLU as the activation function. Batch normalization and dropout with a rate of 0.5 were used in the training. The loss function we used was the weighted cross-entropy loss:

\[
l(p, g) = \sum_{k=1}^{K} \sum_{j=1}^{2} -w_j \cdot g_{k,j} \cdot \log \left( \frac{\exp(p_{k,j})}{\exp(p_{k,1}) + \exp(p_{k,2})} \right)
\]

(1)

where \( p \) is the network output, which is the probability of each class (i.e., PFU or non-PFU) before the SoftMax layer, \( g \) is the ground-truth label (which is equal to 0 or 1 for binary classification), \( K \) is the total number of training samples in one batch, \( w \) is the weight assigned to each class, defined as \( w = 1 - d \), where \( d \) is the percentage of the samples in one class (\( d = 46.1 \% \) for positive class, \( d = 53.9 \% \) for negative class).

The input 4-frame videos were formatted as a tensor with the dimension of \( 1 \times 4 \times 480 \times 480 \) (channel \( \times \) time frame \( \times \) height \( \times \) width). Data augmentation, such as flipping, and rotation were applied when loading the training dataset. The network model was optimized using the Adam optimizer with a momentum coefficient of \((0.9, 0.999)\). The learning rate started as \( 1 \times 10^{-4} \) and a scheduler was used to decrease the learning rate with a coefficient of 0.7 at every 30 epochs. Our model was trained for 264 epochs using NVIDIA GeForce RTX3090 GPU with a batch size of 30. The loss curve, training sensitivity and specificity curves of our training process are provided in Supplementary Figure 5. In these curves, 10\% of the training dataset was randomly selected as the validation dataset. Note that the training and validation datasets (containing holographic videos of the wells) were formed from various wells at different time points of each PFU assay as detailed earlier; therefore, these training and validation sensitivity and specificity curves do not reflect the evaluation of an individual test well that is periodically monitored from the beginning of the incubation. Our blind testing results reported in the Results section, however, were acquired by using the trained PFU detection neural network on individual test wells that were continuously monitored from the beginning of the incubation with a sampling period of 1 hour, achieving >90\% detection rate for PFUs with 100\% specificity in <20 hours.

**Image post-processing**

After getting the PFU probability map and applying the 0.5 threshold, two image post-processing steps were followed to obtain the final PFU detection result: 1) maximum probability projection along time, and 2) PFU size thresholding. The maximum projection was used to compensate for the lower PFU probability values generated from the PFU center when it enters the late stage of its growth. The effect of this maximum projection is illustrated in Supplementary Figure 6. The size threshold on the PFU probability map was set to \( 0.5 \times 0.5 \) mm\(^2\).
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