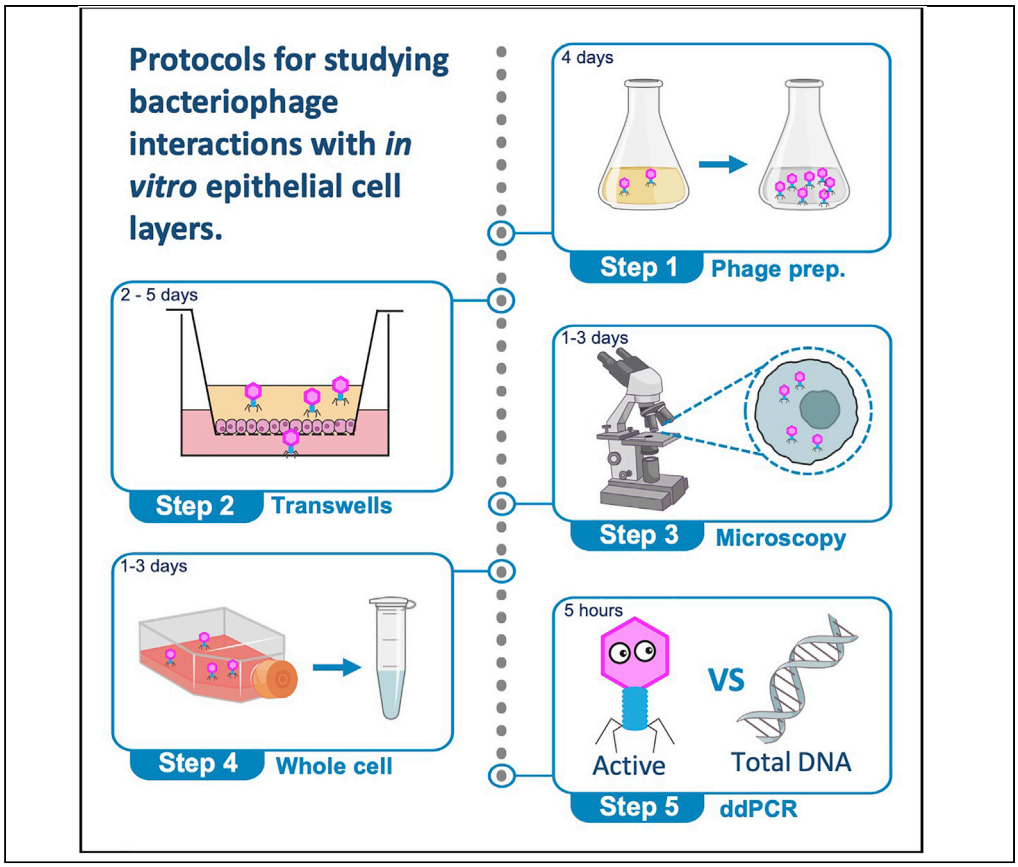
STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocols for studying bacteriophage interactions with *in vitro* epithelial cell layers



Interactions between bacteriophages and mammalian cells are poorly understood. Establishing common methodologies investigating these interactions is important for advancing our understanding in this area. The protocols presented here provide an overview of key approaches investigating interactions between bacteriophages and eukaryotic cells using a variety of techniques, including transwells, microscopy, and whole-cell analysis. These techniques allow for the direct measurement of phage-cellular interactions and characterization of how the presence of phages affects cellular pathways, cell biology, immunology, and the microbiome.

Marion C. Bichet, Ruzeen Patwa, Jeremy J. Barr

jeremy.barr@monash.edu

Highlights

A simplified method for high-titer phage purification and cleanup

Detailed protocol on the use of transwells to study microorganism transcytosis

Comprehensive technique to look at phage-cell interaction under the microscope

Optimized whole-cell analysis for quantification of intracellular phage particles

Bichet et al., STAR Protocols 2, 100697
September 17, 2021
https://doi.org/10.1016/j.xpro.2021.100697

STAR Protocols

CellPress
OPEN ACCESS

Protocol

Protocols for studying bacteriophage interactions with *in vitro* epithelial cell layers

Marion C. Bichet,[1,2] Ruzeen Patwa,[1] and Jeremy J. Barr[1,3,*]

[1]School of Biological Sciences, Monash University, Clayton Campus, Melbourne, VIC 3800, Australia

[2]Technical contact

[3]Lead contact

*Correspondence: jeremy.barr@monash.edu
https://doi.org/10.1016/j.xpro.2021.100697

SUMMARY

Interactions between bacteriophages and mammalian cells are poorly understood. Establishing common methodologies investigating these interactions is important for advancing our understanding in this area. The protocols presented here provide an overview of key approaches investigating interactions between bacteriophages and eukaryotic cells using a variety of techniques, including transwells, microscopy, and whole-cell analysis. These techniques allow for the direct measurement of phage-cellular interactions and characterization of how the presence of phages affects cellular pathways, cell biology, immunology, and the microbiome.

For complete details on the use and execution of this protocol, please refer to Nguyen et al. (2017) and Bichet et al. (2021).

BEFORE YOU BEGIN

All the reagents used in these protocols are essential unless if specified in an attached note.

Phage amplification and purification

Timing: 4 days

Before beginning the experiments, it is important to prepare a purified, high titer phage lysate that is devoid of any bacterial host residues. To reduce experimental error between subsequent assays, we recommend preparing a single stock of high titer (10^9 phages/mL or higher where possible) purified phages that can be used for all subsequent experiments. The protocol presented here is largely adapted from the Phage on Tap protocol (PoT) (Bonilla et al., 2016) and has been optimized for T4 phages.

1. Amplify phages (three days)
   a. Streak your selected bacterial host onto an LB agar plate (or other relevant media). Incubate overnight (between 8–12 h) at 37°C.
   b. Pick a single, isolated bacterial colony from the plate and inoculate in ~15 mL of LB in a 50 mL centrifuge tube. Incubate overnight (between 8–12 h) at 37°C with vigorous shaking (~190 rpm).
   c. The following day, add 10 mM $MgSO_4$ and 10 mM $CaCl_2$ to 200 mL of LB medium in a 500 mL conical flask. Add 2 mL of an overnight culture of the bacterial host to the conical flask. Incubate the culture for one to two hrs at 37°C with vigorous shaking (~190 rpm).

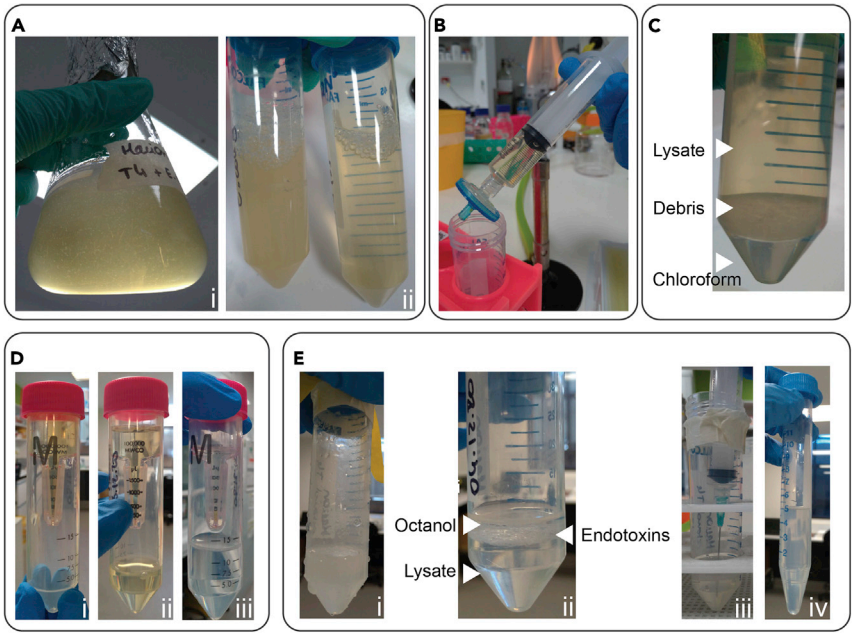


Figure 1. Phage clean-up

(A) Amplifications steps. (i) A flask containing the amplified T4 phages and floating bacterial debris of *E. coli* bacterial host after 6 h of incubation shaking at 37°C. (ii) Tube on the left, before centrifugation, tube on the right is the phage lysate after centrifugation at 3,220 *g* for 10 min at RT.

(B) The filtration process of the T4 lysate, with a 10 mL syringe and a 0.2 μm filter.

(C) T4 lysate after incubation with chloroform and centrifugation for 10 min at 3,220 *g* at RT. The bottom transparent layer is the chloroform; the white middle layer is the bacterial debris trapped in the chloroform and the yellow layer at the top is the clean phage lysate.

(D) Cleaning and concentration steps of the T4 lysate. (i) Lysate in the top chamber of the Amicon Ultra-15 Centrifugal Filter Units 100 KDa filter. (ii) Amicon Ultra-15 Centrifugal Filter Units tube after centrifugation 10 min at 3,220 *g* at RT. (iii) Start the washing process after the concentration steps using SM Buffer.

(E) Endotoxin removal steps using octanol. (i) Lysate after incubation for 1 h with octanol and 15 min in an ice bath. (ii) Lysate after centrifugation 10 min at 3,220 *g* at 4°C. The bottom layer is the lysate, the middle layer is the endotoxins trapped in the octanol and the top layer is the octanol. (iii) Collection of the bottom layer using a 10 mL syringe with an 18 G needle. (iv) Clear final lysate.

Note: The filter sterilised salts ($MgSO_4$ and $CaCl_2$) can be added to the media separately or can be added to the media ahead of time and autoclaved.

d. Once the culture reaches exponential phase (OD600–0.7) add the phage lysate (~10^8 phages/mL) at a Multiplicity of Infection (MOI) between 0.1–1 MOI.
e. Incubate for four to six hrs, shaking vigorously (~190 rpm) at 37°C until bacterial culture clearing is observed (Figure 1Ai).

Note: Depending on the phage and bacterial host, complete clearance of the bacterial host may not be observed. We recommend stopping the amplification of phages after 8 hrs or once bacterial debris is observed floating within the flask.

f. Split the 200 mL of lysate across four 50 mL centrifuge tubes.

2. Spin to remove bacterial debris (15 min)
   a. Centrifuge at 3220 *g* (~4000 rpm) for 10 min.
   b. Carefully transfer the supernatant, being sure not to disturb the bacterial pellet, into new 50 mL centrifuge tubes (Figure 1Aii).

3. Filter-sterilize to produce a bacteria-free lysate (one hour)
   a. Filter-sterilize the phage supernatant using a 0.22 μm filter attached to a 10 mL syringe (a larger volume syringe can also be used) and collect the filtered lysate in new 50 mL centrifuge tubes (Figure 1B).
   b. Regularly change the filter to avoid clogging it with bacterial debris that was not removed by the centrifugation step.

   Note: If the phage concentration is high, it may be hard to pass the lysate through the filter as it will clog easily. Do not push too hard with the syringe to avoid breaking the filter.

   Note: If the phage titre is too high (usually above 10^{10} phages/mL), you can first filter through a 0.45 μm filter to remove the larger debris before filtering through the smaller 0.22 μm filter.

   Note: Alternative options to filter-sterilised the lysate can also be used like larger syringe or vacuum pump and filter bottle.

   ⏸ **Pause point:** The filtered lysate can be stored at 4°C until needed.

4. Add chloroform to the lysate (15 min)
   a. Add 0.1 volumes of chloroform (1 mL / 10 mL of solution) to the phage lysate.
   b. Vortex the lysate and incubate at room temperature (RT; 20°C–25°C) for 15–20 min. Manually shake the tubes every 5–10 min.
5. Spin to pellet the chloroform layer (15 min)
   a. Spin at 3220 *g* (~4000 rpm) for 10 min (Figure 1C).
   b. Carefully collect the supernatant using a serological pipette and without touching the chloroform layer at the bottom of the tube and transfer to new 50 mL centrifuge tubes.

   Note: To remove as much bacterial debris and chloroform as possible, you can repeat step 5 and re-collect the supernatant, making sure there is no remaining chloroform in the lysate.

   ⏸ **Pause point:** The chloroformed lysate can be stored at 4°C until needed.

6. Determine phage titer using phage plaque assay (two days)
   a. Prepare serial dilutions of the lysate (~10^{-1} to 10^{-8}) in LB to obtain a dilution with countable plaques.
   b. Heat LB top agar (final concentration of 0.75% agar) in the microwave until melted completely and let it cool down in a 55°C–60°C water bath.
   c. Add 1 mL of bacterial host in exponential growth phase and 1 mL of the diluted lysate to a glass test tube.
   d. Add 3 mL of the melted top agar to the test tube.
   e. Pour the mixture (5 mL total) on to a pre-made sterile LB agar plate (final concentration of 1.5% agar) and tilt gently to cover the whole plate.
   f. Let the plate sit on the bench until the agar has set.
   g. Invert the plate and incubate overnight (between 8–12 h) at 37°C.
   h. Count the phage plaques and calculate the titer in plaque-forming units (PFU/mL) using the formula:

PFU per mL = (plaques per plate × dilution factor) / volume plated in mL

7. Concentrate phage lysate (adapted from Bonilla et al., 2016) (one day)
   a. Add 15 mL of phage lysate to the Amicon Ultra-15 Centrifugal Filter Units and Spin at 3220 *g* (~4000 rpm) for 5 min. Discard the flow-through and add fresh lysate again to the top of the filter unit (Figure 1Di).

b. Repeat this step until all the lysate is processed (Figure 1Dii).
c. Add SM Buffer to the top chamber of the Amicon Ultra-15 Centrifugal Filter Units and Spin at 3220 *g* (~4000 rpm) for ~2 min to wash the lysate. Repeat this step until the lysate is clear (Figure 1Diii).
d. Vortex the Amicon Ultra-15 Centrifugal Filter Units tube horizontally to detach phages from the filter.
e. Transfer the phage lysate (which will be concentrated in the upper chamber) to a new centrifuge tube.
f. Spin at 3220 *g* (~4000 rpm) for 10 min to remove any debris or aggregates, repeat if necessary.
g. Transfer the supernatant to new 15 mL centrifuge tube.

⚠ CRITICAL: Spin times vary depending on initial phage concentration, as lysates with higher concentrations take longer to pass through the filter. It is important to not spin the centrifugation tube filter dry. It is recommended to start with short < 30 sec spin times and slowly increase the spin time as needed, up to 30 min spins if required, as concentrated lysates may take longer to pass through the filter.

Note: If needed, you can also follow the steps from section four of the PoT protocol from Bonilla et al., 2016. These steps describe how to concentrate a phage lysate in more details.

Pause point: The concentrated lysate can be stored at 4°C until needed.

h. Repeat step 6 to re-titer your lysate to ensure that there was no significant loss of phages during the Amicon concentration step. The titer of the phage lysate should have either stayed the same or increased as the lysate volume should be reduced.

8. Remove endotoxin from phage lysate (adapted from Bonilla et al., 2016) (one day)
   a. Add 0.2 volume of 1-octanol to the lysate and incubate shaking at RT for one hr (Figure 1Ei).

Note: If needed, you can also follow the steps from section five of the PoT protocol from Bonilla et al., 2016. These steps describe how to remove endotoxins from phage lysate with more details.

Note: The 1-octanol solution can easily leak from the tubes, make sure the cap is secured properly to avoid losing any lysate.

⚠ CRITICAL: For steps 8a to 8d, it is important to keep the lysate cold at all times to maintain the octanol in a solidified state.

   b. Transfer the tube to an ice bath for 15 min to solidify the 1-octanol and precipitate the endotoxins.
   c. Spin at 3220 *g* (~4000 rpm) for 10 min at 4°C (Figure 1Eii).
   d. Using a 5-mL syringe and a needle, gently lower the needle through the top organic layer (the octanol) of the tube and slowly collect the bottom layer (aqueous layer) that contains the phages. (Figure 1Eiii). Transfer the lysate to a new 15 mL centrifuge tube (Figure 1Eiv).

Note: Do not collect the top layer to avoid contaminating the lysate with octanol and endotoxins. Be careful with the needle as it may collect some of the octanol top layer while passing through.

   e. These steps can be repeated two or three times to make sure all endotoxins have been removed from the lysate.
   f. Titer the lysate again following step 6. The titer should not have changed.

Note: An endotoxin quantification test is recommended at this step to measure the endotoxin concentration of the samples, we recommend the EndoZyme II kit from Biomérieux. The objective being to have less than 20 EU/mL in any diluted lysates used. For example, following endotoxin removal your lysate may be in the range of ~1000 EU/mL with a titre of 10^{10} phages/mL. By diluting your lysate 100-fold you will have a working stock with a titre of 10^{8} phages/mL and an endotoxin concentration of < 20 EU/mL.

Pause point: The endotoxin-free lysate is stable and can be stored at 4°C until needed.

9. DNase and RNase treat your samples (three hours)
   a. To remove residual bacterial DNA and RNA present in the lysate, incubate 425 µL of the filter-sterilized lysate with 5 µL DNase-I (stock at 1 mg/mL), 50 µL of Ambion DNase Buffer and 20 µL of RNase-A enzyme (stock at 10 mg/mL) for two hrs at 37°C without shaking.
   b. Let the lysate cool down at RT.
   c. Store the lysate at 4°C
   d. It is recommended to check the titer of your phage lysate after this step. See step 6.

⚠ CRITICAL: We do not inactivate the DNase at 75°C as this temperature will damage the phages. We haven't noticed any downstream issues from not inactivating the DNase which should degrade during storage.

Pause point: Once the lysate is DNase treated, it is stable and can be stored at 4°C until needed.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
T4 bacteriophage	ATCC	Cat#11303-B4
E. coli B	ATCC	Cat#11303
Chemicals, peptides, and recombinant proteins		
Eagle's minimal essential medium (MEM)	Life Technologies Australia Pty Ltd	Cat#41090036
Penicillin/Streptomycin (P/S)	Life Technologies Australia Pty Ltd	Cat#15140122
Fetal Bovine Serum (FBS)	Life Technologies Australia Pty Ltd	Cat#10499044
1× Dulbecco's phosphate-buffered saline (DPBS)	Life Technologies Australia Pty Ltd	Cat#14080055
Trypsin 0.25%	Life Technologies Australia Pty Ltd	Cat#25200072
2-(N-Morpholino) ethanesulfonic acid (MES) hydrate	Sigma-Aldrich	Cat#M3671-50G
4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	Sigma-Aldrich	Cat#H3375-25G
$MgSO_4 \cdot 7H_2O$	Merck Pty Ltd	Cat#1058860500
Sodium chloride NaCl	Merck Pty Ltd	Cat#1064040500
1M Tris-HCl pH 7.5	Life Technologies, Australia	Cat#15567-027
Evan Blue Dye (EBD)	VWR International	Cat#A16777.09
Cell-Mask Deep Red Plasma membrane stain (far-red, excitation: 650 nm; emission: 655 nm)	Life Technologies Australia Pty Ltd	Cat#C10046
SYBR Gold (FITC, excitation: 300–495 nm; emission: 537 nm)	Life Technologies Australia Pty Ltd	Cat#S11494
Hoechst (UV, excitation: 350 nm; emission: 461 nm)	Life Technologies Australia Pty Ltd	Cat#62249
Hank's balanced salt solution (HBSS) without phenol red	Life Technologies Australia Pty Ltd	Cat#14025092
Trypan Blue solution	Sigma-Aldrich	Cat#T8154
Paraformaldehyde (PFA) 4%	Sigma-Aldrich	Cat#158127-100G
Ham's F-12K (Kaighn's) Medium (F12K)	Life Technologies Australia Pty Ltd	Cat#21127022
Tris HCl	Bio-Strategy	Cat#0497-1kg
Ethylenediaminetetraacetic acid (EDTA)	Thermo Fisher Scientific Australia	Cat#15576028
Droplet PCR Supermix	Bio-Rad Laboratories	Cat#1863023
Droplet generator oil for probes	Bio-Rad Laboratories	Cat#1863005

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
UltraPure DNase/RNase-Free Distilled Water	Life Technologies Australia Pty Ltd	Cat#10977015
Gibco Bacto Tryptone	Thermo Fisher Scientific Australia	Cat#211705
Yeast extract granulated	Merck Pty Ltd	Cat#1037530500
Agar powder for microbiology	Sigma-Aldrich	Cat#05040-1KG
Magnesium sulfate (anhydrous) $MgSO_4$	Merck Pty Ltd	Cat#1060671000
Calcium chloride dihydrate	Merck Pty Ltd	Cat#1023820500
Deoxyribonuclease I from bovine pancreas (DNase I)	Sigma-Aldrich	Cat#AM2222
DNase I Buffer (1 ng/mL)	Sigma-Aldrich	Cat#AM2222
RNase A (10 mg/mL)	Sigma-Aldrich	Cat#R1253
1-Octanol	Merck Pty Ltd	Cat#8209311000
Sterile Acrodisc Syringe Filters with Supor Membrane, 0.2 μm, 25 mm	Pall Life Sciences	Cat#4612
Whatman Anotop 0.02 μm sterile syringe filters	Fisher Scientific	Cat#09-926-13
Critical commercial assays		
KAM-1325-Antibody Microarray Kits	Kinexus Bioinformatics Corporation	n/a
Experimental models: cell lines		
MDCK-I cells	ATCC	Cat#CRL-2935
A549 cells	From the Hudson Institute of Medical Research and the Oncogenic Signalling Lab	n/a
Oligonucleotides		
Primer gp23 forward: 5′-CTGCAGGTCAGACTTCTG-3′	From Bichet et al., 2021	Micromon Genomics
Primer gp23 reverse: 5′-CATCGGCTGAACACCAC-3′	From Bichet et al., 2021	Micromon Genomics
Probe gp23: 5′-56-FAM/ACTCAGATT/ZEN/GGCCCAGCTGTT/3IABkFQ/-3′	From Bichet et al., 2021	Integrated DNA Technology (IDT)
Software and algorithms		
Fiji	Schindelin et al., 2012	n/a
CellProfiler	McQuin et al., 2018	n/a
Prism	GraphPad Prism version 8.4.2 for macOS GraphPad Software	n/a
QuantaLife	Bio-Rad Laboratories	n/a
Deposited data		
Codes generated in this study	From Bichet et al., 2021	n/a
Other		
Laminar flow hood adapted for tissue culture (TC)	n/a	n/a
Trans Epithelial Electrical Resistance machine (TEER) with chopsticks	Coherent Scientific	EVOM2
TC flask T25 cm^2	Sigma-Aldrich	Cat#CLS430639
12-Well transwells with 3-um pores sizes	Sigma-Aldrich	Cat#CLS3460
96-Wells plates, round bottom	Bio-Strategy	Cat#BDAA353077
ibidi μ-slide 8-well glass-bottom plates	DKSH Australia Pty Ltd	Cat#80827
Cell counting chamber, Malassez pattern	Sigma-Aldrich	Cat#BR719005
Confocal microscope for live microscopy and fixed microscopy Here: inverted Leica SP8 confocal microscope with HC PL APO 63×/1.40 CS2 oil immersion objective	Leica	n/a
Syringe 1 mL	McFarlane Medical	Cat#19032TE
Needle 30 G X 1/2	McFarlane Medical	Cat#19050TE
Cell scraper	Sigma-Aldrich	Cat#C5981
Droplets generator	Bio-Rad Laboratories	n/a
Plate sealer	Bio-Rad Laboratories	n/a
PCR machine	Bio-Rad Laboratories	n/a
Droplet reader	Bio-Rad Laboratories	n/a
ddPCR 96-well deep well	Bio-Rad Laboratories	Cat#12001925
Pipette Tips RT LTS 200μL	Mettler Toledo	Cat#30389239
PCR Plate Heat Seal, foil, pierceable	Bio-Rad Laboratories	Cat#1814040

(*Continued on next page*)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
DG8 gasket, ddPCR	Bio-Rad Laboratories	Cat#1863009
DG8™ Cartridges	Bio-Rad Laboratories	Cat#1864008
90 mm Petri dish	Techno Plas	Cat#TPG9014
Amicon Ultra-15 Centrifugal Filter Unit	Merck Pty Ltd	Cat#UFC910024

⚠ CRITICAL: Chloroform, 1-Octanol and PFA are toxic and should only be used within a chemical hood and with appropriate PPE.

MATERIALS AND EQUIPMENT

Complete F12-K for A549		
Reagent	Final concentration	Volume
F12-K	n/a	445 mL
P/S (v/v)	1%	5 mL
FBS (v/v)	10%	50 mL
Total		**500 mL**
Stored at 4°C for two months		

HBSS pH 6		
Reagent	Final concentration	Volume
HBSS (with Phenol red)	9.8 g/L	9.8 g
2-(N-morpholino) ethanesulfonic acid (MES) Hydrate	1.95 g/L	1.95 g
ddH_2O	n/a	1000 mL
Adjust to pH 6	n/a	n/a
Total		**1000 mL**
Stored at 4°C for one year		

HBSS pH 7.5		
Reagent	Final concentration	Volume
HBSS (with Phenol red)	9.8 g/L	9.8 g
4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES)	2.35 g/L	2.35 g
ddH_2O	n/a	1000 mL
Adjust to pH 7.5	n/a	n/a
Total		**1000 mL**
Stored at 4°C for one year		

SM Buffer		
Reagent	Final concentration	Volume
$MgSO_4 \cdot 7H_2O$	2.0 g/L	2.0 g
NaCl	5.8 g/L	5.8 g
Tris-HCl pH 7.4	5%	50 mL
ddH_2O	n/a	950 mL
Total		**1000 mL**
Stored at 20°C–25°C for one year		

Lysis Buffer		
Reagent	Final concentration	Volume
Tris HCl	1%	10 mL
EDTA	0.5 M	146.12 g
ddH_2O	n/a	990 mL
Total		**1000 mL**
Stored at 20°C–25°C for six months		

LB Buffer		
Reagent	Final concentration	Volume
Bacto Tryptone	10 g/L	10 g
Yeast	5 g/L	5 g
NaCl	10 g/L	10 g
ddH_2O	n/a	1000 mL
Total		**1000 mL**
Stored at 20°C–25°C for two months		

LB Agar for plate		
Reagent	Final concentration	Volume
Bacto Tryptone	10 g/L	10 g
Yeast	5 g/L	5 g
NaCl	10 g/L	10 g
Agar	15 g/L	15 g
ddH_2O	n/a	1000 mL
Total		**1000 mL**
Stored at 4°C for one month		

Top Agar		
Reagent	Final concentration	Volume
Bacto Tryptone	10 g/L	2 g
Yeast	5 g/L	1 g
NaCl	10 g/L	2 g
Agar	7.5 g/L	1.5 g
ddH_2O	n/a	200 mL
Total		**200 mL**
Stored at 20°C–25°C for one month		

STEP-BY-STEP METHOD DETAILS

Transcytosis of phages using transwells

Timing: 2–5 days

Within the human body, particularly within the gut, phages continually interact with eukaryotic cell layers and it is known that phage particles can be internalized by mammalian cells (Nguyen et al., 2017; Bichet et al., 2021). Transwells are a quick and reliable method to study the active uptake and transport of particles, drugs and infectious agents across confluent cell layers grown on a permeable membrane. The Transwell set-up mimics a confluent monolayer of cells exhibiting tight junctions that limit paracellular transport and thereby selectively measure transcellular transport processes. Trans Epithelial Electrical Resistance (TEER) measures allow for live monitoring of cell

layer confluency and detection of any disruptions and leaks. By applying particles, drugs or microorganisms, such as viruses, to either side of the cell monolayer, it is possible to quantify their subsequent transcellular uptake and transport. Transwells can be used to study immunological responses, drug transfer, cell metabolism and pathogen interactions with eukaryotic cells (Tugizov, Herrera and Palefsky, 2013; Nguyen et al., 2017; Otero et al., 2019). Inhibitors, drugs or other chemicals can be further used to assess their effect on the uptake and transport of particles. Here we describe the setup used in Nguyen et al., 2017 research paper to demonstrate the directional transcytosis of phages across confluent cell layers.

1. Prepare transwells (one to five days)

Note: As this assay uses live cells, it cannot be paused before the collection of the samples.

Note: MDCK-I is considered the gold standard cell line for the study of transcytosis processes and is recommended for use here, but any other cell line forming a confluent cell layer can also be used for this assay.

Note: TEER measures the electrical resistance between the upper and lower chambers in the transwells. If there is a gap in the cell layer then the resistance measurement will be low. If there are no gaps in the cell layer then the resistance will be higher, indicating that the cell layer is confluent and is ready to be used. Note that the final TEER measurements will differ depending on the cell line used.

a. Seed MDCK-I cells, at a passage number between 10 to 30 (or any cell line of your choice that can form a confluent cell layer) into the top chamber of the transwell at a cell density of between 5×10^4 to 1×10^5 cells/mL, in a total volume of 500 µL per well. The bottom chamber should contain 1000 µL of media with 10% FBS only (Figure 2Ai–2Aiii). To seed the cells in the top chamber, carefully place the pipette tip on the side of the well and avoid touching or damaging the membrane (Figure 2B). Incubate cells at 37°C and 5% CO_2 until they reach confluency (usually between two to five days depending on the cell line).

Note: We recommend the use of 12 wells transwells with 3 µm pores sizes (Sigma) as smaller pore sizes reduced transport of phages across the filter (Figure 2Ai).

Note: Ensure a homogenous distribution of cells by gently rotating the plate horizontally following an infinity shape (∞) to avoid accumulation of cells on the side or in the middle of the plate. Avoid circular shapes as this will push the cells to the side of the well.

Note: Visual inspection of the cell layer should be done every day to ensure proper cell growth, density and homogeneity.

Note: Due to the thickness of the transwell membrane, cell confluency is difficult to determine using light microscopy (see confluency controls below).

b. Replace cell culture media every other day. To do so, tilt the transwell plate at 45° angle, gently place a pipette tip on the side of the inner well and carefully remove the media above the cell layer without touching the membrane as this can disrupt the cell monolayer (Figure 2B). Add fresh media to the chamber following the same process, do not add media directly into to cells but rather on the wall of the chamber and let it drip slowly on the cells.

Note: Do not attempt to remove all liquid from the well as this may disrupt the cell monolayer. Always leave a few microliters of media in the well.

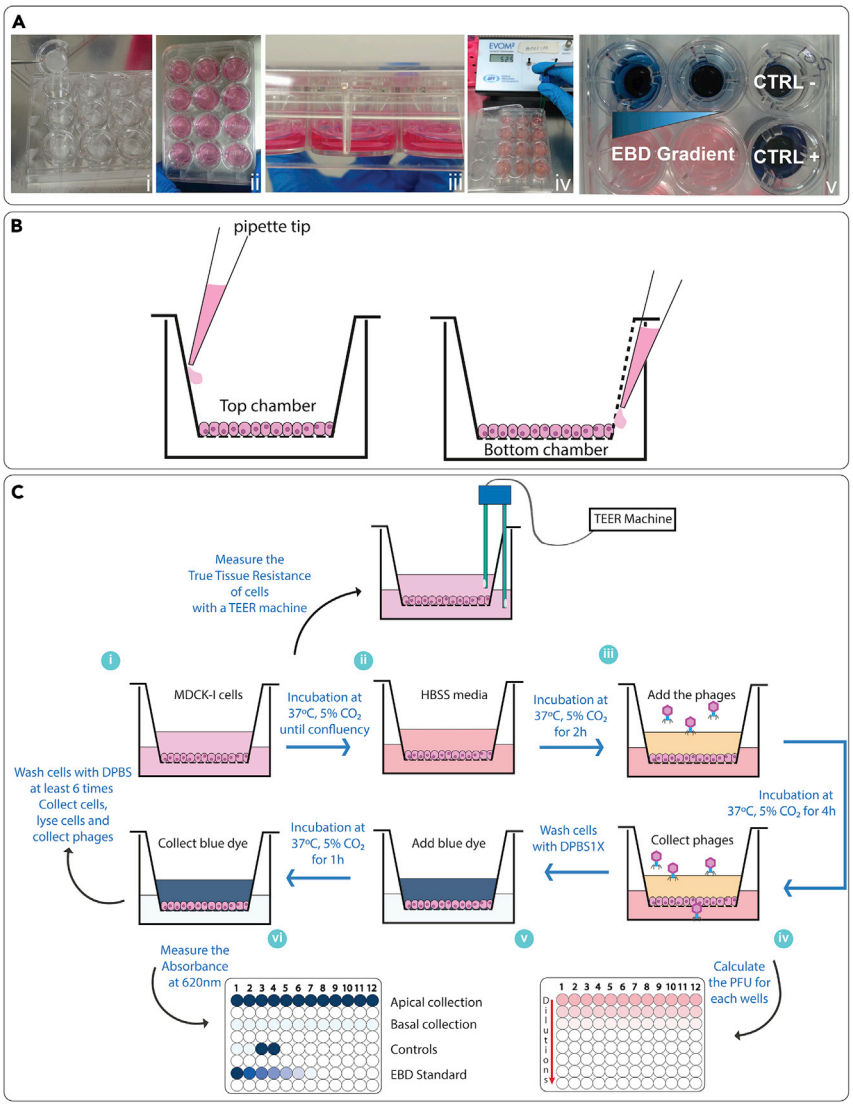


Figure 2. Transwells

(A) Steps to work with a transwell. (i) Top view of a transwell 3 μm pores, empty. (ii) Top view of a transwell 3 μm pores, with media and cells. (iii) Side view of the transwell with the bottom and top chambers filled with cell media. (iv) Measurement of TEER using the EVOM^2 machine. (v) Top view of a transwell with a gradient of EBD transfer.

(B) Scheme representing the positions of the pipette tip in the transwell chambers to add and/or remove media without disturbing the cell layer.

(C) Transwell steps. (i) Grow cells in the top chamber of the transwell and check for the cell layer resistance. (ii) Once the cell layer reached confluency, wash the cells and incubate in HBSS media. (iii) Add the phages to the top chamber. (iv) After incubation with the phages, collect the top and bottom samples in a 96 well plate. (v) Control cell confluency by adding EBD dye to the top chamber. (vi) After incubation collect samples from both chambers in a 96 well plate, the cells can be collected after these steps and washes.

c. Return the transwell plate to the incubator and continue growing the cells until the required cell resistance is obtained.

d. Use the TEER machine following the manufacturer's recommendations, record daily cell resistances measures until cells reach > 1000 Ω × cm^2 for MDCK-I cells (Figures 2Aiv and 2Ci).

Note: The TEER values at confluency will vary depending on the cell type. For MDCK-I cells 1000 Ω × cm^2 is typical for a confluent cell layer that can be used for the assay.

2. Prepare transwells for transcytosis experiment (five hours)
   See Figure 2C for a visual overview of the following steps.
   a. Using the same technique as described above in step 1a (see also Figure 2B), wash the top and bottom chamber of the transwell with 1 mL of 1× DPBS, to remove any traces of FBS.
   b. Once the cells are washed and all media and DPBS is removed, add 500 μL of Hank's balanced salt solution (HBSS) + HEPES (pH 7.4) (see media preparation in the key resources table and materials and equipment sections) to the top and bottom chambers (Figure 2Cii), following previous recommendation and being careful not to touch the cell layer with the pipette tips (Figure 2B).

      Note: Replacing media with HBSS buffer serves to both starve the cells of serum before the experiment and to accustom the cells to this new buffer.

   c. Incubate the transwells for two hrs at 37°C and 5% CO_2 to allow the cells to become accustomed to the new media.
   d. Prepare a solution of phages in HBSS-MES at pH 6 (see media preparation in key resources table and materials and equipment sections) at the desired phage concentration. We recommend preparing a minimum of 3 mL cleaned phage lysate for a 12 well plate assay, with each well containing 250 μL of the phage solution per well.

      Note: If measuring phage transcytosis across cell layers, we recommend using a final concentration between 10^7 - 10^9 PFU/mL.

   e. Discard media from both chambers without touching the membrane (Figure 2B).
   f. Apply 250 μL of HBSS-MES-pH 6 phage solution to the top chamber and 250 μL HBSS-HEPES at pH 7.4 to the bottom chamber (Figure 2Ciii).
   g. Incubate transwell plate for two to four hrs (it is also possible to leave the incubation for longer, even overnight (between 8-12 h) if the cell media is complemented with 10% FBS) at 37°C and 5% CO_2.

      Note: The cells are without any FBS at this stage of the experiment, and incubation for more than four hours may cause the cells to shrink, thereby creating leaks in the cell layer.

      Note: The differences in media and pH between the top and bottom chambers is to mimic the pH-dependent uptake between the basal and apical sides of the cells. Media can be switched to induce uptake in the opposite direction (Nguyen *et al.*, 2017).

   h. Collect a minimum of 150 μL of solution from both the top and bottom chambers and store in a labeled 96 well plates until analysis (Figure 2Civ).
   i. For phage quantification, serially dilute the samples collected in the 96 well plate using SM Buffer, and follow step 6 in the "before you begin" section for phage quantification.

3. Check the integrity of the cell layer (two hours)
   Evans Blue Dye (EBD) is used post-assay to check the permeability of the cell layer and ensure none leaked during the experiment. EBD is a non-permeating dye that enters plasma-membrane damaged cells. The dye will only pass to the opposite chamber of the transwells if the cell layer was damaged cells or contained leaks.
   a. Prepare a 5 mL solution of 0.1% (w/v) of EBD in 1× DPBS.
   b. Add 250 μL of this 0.1% EBD solution to the top chamber being careful not to touch the membrane, followed by the addition of 250 μL of 1× DPBS to the bottom chamber (Figures 2Av and 2Cv).
   c. Incubate at 37°C and 5% CO_2 for one hr.

d. Collect 100 μL of the solution from both the top and bottom chambers and transfer to a new labeled 96 well plate (Figure 2Cvi).
e. Measure the absorbance at 620 nm using a spectrophotometer.
f. Plot an EBD standard absorbance curve by diluting from 0.1 μL to 3 μL of 0.1% EBD (w/v) in 100 μL of 1× DPBS in a 96 well plate and measure the absorbance at 620 nm. The presence of the dye in the bottom chamber is an indication of a non-confluent cell layer with likely paracellular transport and these wells should be discarded from the analysis.

Note: The cut-off value we used to determine the failure of a well is of an absorbance at 620 nm higher than 0.05.

Note: The 96 well plate can be stored at 4°C up to one month if sealed with parafilm.

Note: This technique cannot detect small leaks. It is important to combine these results with the TEER measurements.

⚠ CRITICAL: Here we are ensuring that the confluency of the cell layer was maintained throughout the experiment. If the dye applied in the top chamber does not leak to the bottom chamber, then the integrity of the cell layer was maintained throughout the experiment (Figure 2C). If the dye leaks then the samples from that well should not be taken into account during the analysis as the cell layer has been disrupted at some point during the experimentation (see potential problem 1 in the troubleshooting section).

Pause point: Now that the samples have been collected, it is possible to store the phages and EBD samples at 4°C before analysis.

4. Data collection (one day)

Note: As the collected volume will likely be low (~100 μL), you may need to apply a multiplication factor to calculate PFU/mL.

Note: The concentration of phages applied in the top chamber is usually known whereas the phages transferred to the bottom chamber is unknown.

a. To measure the concentrations of phages in the applied wells (from the top chamber), serially dilute the samples and quantify PFUs via plaque assay, see step 6 in the "before you begin" section.

Note: Phages or particles can be applied to either the top or the bottom chamber depending on the experimental needs, here phages were added to the top chamber as demonstrated by our previous study (Nguyen et al., 2017) where phages were found to have higher rates of transcytosis from apical to basal side of the cells, rather than in the opposite direction.

b. To measure the concentrations of phages in the transferred wells (here the bottom chamber), it is recommended to quantify an undiluted sample and serial dilutions. If there are too many plaques, then plate at a higher dilution. If there are no plaques, then plate the undiluted sample being sure to factor in the correct dilution factor. See step 6 in the "before you begin" section.

Note: Due to the small sample size (100 μL), the detection limit of the assay is 10 phages/mL.

Visualization of phages and cells under the microscope

Timing: 1–3 days

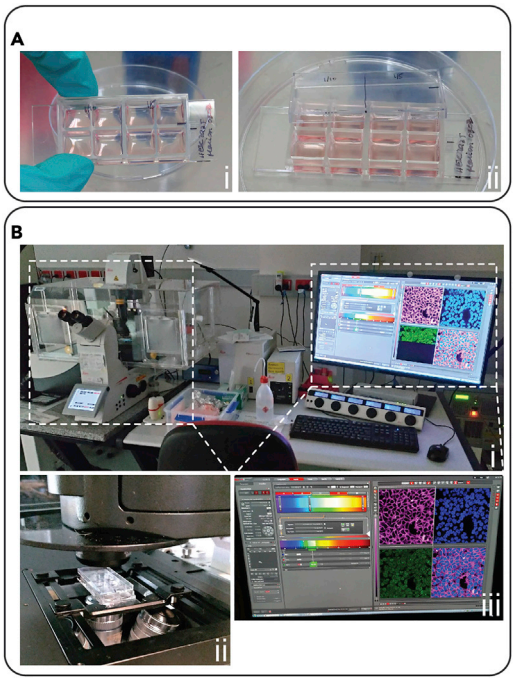


Figure 3. Microscopy

(A) 8 well slide setup. (i) μ-slide 8 well, glass-bottom from Ibidi with cells. (ii) Top view of the μ-slide 8 well with the lid open.

(B) Microscopy setup. (i) Picture of the full microscopy station, Leica SP8 confocal inverted microscope on the right and the computer with the LAS-X software open on the left. (ii) Zoom-in of the 8 well slides in the microscope setup. (iii) Zoom-in of the LAS-X software.

Live microscopy is commonly used to visualize how cells and different microorganisms interact over time. Little is known about the interaction between phages and mammalian cells and real-time visualization is an important tool to improve our understanding. Here we use this method to visualize phages interacting with tissue culture cells. Utilizing different dyes and labels, phage particles, which are extremely small in comparison to the mammalian cell, can be visualized along with their uptake and intracellular transport. This technique has been extensively used in Bichet et al., 2021 with the real-time visualization of phage uptake by different cell types under both static and flow rate conditions. The results demonstrated that the rate of phage uptake was highly dependent on both phage and cell type.

Note: As this assay uses live cells, it cannot be paused prior to acquisition of images.

5. Cells preparation (one to three days)
   a. Seed A549 cells onto any microscopy-compatible surface, preferably glass-bottom coverslips/slides. For ease of use we recommend μ-Slide 8 Well glass bottom from Ibidi. Seed cells at a density between 5×10^4 to 10^5 cells/well in a total volume of 200 μL/well of F-12K supplemented with 10% FBS media (Figure 3A).
   b. Incubate cells at 37°C and 5% CO_2 for two to three days until the cells reach the desired density for the experiment, here we used ~90% confluency (i.e., a subconfluent cell layer).

Note: Here we use A549 cells, but any other adherent cell line may also be used.

Note: Some cells auto-fluoresce; therefore, it is important to have correct controls for the microscopy experiment as phage particles can be hard to distinguish from the background fluorescence. As a control, we recommend the use of both unstained cells as well as cells incubated with SYBR Gold-stained, phage-free media.

Note: It is important to use cells from early passages, not above passage 30, for consistent results.

Note: It is recommended to perform regular screens for mycoplasma infection as this can interfere with cell activity.

6. Fluorescence labeling of phages (two hours)
   a. To fluorescently label phage particles, add SYBR-Gold stain (1× final concentration) to 1 mL of phage lysate at the desired titer. We recommend a final titer of 10^9 phages/mL.
   b. Incubate the solution in the dark at 4°C for a minimum of one hr or overnight (between 8–12 h).

   Note: Any stain or dye that can label the phages can be used. However, it is important to ensure there are no overlaps between fluorescence wavelengths of other stains used.

   Note: Some dyes can stain the phage capsid rather than staining the DNA inside it. Both stains can be used depending on the experimental need. Keep in mind that the SYBR stain does not modify the exterior of the phage.

   c. Following incubation, transfer the stained phages to the upper reservoir of a 4 mL - 100 kDa Amicon Ultra centrifugal unit to wash and remove the excess of SYBR Gold stain (see potential problem 2 in the troubleshooting section).
   d. Spin at 3220 g (~4000 rpm) for between 30 s to two min, ensuring not to spin the filters dry.
   e. Between each centrifugation discard the solution from the lower chamber and add fresh HBSS (without phenol red, compatible with microscopy) to the upper chamber.
   f. Repeat steps 6d-e four times or more as necessary. Stained lysate should appear clear to the eye after washing.

   ⚠ CRITICAL: Spin times vary depending on initial phage concentration, as lysates with higher titres take longer to pass through the filter. It is important to not spin the centrifugation tube filter dry. Recommended to start with short < 30 s spin times and slowly increase the spin time if needed.

   Note: We use HBSS media to wash phages as it is both compatible with live-cell microscopy and tissue culture cells stay viable for longer in HBSS without serum compared to other buffers, such as DPBS or SM where the cells begin to shrink and die during the fluorescence acquisition.

   g. Following four volume exchanges (the volume of phages being washed four times in HBSS), collect the SYBR gold labeled and washed phages from the inner filter by pipetting back and forth against the membrane to dislodge any phages that may be stuck.
   h. Transfer the filtrate to a new 1.5 mL microcentrifuge tube (around 200 μL) and resuspend up to 1 mL with fresh HBSS. The titer of this clean SYBR-stained phage solution should be close to the initial titer, but it is recommended to check this via plaque assay.

   ⏸ Pause point: This stained phage solution can be stored at 4°C in the dark for up to two weeks, but the fluorescence will be stronger and sharper when the solution is freshly prepared (ideally acquire images on the same day as the staining of the phages).

7. Live microscopy (three to five hours)
   a. Once cells have reached the desired confluency, wash the cells three times with 500 μL of warm 1× DPBS per well.
   b. Discard the 1× DPBS and add 200 μL of warm F-12K media without FBS to each well and incubate for two hours at 37°C and 5% CO_2 to serum starve the cells.
   c. Discard the media from wells.

d. Add 200 μL per well of 1× Cell-Mask (stains the plasma-membrane) and 1× Hoechst (stains the nucleus) diluted in warm F-12K media without FBS. Incubate again for 20 min at 37°C and 5% CO_2.

Note: At this step, any other label or dye can be used to stain the cells or any of their components, as long as the fluorescence wavelengths do not overlap with the stain used to visualize the phages (here SYBR-Gold).

Note: It is also possible to start the fixed microscopy at this point of the protocol, the phages can be added to the well and incubated for the desired time before fixing the cells, see step 8 below.

Note: If the live microscopy is not needed, the nuclei can be stained post-fixation with the DAPI stain which is stronger than the Hoechst but not compatible with live microscopy.

e. Discard the media and wash cells three times with 500 μL of warm 1× DPBS per well.
f. Add 200 μL per well of warm HBSS to the cells.

Note: At this point, the cells are starving, if the experiment must go for longer than 4 h, we recommend adding at least 1% FBS to the cell media (see potential problem 3 in the troubleshooting section).

g. Warm up the microscope chamber at 37°C and 5% CO_2, at least two hours before the acquisition.
h. At the confocal microscope, place the slide under the microscope and focus on a field of view, set the laser's parameters to the optimal set-up (Figure 3B).
i. We used an inverted Leica SP8 confocal microscope with HC PL APO 63×/1.40 CS2 oil immersion objective with a hybrid detector (HyD) used in sequential mode to visualize SYBR-stained phage DNA. Set the HyD system for the phages channel (480 nm channel) with the highest Line Acquisition number.

Note: The optimal microscope set-up will depend on the microscope type and operating system.

Note: Do not set the laser power too high otherwise you might damage your cells and bleach the dyes. See potential problem 3 in the troubleshooting section.

Note: Each acquisition depends on what you are looking for, your cell types and the organism/particles you are applying to the cells. Each of the parameters above can be modified and adapted to each particular assay.

Note: You can add a refocus step between each frame, as the cells might move slightly during the acquisition. But by doing so you increase the time required between the acquisition of each image.

Note: Depending on the need, image acquisition can be less than one image per minute and can also be manually acquired if needed.

j. Prepare the acquisition to get one image every 60 s to 5 min (ideally choosing the shortest time possible without bleaching the sample) for up to 4 h.
k. Once an optimal cell section has been chosen, turn off the lasers, carefully discard the HBSS from the well using a pipette and add 200 μL of the SYBR-stained phages solution at 10^9 phages/mL, pre-warmed to the microscope temperature.

Note: For this step, carefully add the SYBR-stained phages without touching the wells to maintain the focus on the cell layer.

l. Close the microscope chamber to keep the humidity and temperature stable for the image acquisition. After adding the phages to the well, quickly re-check the focus and begin image acquisition.
m. Once the acquisition has finished, the slide can be either used for fixed microscopy see step 8 below or can be discarded as cells are no longer sterile.

8. Fixed microscopy (two hours)
Microscopy is an excellent tool to visualize where particles are localized within cells. Fixed microscopy has a better resolution than live microscopy as it can acquire high-resolution images with a high degree of precision.
a. After the live imaging experiment, wash the cells three times with 1× DPBS to remove as many extracellular phages as possible.

Note: It is also possible to start the fixed microscopy before live microscopy. Instead of real-time monitoring of phage uptake, phages can be added to the wells, incubated for the desired time, followed by washing the samples and fixing as described in steps 8a-c.

Note: Fluorescence is not stable over time. To get the best images from the samples it is recommended to acquire images from new slides.

b. To fix the cells, remove the DBPS from each well and add 50 μL of 4% paraformaldehyde (PFA) within a chemical hood and incubate 20 min on ice or at 4°C.
c. After incubation, carefully wash cells three times with 1× DBS to remove any trace of PFA and leave each well in 200 μL of 1× DPBS.

⚠ CRITICAL: Paraformaldehyde is a toxic chemical and should only be used in a chemical fume hood and waste should be collected and discarded appropriately.

Pause point: Now that samples have been fixed, they can be stored protected from light at 4°C for a few weeks before being imaged, however, the fluorescence will decrease over time.

9. Data collection
Different software can be used for image analysis. Here we use the software FIJI (Schindelin et al., 2012).
a. Open the image stack on FIJI.
b. Arrange the channels, for the nucleus to be blue, the phages green, and the plasma membrane to be magenta (or as desired).
c. Split the channels and remerged the channels to fit the colors chosen.
d. Add the scale bar to the images on top of the overlay.
e. Add the time stamp by setting the time between each frame as you set it up at the microscope.
f. Save the movie with 12 frames per seconds (fps) (or as desired) (see microscopy analysis section for further details on image analysis).

Intracellular phage preparation for whole-cell analysis and phage quantification

Timing: 1–3 days

Understanding the fate of intracellular phages is important when considering phages interactions with the eukaryotic cells. By collecting whole-cell lysates following phage exposure it is possible to investigate both broader whole-cell analysis while also quantify intracellular phages using both

traditional plaque assays and ddPCR (method described in the following section). A number of techniques are available to analyze a wide range of cellular responses, including protein, RNA, cytokine and metabolite screens, in the cells pre- and post-incubation with phages. Using the same samples for these whole-cell analyses and phage quantification is a good way to ensure the parameters are consistent across the range of experiments. Here we describe a method used to collect cell lysates that can be used for a range of whole-cell analysis as well as intracellular phage quantification.

Note: This assay uses live cells and cannot be paused before the sample collection.

Note: It is recommended to use high phage concentration to ensure phage uptake by the cells and phage detection by the ddPCR and PFU assays.

Note: Other molecular methods can also be used (*e.g.*, semi-quantitative PCR, qPCR) although due to small number of intracellular phages, limit of detection may be an issue. As such, we recommend ddPCR as the most accurate quantification method (method described in the following section).

Note: In the Bichet et al., 2021 research paper, we used 30 secs of incubation of the phages with the cells as control of surface-bound versus intracellular phages when compared to 18 hrs incubation. Phages can be incubated for any desired times as long as cells stay healthy.

10. Cell preparation for whole cell assay and intracellular phage quantification (one to three days)
This initial sample preparation is the same for whole cell assay and intracellular phage quantification but the two experiments cannot be combined as some of the steps for the whole cell assay might damage active phages. Once the cells ready, you can choose to either process the cells for the whole cell analysis, or to process them for the intracellular phage quantification.

Note: If both assays need to be performed, we recommend using two sets of separate flasks in parallel to keep the conditions as similar as possible between the two experiments.

a. It is important to compare samples incubated both with and without phages. We recommend using 'filtered lysate' as a no-phage control sample whereby phage particles have been removed from the sample using a 0.02 μm filter. It is important to pass the phage lysate multiple times through the 0.02 μm filter to ensure the complete removal of phages. The 'lysate' obtained after filtration should not contain phage particles and will be referred to as 'filtered lysate'.

Note: It is good to test the presence of phages in the 'filtered lysate' using the PFU method described in step 6 of the "before you begin" section.

Note: Different concentrations of phages can be used, but from our experience higher titres give better results for cellular uptake.

b. Grow cells in a T25 flask with 6 mL of complete media until cells are confluent. We recommend a concentration of 8×10^4–1×10^5 cells/mL for A549 cells (Figure 4i).
c. Once cells are confluent, prepare a phage solution in cell media of 10^7 - 10^9 phages/mL.

Note: Bigger flasks like T75 or smaller volumes like six well plates can also be used for this assay depending on the specific needs. The volumes and concentrations will need to be adjusted to the adequate flask or well.

d. Add 3 mL of phages or 'filtered lysate' at the desired concentrations for each of the experimental condition flasks (Figure 4ii).

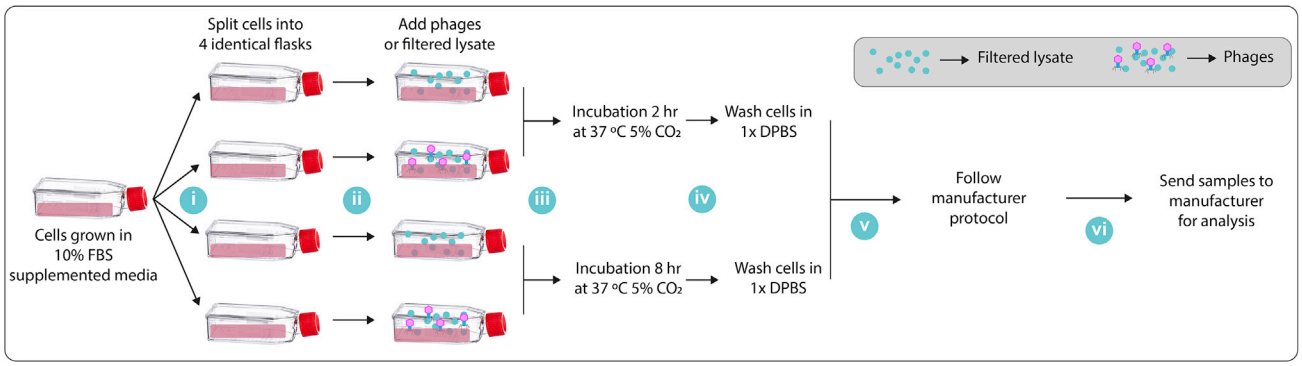


Figure 4. Whole cell analysis

Scheme representing the steps to collect samples for whole-cell analysis. (i) Split cells into 4 identical flasks. (ii) Add phages or control sample to the flasks. (iii) Incubate the samples for the desired time. (iv) Wash the cells with DPBS to remove the excess phages. (v) Follow the manufacturer instruction for the assay chosen. (vi) Send the samples back to the manufacturer for analysis.

e. Incubate the cells and phages together for two hrs or overnight (between 8–12 h) (or as long as desired) at 37°C and 5% CO_2 (Figure 4iii).
f. *Optional step* - For the control flask, add phages only 30 s before the start of washing.
g. Wash the cells at least three times or more with ice-cold 1× DPBS to remove the excess phages (Figure 4iv).

Note: The control flask is useful to measure phage adherence to the cell layer as the 30 secs time frame is too short to facilitate the internalization of phages by the cells (Bichet et al., 2021). The earliest time to see phage internalization is ~10 min as described in Bichet et al., 2021 with the A549 cells. We recommend incubation times longer than 30 min to ensure sufficient phage uptake and detection by downstream assays.

⚠ CRITICAL: From steps 10g to 11e, it is essential to keep the cells cold to avoid the degradation of any cellular products, like proteins or RNA, which are sensitive to cellular enzymes.

11. Collect cells for whole cell assay (one hour)

This subsection is for whole cell analysis, the use of strong lysis buffer in step 11a and the sonicator in step 11d prevent the quantification of active phages (to collect active intracellular phages, follow the next subsection "collect phages for intracellular phage quantification," step 12). Intracellular phage DNA can be quantify using this technique.

a. Remove as much 1× DPBS as possible and add between 150 to 200 µL of ice-cold Lysis Buffer (alternatively you can use a lysis buffer provided by a commercial kit, for example the KAM-1325 kit from Kinexus for a protein micro-array) (Figure 4v).
b. Scrap the cells from the flask with a cell scraper.
c. Collect the cells in a labeled 1.5 mL microcentrifuge tube.
d. To lyse the cells, either pass three times through a 30 G needle using a syringe or use a cell sonicator four times with between 10 to 15 s intervals.
e. Once the cells are lysed the samples can be processed for a range of downstream whole-cell analysis techniques (Figure 4vi) (see potential problem 4 in the troubleshooting section).

Note: This protocol is highly adaptable to a range of whole-cell analysis techniques, including; protein microarray, cytokine array, transcriptomics, etc.

Note: To collect and lyse the cells we recommend following the manufacturer's instructions to avoid using incompatible buffers.

Note: Sonication might damage phages.

Note: Some reagents used to lyse the cells, like Tx-100, may not be compatible with specific arrays or kit. Before lysing the sample make sure all the reagent used are compatible with the commercial array/kit you plan to use.

12. Collect phages for Intracellular phage quantification (one hour)
    Continue previous protocol from step 10 g.
    This subsection is to collect and quantify intracellular active phages. It uses only gentle lysis buffer and will only break the cells membrane and not the phages.
    a. Trypsinize the cells with 1 mL of trypsin at 37°C and 5% CO_2 until the cells detach (around 3–5 min for A549 cells).
    b. Once detached, collect the cells in 5 mL of 1× DPBS and spin the cells for 3 min at 453 *g* (∼1500 rpm) and discard the supernatant.
    c. To remove any non-internalized phages or non-adherent phages, resuspend the cells in 5 mL of 1× DPBS and repeat the step above two more times.
    d. Collect 1 mL of the supernatant after the last centrifugation in a 1.5 mL microcentrifuge tube, as a wash control.
    e. Resuspend the cells in 1 mL of Lysis Buffer (see media preparation in the key resources table and materials and equipment sections) and transfer to a fresh 1.5 mL microcentrifuge tube, incubate 20 min at RT (Figure 5Ai).
    f. Pass the cells three to five times through a 30 G needle with a 1 mL syringe to ensure complete lysis (Figures 5Aii and 5Aiii). Some cell lines are harder to lyse with the Lysis Buffer, this step is to ensure all the cells are properly lysed before the next step.
    g. The samples are ready for intracellular phage quantification, follow steps using ddPCR described in the subsection bellow to quantify total phage DNA and/or plaque assay in step 6 in the "before you begin section" to quantify active phages.

Note: To ensure that the cells have lysed, place a few microliters on a glass microscope slide with a glass coverslip on top of the drap and check under a light microscope at 10× objective (Figure 5A). Only nuclei and cell debris should be present (Figure 5Aiii). If not, continue to pass the cells through the syringe until all the cells have lysed.

Note: It is recommended to quantify the phages via PFU and ddPCR as quickly as possible as phages may not be stable in lysis buffer over a long time.

13. ddPCR setup to quantify total DNA copies (five hours)
    Following cell collection and lysis from the subsection above, intracellular phages can be quantified using either traditional plating methods, step 6 in the "before you begin section" to quantify active phages or absolute number of phages DNA copies measured using droplet digital polymerase chain reaction (ddPCR). While ddPCR is a useful method to quantify the absolute number of DNA copies in a sample, a range of other molecular or microbiology assays (e.g., semi-quantitative PCR, qPCR, plaque assay) may also be used. The principle of ddPCR is based on traditional PCR amplification coupled with fluorescence probe-based detection to provide absolute quantification of DNA copies in the sample. In this technique, the PCR sample is partitioned into about 20,000 water-oil emulsion droplets that separate DNA molecules, with each droplet containing between zero to five DNA copies. PCR amplification is carried out within each droplet and droplets that contain DNA are detected by fluorescence and scored as positive, while the droplets without the fluorescence are scored as negative. The ratio between positive and negative droplets is calculated by the software and the Poisson Distribution statistics used to quantify the total number of DNA copies present within a sample.

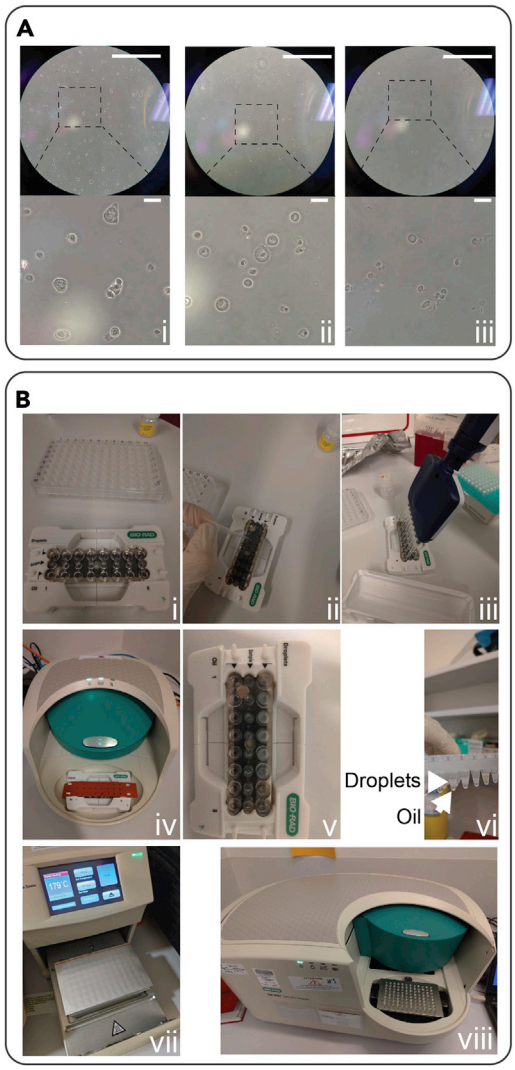


Figure 5. Intracellular phages

(A) A549 cells during the lysis steps. (i) A549 cells before adding the lysis buffer, with zoom-in. (ii) A549 cells after the incubation 20 min at RT with the lysis buffer, with zoom-in. (iii) A549 cells passing three times through a 30 G needle, with zoom-in. Scale bar represents 1 mm and 0.1 mm for the top images and enlarged images respectively.

(B) ddPCR steps. (i) Sample transferred from the 96 well plate to the middle row of the cartridge. (ii) Removing bubbles using a pipette tip. (iii) Adding ddPCR oil to the bottom row of the cartridge. (iv) The cartridge with a rubber gasket placed in the droplet generator. (v) Droplets appear in the top row of the cartridge and transfer back to the 96 well plate. (vi) Droplets are floating on top of the sample. (vii) Plate sealed with an aluminum foil cover. (viii) After the PCR cycles, the plate is placed in the droplet reader to quantify the DNA concentration in the sample.

Note: ddPCR will quantify absolute DNA copies which include both active phages, being those able to infect their bacterial host, and phages that have been inactivated during cellular uptake and are no longer able to infect their bacterial host.

Note: The droplet generator is set up to run eight samples at a time, it cannot do more or less per run. Always ensure you have multiples of eight to maximise your samples per run.

Note: There are two existing methods to measure DNA with the ddPCR, the SYBR-green method, where you use a non-specific fluorophore that binds to double-stranded DNA or

Table 1. ddPCR mix

Super mix ddPCR	Final concentration	Volume
SuperMix for 2× Probe	1×	10 μL
gp23 Forward Primer (10 μM)	0.1–1 μM	1.8 μL
gp23 Reverse Primer (10 μM)	0.1–1 μM	1.8 μL
20× Probe	1×	1 μL
ddH_2O	n/a	5.4 μL
Sample	n/a	2 μL
Total		**22 μL**

Description of all the reagents needed for the ddPCR mix and their concentrations, adapted for detection of T4 phages using gp23 primers.

the probe system with a dark quencher that will be removed during the elongation step of the PCR. Either method can be used to measure intracellular phages. The probe method is known to have more precision, but is more expensive and requires the design of a targeted PCR probe for your sample of interest. Here we describe the probe method.

Note: When making the primers and/or the probe make sure to follow the manufacturer's instruction. It is further recommended to check the primers by traditional PCR before using them with ddPCR.

a. Prepare the probe Super Mix (Table 1). This mix is for one reaction only (22 μL /reaction) and will need to be multiplied for the number of reactions needed:

Note: Here we used specific primers targeting the gp23 gene on the T4 phage. Other primers following the manufacturer requirements can be designed to detect any microorganism desired.

b. Add the 22 μL per reaction in each well of the ddPCR deep well 96-well microplate (see Bio-Rad instruction), set up the eight reactions on the far-right column of the microplate.
c. Transfer 20 μL per reaction to the middle row of the droplet cartridge (Figure 5Bi).

Note: Take note of the sample identification and localisation in the plate to avoid mixing the samples.

Note: Avoid creating bubbles during pipetting of the samples and during the addition of oil as these may prevent the generation of droplets by blocking the micro-channels (Figure 5Bii).

d. Add 70 μL of the droplet generation oil to the cartridge row labeled 'oil' (Figure 5Biii). Close the cartridge with the rubber gasket.
e. Set the cartridge in the droplet generator, check if the lights are green, if yes, close the machine and let it run (Figure 5Biii). Once the droplet generation is complete, remove the cartridge from the machine and discard the gasket.
f. With a 50 μL pipette and Rainin tips collect 40 μL of droplets from each well (last row of the cartridge) and place the droplets in the far-left column of the 96 wells microplate that was used at the start (FigureS 5Biv–5Bvi).

⚠ CRITICAL: The droplets are very fragile and can break easily, it is recommended to pipette slowly and steadily to transfer the droplets from the cartridge back to the 96 well plate without losing their integrity (around 5 secs per pipetting) (Figures 5Bv and 5Bvi).

Table 2. PCR cycles

PCR cycling conditions			
Steps	Temperature	Time	Cycles
Initial Denaturation	95°C	10 min	1
Denaturation	94°C	30 s	40
Annealing	60°C	1 min	
Extension	72°C	1 min	
Final extension	98°C	10 min	1
Hold	4°C	Forever	

Description of the PCR cycle for the ddPCR T4 gp23 primers.

Note: The Rainin tips have been tested for droplet quality and shown to create less droplet shearing than other tips. Different tips may be used, although there is a risk of droplet loss during the pipetting steps.

Note: The droplets are visible, and form a cloudy layer on top of the clear oil layer (Figure 5Bvi).

g. Seal the plate using an aluminum sheet and a plate sealer (Figure 5Bvii).

Note: Be careful to use only cold heating blocks (a PCR block that has not been recently used and is at RT) as you don't want to warm up your samples at this step, that way only the top of the microplate will be heated for the sealing and not the whole plate.

h. Once sealed, place the plate in the thermal cycler using the guidelines below for T4-gp23 amplification.

Note: This PCR cycle (Table 2) is specific for the T4-gp23 (113 pb amplicon) amplification but can be adapted for any set of primers that fits the manufacturer recommendations. This cycle can also be used for regular PCR as well as ddPCR.

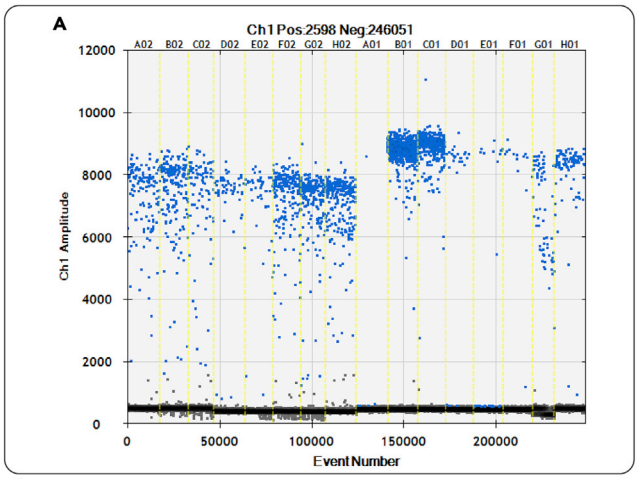
Pause point: After running the PCR cycles, the plate can be stored in the dark, at 4°C for a couple of days before analysis with the droplet reader as the droplets are stable at 4°C.

i. Following the PCR amplification, place the plate in the droplet reader (Figure 5Bviii). Follow the instructions of the QuantaLife software from Bio-rad. Once the run has finished, discard the plate and save the data.

Note: Make sure the plate is placed flat in the droplet reader as the needle used to read the droplets will pierce the foil and aspirate the samples, if the plate is tilted, the needle will bend and break.

Note: The plate should be empty at the end of the analysis. If the plate is not empty, it means the droplet reader didn't correctly read the wells that are not empty and the experiment should be repeated (see potential problem 5 in the troubleshooting section).

j. For each well selected check the automatic threshold and manually adjust if necessary, between the positive (top/blue) or negative (bottom/black) droplets (see ddPCR analysis section and Figure 6A).



B

	A	B	C	D	E	F	G	H	I
1	Sample	Target	Status	Concentratio	CopiesPer20uLWell	Positives	Negatives	AcceptedDroplets	Threshold
2	A549 30 sec	gp23	CHECK	No Call		72	11676	11748	1466
3	A549 18 hrs	gp23	OK	9.4	188	140	17445	17585	1564
4	Water	gp23	OK	0.34	6.8	5	17237	17242	578
5	Applied A549 30 sec	gp23	OK	57.7	1154	783	15567	16350	1587
6	Applied A549 18 hrs	gp23	OK	33	660	413	14502	14915	1298
7	wash A549 30 sec	gp23	OK	2	40	26	15304	15330	574
8	wash A549 18 hrs	gp23	OK	2.4	48	32	15908	15940	546

Figure 6. ddPCR analysis

(A) ddPCR droplet visualization. Example of visualization of positives and negatives droplets in blue and black respectively, with an automatic threshold from the QuantaLife software.

(B) ddPCR droplet analysis. Example of ddPCR analysis with a "NO CALL" sample.

k. With the excel spreadsheet given at the end, you can calculate your initial sample concentration depending on the dilutions used for the experiment (see ddPCR analysis section and Figure 6B).

Note: It is commonly seen that the control sample made with ultra-pure water instead of lysate won't be entirely negative and will contain some false positive. To overcome this issue, the false positives droplets are subtracted from all the other samples as a blank sample would (see ddPCR analysis section).

EXPECTED OUTCOMES

These assays allow for a broader understanding of the interactions between phages and eukaryotic cells. Adapting these methods to a range of different phages, cell types and experimental techniques can help elucidate the diverse ways in which phages may directly interact with the human cell layers and tissues. These methods combine cell biology, microbiology, and molecular assays with classical phage biology, allowing us to quantify and visualize phage-eukaryotic cell interactions. This work provides a standardized methodology to investigate, characterize, and better understand these interactions. These approaches are amenable to more advanced work, including cellular biology, phage therapy and gene and drug delivery.

The microscopy experiment allows for direct visualization of phage-cell interactions. In Bichet et al., 2021, it was found phage uptake was dependent on both the cell type used and phage morphology, with both macropinocytosis rates for each cell type and phage size and morphology influencing the uptake of phages. The quantification of intracellular phages following incubation with the cells provides opportunities to probe how phages and cells interact. In the Bichet et al., 2021 paper, it was found that phages were rapidly adhering to the cell layer and being inactivated in less than 30 s. However, after 18 h of incubation with phages we found an accumulation of active phages inside

the cells. Further work is required to understand the diverse interactions between internalized phages and their ultimate fate within the mammalian cell.

QUANTIFICATION AND STATISTICAL ANALYSIS

Microscopy analysis

For the microscopy analysis, the total number of cells and the number of cells with intracellular SYBR-stained phages were manually counted to obtain a percentage of cells with intracellular phages, as described in Bichet et al., 2021. We used CellProfiler program to quantify green fluorescence signal in cells and assess the phage uptake. The program was adapted to automatically detect nuclei using the Hoechst channel and remove any SYBR fluorescence coming from the nuclei area. You can find further details of the analysis in Bichet et al., 2021 (see also problem 2 in the troubleshooting section).

ddPCR analysis

Using the QuantaLife software from Biorad we calculated DNA copies per mL for each of our samples (Bichet et al., 2021). Figure 6A is an example visualization of the positive droplets in blue and negative in black. The software will automatically calculate the threshold between positive and negative droplets, but this can be manually adjusted if needed. When no threshold between positive and negative droplets is found, the sample is call "NO CALL" (Figure 6B). Two reasons can explain why no threshold is found, either there is too many DNA and only positive droplets or there is not enough DNA and only negative droplets are detected. In both of these options the ratio between positive and negative droplets cannot be achieve and the DNA concentration can't be calculated. The best range to detect phage DNA using ddPCR is between 10^3 to 10^5 phages/mL. These samples cannot be used for the downstream analysis. Subtract the water sample (blank) to the other samples. The concentration is calculated either per 20 μL or per μL, with further dilutions being taken into account when calculating the final concentration per mL (Figure 6B).

LIMITATIONS

The techniques described in this article are designed to provide a broad array of methods that can be used to study phages-eukaryotic interactions. However, while the techniques have been adapted and tested for the cells and phages reported here, the methods have not been broadly tested for other combinations of phages and cell types. As such, it is necessary to apply critical thinking when using these techniques to be able to adapt each method to best fit the cellular model being studied (e.g., the incubation time, the media used and the concentrations of phages and cells tested).

A major limitation for these assays is the requirement of high titer and purified phages. Obtaining pure and high titer phage lysates is essential for repeatable and coherent results when investigating phage-eukaryote interactions. Some of the interactions between cells and phages are minimal and can be challenging to detect. This is especially evident when using confocal microscopy, where the use of the HyD detector is recommended as it can detect low fluorescence signals, like those emitted from phages, with higher precision than a regular confocal detector.

For molecular assays, it is essential to design primers that can efficiently and specifically detect the phage DNA being used, hence a complete genome of the phage is required. This could be a limitation when using novel or uncharacterized phages that lack whole-genome sequence data.

As described in Bichet et al., 2021, cell lines do not uptake phages at the same rate. Depending on the cells of interest, the analysis of intracellular phages could be limited or absent. It was further demonstrated that phage size influences cellular uptake. If the phages of interest are larger than 200 nm, it may slow the uptake rate and reduce the number of detectable intracellular phages.

Transwell assays are highly dependent on cell confluency and the ability to form tight junctions. Some cells are not able to form tight junctions, leading non-confluent cell layers that leak. If the cell layer is leaking then the transcytosis of phages cannot be accurately assessed as phages crossing the cell layer will largely originate from the leaks between the cells and not from transcytosis through the cells.

In the ddPCR protocol described above (section 13), the limit of detection is dependent on the volume of the sample tested. Only 2 μL of the sample are tested with the ddPCR assay. If the concentration of phages is lower than 1 phage per 2 μL (~5×10^2 phages/mL), it may not be possible to accurately detect phages via this assay. Following the manufacturer instructions, the protocol can be adapted to test up to 10 μL of sample per reaction.

TROUBLESHOOTING

Problem 1

The transwells are not forming a tight, confluent layer of cells and the control dye EBD is leaking to the opposite chamber (refers to the "transcytosis of phages using transwells" section, step 3).

Potential solution

Transwells are suitable when used with cell lines forming tight junction, such as the MDCK-I cell line (TEER value when MDCK-I cells reach full confluency $\geq$ 1000 $\Omega \times cm^2$). TEER values indicate the integrity and permeability of the cell monolayer before they are evaluated. The tighter the cell layer, the higher the TEER values will be. For the MDCK-NBL2 cell line, the TEER value is much lower than the parental MDCK-I cell line (MDCK-NBL2 TEER at confluency $\simeq$ 150–200 $\Omega \times cm^2$) as the tight junctions between cells are not as strong.

The best way to reach confluency quickly is to make sure the cells are evenly distributed across the surface of the transwell and not clustered in the middle of each well. If cells are not homogenously spread across the well, cell confluency will not be reached and cells will begin growing on top of each other. To ensure homogeneity, it is best to gently rotate the plate horizontally following an infinite shape (∞), a few minutes after seeding the cells.

An additional reason for leakage of these transwells could be the improper manipulation of the wells, such as the touching of the end of the pipette tip to the membrane leading to a disruption to the cell layer, creating leaks.

Finally, it is essential to wait for the cell layer to reach confluency before starting the experiment. If sufficient TEER is not reached, then the layer won't be fully confluent and this can lead to leakages.

Problem 2

The SYBR-Gold dye from the SYBR-stained-phages is also staining the DNA inside the nucleus of cells during the microscopy acquisition (refers to the "visualization of phages and cells under the microscope" section, step 6).

Potential solution

One of the solutions for this problem is to increase the number of washes with the Amicon Ultra-4 Centrifugal Filter Units 100 KDa after the staining of the phages with SYBR-Gold. More washes will allow excess free dye to be removed from the lysate and be replaced by the buffer. Additionally, the more the phages are concentrated, the more washes will be necessary to get rid of all the extra dye. The SYBR stained phages should not leak into the nucleus if the phages are thoroughly washed. Unfortunately, this is only apparent following microscopy acquisition.

It is not always possible to predict if the samples are properly clean or not. To be able to still use the images acquired with leakage of the SYBR stain to the nucleus, we used the CellProfiler

(McQuin et al., 2018) program to differentiate the signal coming from phages and the one coming from the nucleus of the cells (Bichet et al., 2021). With this program, it is possible to subtract the nucleus fluorescence from the actual phage fluorescence and obtain an actual quantification of the fluorescence coming from the phages.

Problem 3

Cells are dying during the microscopy acquisition (refers to the "visualization of phages and cells under the microscope" section, step 7).

Potential solution 3

Under the microscope, cell death can occur for multiple reasons. This could be due to the FBS starvation steps during image acquisition. If cells are starved for too long and prone to stress from laser excitation or buffer changes, this can result in cell death. A simple solution to this issue is to keep a low amount of FBS in the media at all times. In Bichet et al., 2021, 1% FBS was used in the microscopy media to ensure the wellbeing of the cells during the real-time image acquisition. Another reason for cell death is laser power and acquisition time. If cells are too sensitive to the laser power, try increasing the time between image acquisition or decrease the laser power. This will allow cells additional time to recover between each image and the experiment can last longer.

Problem 4

Not enough proteins to run a whole cell analysis on protein content (refers to "intracellular phages preparation for whole cell analysis and phage quantification" section, step 11).

Potential solution 4

To run a whole cell analysis, it is usually recommended to have a lot of cells for sample collection. For the protein microarray, it is typically recommended to collect a minimum of 300 μg of protein per sample. After running a commercial Bradford assay to measure the protein concentration, if the protein concentration is lower than the recommended minimum amount, we recommend to repeat the experiment with a larger volume. For this type of assay a T25 or T75 flasks for each condition should be sufficient. We also suggest to have the cells confluent to increase the final quantity of protein collected. Additionally, we recommend preparing extra samples for downstream controls like Western-Blot or Transcriptomics.

Problem 5

The wells are not emptied after the ddPCR droplet reading (refers to the "ddPCR setup to quantify total DNA copies (five hours)" section, step 13).

Potential solution 5

When wells are not emptied after the droplet reading run, this can be due to several things. The first possibility is that the waste bottle inside the machine is full preventing the reading of the samples. The second possibility is that the plate is not properly flat in the machine preventing the needle from reaching the sample at the bottom of the deep well plate. Finally, the third possibility is that the plate template is not updated and does not correspond to the actual sample location in the plate. If this problem occurs, depending on the reason, we suggest to run the samples a second time, making sure everything is set properly or we suggest to repeat the experiment.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Jeremy J. Barr (jeremy.barr@monash.edu).

Materials availability

This study did not generate new unique reagents.

Data and code availability

This study did not generate any unique datasets.

Codes generated in this study can be found at https://doi.org/10.1016/j.isci.2021.102287.

ACKNOWLEDGMENTS

This work, led by M.C.B., was supported by the Monash Graduate Scholarship (MGS), including the efforts of J.J.B., funded by the Australian Research Council DECRA Fellowship (DE170100525), National Health and Medical Research Council (NHMRC: 1156588), and the Perpetual Trustees Australia award (2018HIG00007). We thank the following labs for kindly providing cell lines: Hudson Institute of Medical Research and the Oncogenic Signalling Lab for providing the A549 cell line. We thank the following facilities for kindly providing equipment and guidance: Monash Micro Imaging Facility for help with microscopy acquisition and Department of Biochemistry & Molecular Biology (Monash Biomedicine Discovery Institute) Imaging Facility for providing access to ddPCR equipment.

AUTHOR CONTRIBUTIONS

Conceptualization, M.C.B. and J.J.B.; methodology, M.C.B.; resources, J.J.B.; writing – original draft preparation, all authors; writing – review and editing, all authors; supervision and funding acquisition, J.J.B.

DECLARATION OF INTERESTS

The authors declare no competing interests.

REFERENCES

Bichet, M.C., Chin, W.H., Richards, W., Lin, Y.W., Avellaneda-Franco, L., Hernandez, C.A., Oddo, A., Chernyavskiy, O., Hilsenstein, V., Neild, A., et al. (2021). Bacteriophage uptake by mammalian cell layers represents a potential sink that may impact phage therapy. iScience *24*, 102287.

Bonilla, N., Rojas, M.I., Netto Flores Cruz, G., Hung, S.H., Rohwer, F., and Barr, J.J. (2016). Phage on tap–a quick and efficient protocol for the preparation of bacteriophage laboratory stocks. PeerJ *4*, e2261.

McQuin, C., Goodman, A., Chernyshev, V., Kamentsky, L., Cimini, B.A., Karhohs, K.W., Doan, M., Ding, L., Rafelski, S.M., Thirstrup, D., et al. (2018). CellProfiler 3.0: Next-generation image processing for biology. PLoS Biol. *16*, e2005970.

Nguyen, S., Baker, K., Padman, B.S., Patwa, R., Dunstan, R.A., Weston, T.A., Schlosser, K., Bailey, B., Lithgow, T., Lazarou, M., et al. (2017). Bacteriophage transcytosis provides a mechanism to cross epithelial cell layers. mBio *8*, e01874–17.

Otero, J., García-Rodríguez, A., Cano-Sarabia, M., Maspoch, D., Marcos, R., Cortés, P., and Llagostera, M. (2019). Biodistribution of liposome-encapsulated bacteriophages and their transcytosis during oral phage therapy. Front. Microbiol. *10*, 689.

Schindelin, J., Arganda-Carreras, I., Frise, E., Kaynig, V., Longair, M., Pietzsch, T., Preibisch, S., Rueden, C., Saalfeld, S., Schmid, B., et al. (2012). Fiji: an open-source platform for biological-image analysis. Nat. Methods *9*, 676–682.

Tugizov, S.M., Herrera, R., and Palefsky, J.M. (2013). Epstein-barr virus transcytosis through polarized oral epithelial cells. J. Virol. *87*, 8179–8194.