

Bichet et al. 2020

1 **Bacteriophage uptake by Eukaryotic cell layers represents a major sink for**
2 **phages during therapy**

3

4 **Authors**

5 Marion C. Bichet¹, Wai Hoe Chin¹, William Richards¹, Yu-Wei Lin², Laura Avellaneda-
6 Franco¹, Catherine A. Hernandez³, Arianna Oddo⁴, Oleksandr Chernyavskiy⁵, Volker
7 Hilsenstein⁵, Adrian Neild⁶, Jian Li², Nicolas Hans Voelcker⁴, Ruzeen Patwa¹ & Jeremy J.
8 Barr^{1*}

9 ¹ School of Biological Sciences, Monash University, Clayton Campus, VIC, 3800, Australia

10 ² Biomedicine Discovery Institute and Department of Microbiology, Monash University, Clayton, VIC,
11 Australia

12 ³ Department of Integrative Biology, University of California, Berkeley, Berkeley, CA, USA

13 ⁴ Faculty of Pharmacy and Pharmaceutical Sciences, Monash University, Parkville Campus, VIC, 3800,
14 Australia

15 ⁵ Monash Micro Imaging, Monash University, Clayton Campus, VIC, 3800, Australia

16 ⁶ Department of Mechanical and Aerospace Engineering, Monash University, Clayton Campus, VIC,
17 3800, Australia

18

19 ***Corresponding author**

20 Jeremy J. Barr

21 25 Rainforest Walk, School of Biological Sciences, Monash University, Clayton Campus, VIC,
22 3800, Australia

23 jeremy.barr@monash.edu

Bichet et al. 2020

24 **Abstract**

25 For over 100 years, bacteriophages have been known as viruses that infect bacteria. Yet
26 it is becoming increasingly apparent that bacteriophages, or phages for short, have tropisms
27 outside their bacterial hosts. During phage therapy, high doses of phages are directly
28 administered and disseminated throughout the body, facilitating broad interactions with
29 eukaryotic cells. Using live cell imaging across a range of cell lines we demonstrate that cell
30 type plays a major role in phage internalisation and that smaller phages (< 100 nm) are
31 internalised at higher rates. Uptake rates were validated under physiological shear stress
32 conditions using a microfluidic device that mimics the shear stress to which endothelial cells
33 are exposed to in the human body. Phages were found to rapidly adhere to eukaryotic cell
34 layers, with adherent phages being subsequently internalised by macropinocytosis and
35 functional phages accumulating and stably persisting intracellularly. Finally, we incorporate
36 these results into an established pharmacokinetic model demonstrating the potential impact of
37 phage accumulation by these cell layers, which represents a major sink for circulating phages
38 in the body. Understanding these interactions will have important implications on innate
39 immune responses, phage pharmacokinetics, and the efficacy of phage therapy.

40

41 **Introduction**

42 Phages, short for bacteriophages, are viruses that infect bacteria and are the most abundant life
43 form on the planet (1–3). Phages are found ubiquitously in the environment and are a major
44 contributor to global genetic diversity (4–6). Our bodies harbour a large number of phages,
45 and, together with their bacterial hosts, they constitute a key component of our gut microbiome
46 (7). The gut carries the largest aggregation of phages in the body, with an estimated 2×10^{12}
47 phages present in the average human colon (4,8,9). These phages are constantly interacting
48 with gut bacteria, as well as the epithelial cell layers of the gut (10). Phages are detected in the
49 circulatory systems of the body, suggesting they are capable of translocating from the gut and
50 penetrating throughout the body (8,11). Once past the gut barrier phages are able to penetrate
51 cell layers and major organs of the body; being found in classically sterile regions such as the
52 blood, serum, organs and even the brain (8,10–19). Numerous mechanisms pertaining to the
53 transport of phages across epithelial barriers have been proposed (11,16), including the ‘leaky
54 gut’ where phages bypass cell barriers at sites of damage and inflammation (20,21), and
55 receptor-mediated endocytosis (22,23). Recently, a non-specific mechanism for phage uptake
56 and transport across epithelial cell layers was proposed by Nguyen and colleagues, whereby
57 epithelial cells uptake phages via macropinocytosis and preferentially transcytose phages from
58 the apical surface toward the basolateral side of the cell (8,24). Macropinocytosis is a broad
59 mechanism describing the enclosure of media within ruffles in cells’ membrane, prior to
60 internalising the media, and any phages it may contain, within the cells. Despite their
61 prevalence in the human body, phage’s capacity to interact with and influence eukaryotic cells
62 remains largely unknown. These interactions can have important implications during phage
63 therapy.

64

65 Phage therapy is a promising alternative to treat pathogenic bacterial infections. In Eastern
66 Europe, phage therapy was widely used since its discovery in 1917 (1), whereas in Western
67 countries, phage therapy was largely abandoned in favour of antibiotics (25–28). However,
68 with the rise of antimicrobial resistance as one of the greatest threats to human health, phage
69 therapy is being re-established as a potential treatment option for difficult-to-treat, antibiotic
70 resistant, bacterial infections (25). In order for phage therapy to be effective, phages must first
71 be administered to the site of infection. Administration routes include, intravenous (IV) or
72 intraperitoneal (IP) to treat septicemia; orally to treat gastrointestinal infections; intranasal or
73 inhalation to treat respiratory infections; or topically for cutaneous infections (29,30). The

Bichet et al. 2020

74 administration route and bioavailability of phages needs to be carefully taken into account in
75 order to achieve favourable efficacy *in vivo*.

76

77 In contrast to conventional drugs, phages are unique therapeutic agents capable of self-
78 replicating and maintaining titres in the body (30–33). As such, there is a lack of knowledge
79 regarding phage pharmacokinetics and pharmacodynamics (31). Following administration, two
80 major pharmacokinetic factors important for the efficacy of phage therapy are accessibility and
81 clearance. First, natural barriers such as cell layers and mucus can decrease accessibility of
82 phages to sites of infection, thereby necessitating the administration of higher doses to achieve
83 a favourable therapeutic effect. Second, phage clearance has been reported to occur rapidly;
84 sometimes within just minutes to hours following parenteral administration in animal models
85 and patients (14,34–40). Phage clearance within the body is thought to be mediated by three
86 main components: 1) Phagocytic cells (41), 2) The Mononuclear Phagocytic System (MPS;
87 which was also previously called the reticuloendothelial system, or RES) which includes the
88 liver and spleen that filter out and remove phages from circulation (42), and 3) Phage
89 neutralizing antibodies, although it is still unclear how effective and rapidly produced these
90 anti-phage antibodies are (31,43). Due to these complications, it is difficult to predict how
91 phages will behave in the body when administered and ultimately whether phage therapy will
92 be successful.

93

94 One underexplored aspect of phage therapy is the non-specific interactions between phages
95 and the cell layers of the body. During therapy, large quantities of phage monocultures or
96 cocktails are administered to patients in order to maintain a killing titre to combat a bacterial
97 infection. Once within the body, these phages can have very short half-lives and are actively
98 removed or inactivated by the body (38–40,44). Following administration during phage
99 therapy, epithelial and endothelial cell layers are amongst the first and most abundant phage-
100 eukaryote interactions. Here, we present new insights into phage-mammalian cell adherence,
101 uptake, and trafficking, via *in vitro* tissue culture cell layers. Our results suggest that cell layers
102 of the human body represent a major and unaccounted sink for exogenously administered
103 phages. Put within the context of phage therapy, the interaction between eukaryotic cells and
104 exogenous phages have important implications for phage administration, dosing, and
105 pharmacokinetics.

106

107 **Results**

108 **The rate of phage uptake varies depending on the cell type.** To better understand phage-
109 eukaryote interactions, we used seven *in vitro* tissue culture cell lines that were selected to be
110 broadly representative of different tissues types within the body, and examined their
111 interactions with ultrapure high titre monocultures of T4 phage. Using real time live-cell
112 imaging on a confocal microscope with a sensitive hybrid detector (HyD), we visualised the
113 interaction and subsequent internalisation of phage particles within eukaryotic cells. Cells were
114 first grown on glass bottom slides for two days to generate an ~ 80% confluent cell layer,
115 followed by fluorescence staining of the nucleus and plasma membrane. T4 phages were
116 prepared using the Phage-on-Tap method (45), labelled using SYBR-Gold, subsequently
117 washed to remove residual stain, and then directly applied to cell layers observed using live
118 cell imaging.

119
120 Phages were visualised in real time being engulfed and trafficked through all seven of the
121 different cell lines over a two-hour period (Fig. 1a and Supplemental movies SM1-7). The cell
122 types tested include: epithelial cells - HeLa, A549 and HT29, from the cervix, lung and colon,
123 respectively; fibroblast cells - MDCK-I and BJ, from dog kidney and human skin, respectively;
124 the endothelial cell line - HUVEC from umbilical vein; and monocyte-induced macrophages -
125 THP-1 cells (Fig. 1b). The increase in green fluorescence over time corresponds to the uptake
126 and accumulation of fluorescently labelled T4 phages by the cells. We saw the first evidence
127 of phage accumulation within cells occurring around 30 minutes, with continued accumulation
128 over the following 90 minutes.

129
130 We observed large variation in the uptake of phages across the seven cell types investigated.
131 To quantify this, cells containing intracellular phages were manually counted and compared
132 with the total number of cells in the field of view (FOV) at the two-hour time point for each
133 replicate. Cells were then categorised as either high, intermediate, or low phage-uptake using
134 univariate clustering analysis (Fig. 1c and Supplemental Fig. 1) (46). A549 lung epithelial cells
135 showed the highest accumulation of phages, with a median of cells containing fluorescently-
136 labelled phages at the two-hour time point of 99% ($\pm 2\%$, mean \pm standard deviation [SD];
137 Field of View [FOV] $n = 8$; coefficient of variation [CV] = 2%). Next, HUVEC, MDCK-1,
138 and HeLa cells, representing endothelial, fibroblast and epithelial cell types all showed
139 intermediate levels of phage accumulation at the two hour time point, with medians of 44% (\pm

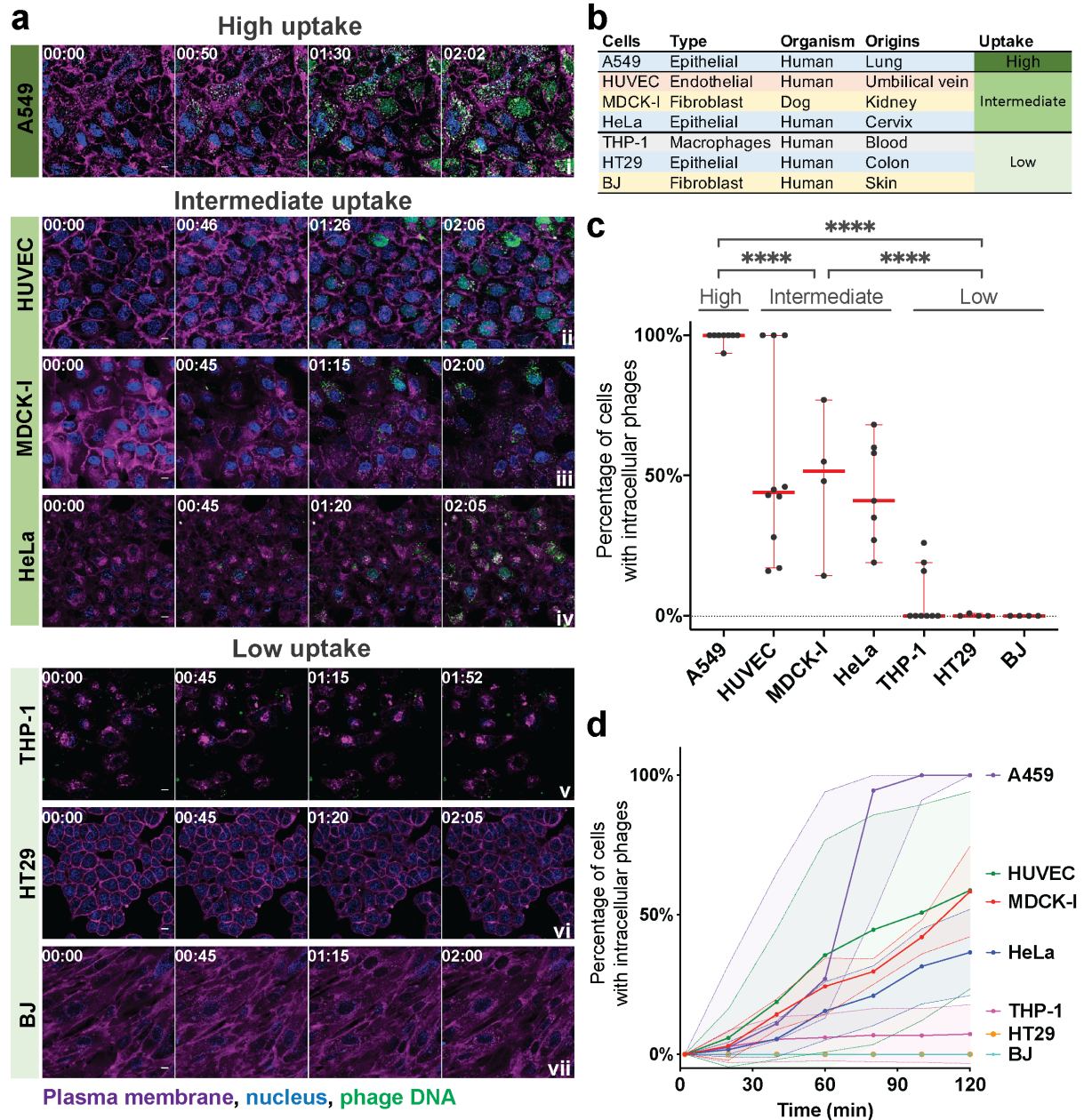
Bichet et al. 2020

140 34% SD; FOV $n = 10$; CV = 63%), 51% ($\pm 26\%$ SD; FOV $n = 4$; CV = 54%), and 41% ($\pm 18\%$
141 SD; FOV $n = 7$; CV = 42%) of phage-positive cells, respectively. Finally, THP-1, HT29, and
142 BJ cells, representing macrophages, epithelial and fibroblast all showed little to no
143 accumulation with medians of 0% ($\pm 11\%$ SD; FOV $n = 9$; CV = 155%), 0% ($\pm 0.5\%$ SD; FOV
144 $n = 4$; CV = 200%), and 0% ($\pm 0\%$ SD; FOV $n = 4$; CV = 0%) phage-positive cells at two
145 hours, respectively. We further quantified the rate at which cells internalised phages by
146 manually counting the number of cells per frame of interest containing fluorescently-labelled
147 phages for each of the field of view per cell lines (Fig. 1d). Most cells showed large variability
148 in the uptake rate over the two-hour period. For A549 cells, which had the highest accumulation
149 of phages, we saw large variation in the rate of uptake, with a median of 27% ($\pm 36\%$ SD; FOV
150 $n = 6$; CV = 83%) of cells that contained phages at one hour of incubation compared to 100%
151 ($\pm 0\%$ SD; FOV $n = 6$; CV = 0%) of cells at two hours. Comparatively, HUVECs, which were
152 classified as intermediate accumulation of phages showed extensive variability in their uptake
153 rates, with a median of 12% ($\pm 41\%$ SD, FOV $n = 8$; CV = 116%) and 46% ($\pm 35\%$ SD; FOV
154 $n = 8$; CV = 60%) of cells containing phages at one and two hours, respectively.

155
156 To confirm that phages were internalised and not simply attached to the cell surface, we created
157 a three-dimensional (3D) reconstruction using a Z stack to visualise the intracellular
158 localisation of phages. After the live cell imaging of MDCK-I cells incubated with
159 fluorescently-labelled T4 phages for one hour (Supplemental movie SM8), we acquired a high-
160 resolution Z stack image of one chosen field of view. We reconstituted the 3D volume of the
161 cell to visualise phage repartition in the cytoplasm (Fig. 2 top left corner and Supplemental
162 movie SM9). Finally, we looked at the localization of phages in 3D using the XY cross section
163 (Fig. 2). The 3D reconstruction of the cell confirmed that phages internalized by the cells
164 (visualised as green fluorescent particles) lie in the same focal plane as the nucleus (Fig. 2).
165 Phages were distributed throughout the cell cytoplasm and appeared to be localised within
166 membrane-bound vesicles surrounding the nucleus.

167

Bichet et al. 2020

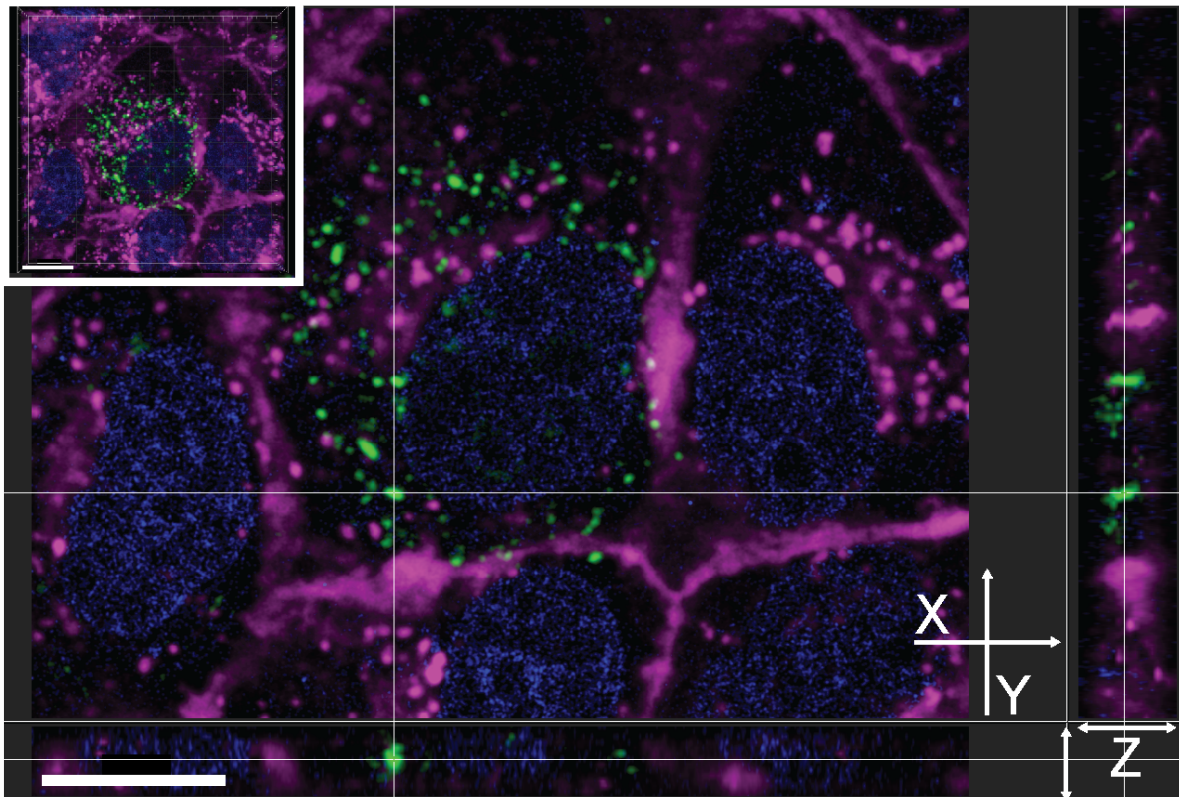


168
169

170 **Figure 1. Uptake and internalisation of phages varies across cell type.** (a) T4 phage was
 171 fluorescently labelled, applied to different cell lines and incubated for two hours on a glass
 172 bottom slide. Cells were stained with Hoechst 33342 nucleus stain (blue), CellMask plasma
 173 membrane stain (magenta) and T4 phages labelled with SYBR-Gold DNA-complexing stain
 174 (green). Using real-time microscopy, one image was acquired every two minutes. Scale bar:
 175 10 μ m; Timing: hours:minutes. (b) Table of cell lines used in this study, their cell type,
 176 organism, organ origins and category of uptake. Cells lines are ranked as high, intermediate,
 177 and low uptake. (c) Percentage of cells containing intracellular phages at the two-hour time
 178 point. Scatter plots show medians of percentage of cells with intracellular phages; error bars
 179 represent 95% confidence intervals; each dot represents one Field of View (FOV). Cells lines
 180 are ranked as high, intermediate and low uptake. P-values between the different groups
 181 calculated from a one-way ANOVA, shown as stars ($F(2, 43) = 71.23$; $P < 0.0001$: ****). (d)
 182 Percentage of cells containing phages represented across time. For each video the number of
 183 cells with and without intracellular phages in a FOV were manually counted every ten minutes
 184 (A549 $n = 6$; HUVEC $n = 8$; MDCK-I $n = 3$; HeLa $n = 6$; THP-1 $n = 8$; HT29 $n = 3$; BJ $n = 3$).

Bichet et al. 2020

185 Curve plots show medians of percentage of cells with intracellular phages; error bars represent
186 95% confidence intervals in the shaded area for each curve; each point represents one video
187 over time.
188



189 Plasma membrane, nucleus, phage DNA 190

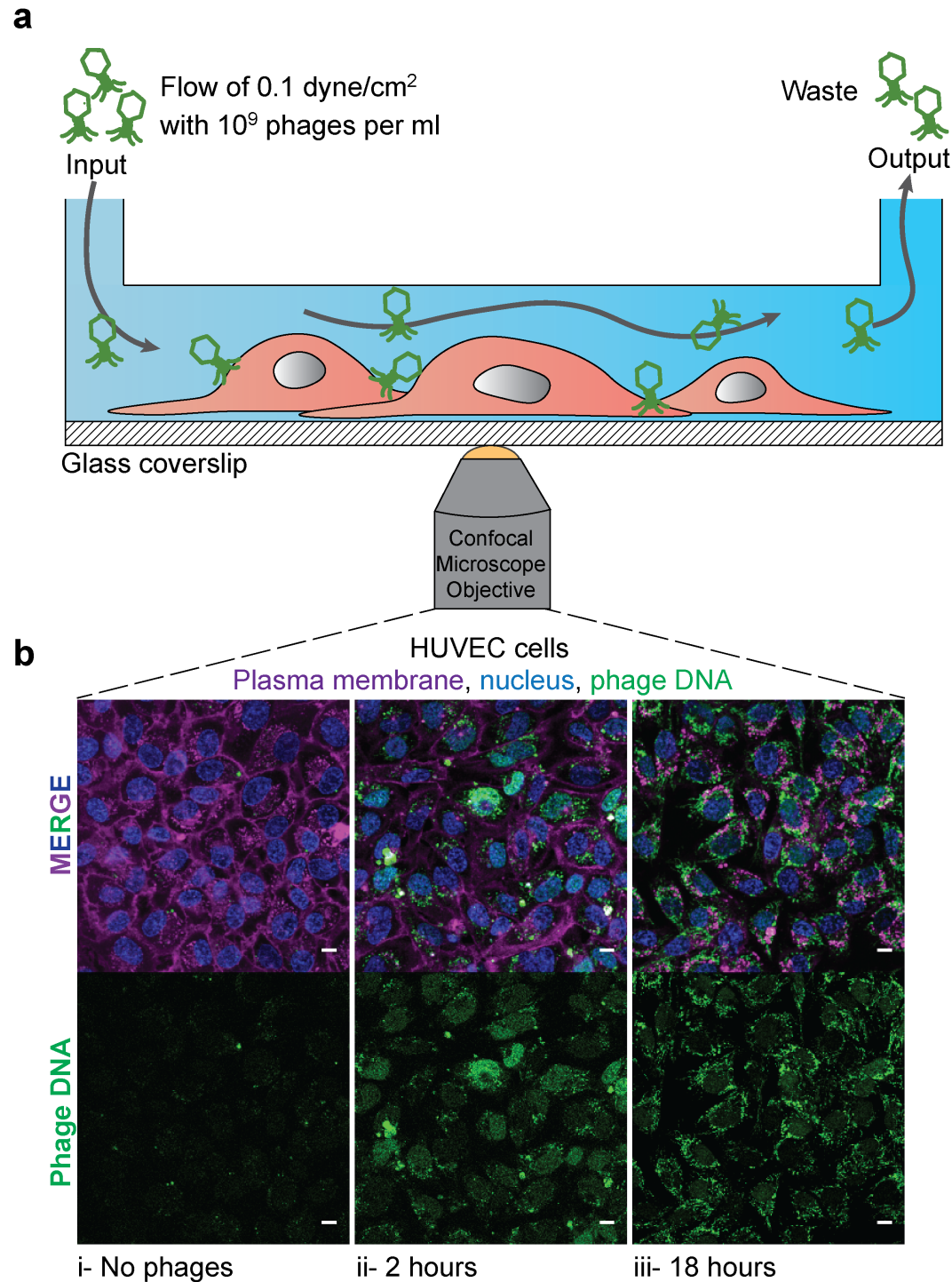
191 **Figure 2. 3D reconstruction of intracellular phages.** MDCK-I cells were incubated for an
192 hour with T4 phages on glass bottom slide before acquisition of a high-resolution Z stack to
193 visualise phage dispersion inside of cells. Cells were stained with Hoechst 33342 nucleus stain
194 (blue), CellMask plasma membrane stain (magenta) and T4 phages labelled with SYBR-Gold
195 DNA-complexing stain (green). Images were acquired in real-time on live cells. XY cross
196 section made using Imaris software with an embedded 3D projection of the cell in the top left
197 corner of the image. The cross in the centre of the image shows a cluster of internalised phages
198 with its repartition in the Z dimension (depth) of the image represented in the X and Y sides
199 views. Scale bar: 10 μm .
200

Bichet et al. 2020

201 **Phage uptake occurs at comparable rates under both static and flow conditions.** The
202 previous experiments were all conducted under static conditions, where phages were directly
203 applied to the cell culture media and phage-cell encounters driven purely by diffusion.
204 However, in the context of phage therapy, phages administered to the body are likely to
205 encounter dynamic environments and active fluid flow, such as in the circulatory and lymphatic
206 system. These dynamic conditions may lead to increased phage-cell encounter rates or altered
207 cellular uptake (47–52). We investigated whether phage uptake rates under static conditions
208 were comparable with uptake rates under fluid flow and shear conditions that mimic the
209 circulatory systems of the body (Fig. 3a). We chose HUVECs to use in our flow experiment
210 for two main reasons, the first one is that they are part of the intermediate uptake group
211 determined in our first figure and are not part of one of the two other extremes. Secondly,
212 HUVECs are endothelial cells and would be amongst the first type of cells to be in contact with
213 circulating phages in the human body. Using an in-house fabricated microfluidic device
214 mounted on a glass coverslip adapted for confocal microscopy (53,54), we seeded the device
215 with HUVECs and incubated it under static conditions for 12 hours to ensure sufficient cellular
216 attachment to the substrate. Cell layers were allowed to establish within the device under a low
217 flow rate of 0.66 $\mu\text{l}/\text{min}$ for one day, before increasing to a final flow rate of 8 $\mu\text{l}/\text{min}$ until
218 cells reached confluency. Physiological shear stress values observed in the human body ranges
219 from 0.1 dyne/cm^2 in the microcirculation, reaching higher rates of 50 dyne/cm^2 found in larger
220 circulatory vessels (55–59). Due to the volumes of media and quantity of phages applied to the
221 chip, we chose a flow rate of 8 $\mu\text{l}/\text{min}$, which is equivalent to a shear stress of 0.1 dyne/cm^2 in
222 our chip and was at the lower end of physiological circulatory range (Supplemental Table 1).
223 We perfused the chips with media containing 10^9 phages/ml, with phage uptake visualised as
224 previously described at two and 18 hours timepoints. Even though the volumes and quantity of
225 phages seen by the cell layer in the static (200 μl) and flow (960 μl) conditions are different,
226 we still observe similar rates of T4 phage uptake after two hours (Fig. 1a & 3b. ii and
227 Supplemental Fig. 3). After 18 hours incubation under shear stress, we observed a significant
228 increase in the fluorescence intensity compared with two hours incubation (unpaired t-test, P
229 < 0.001) (Supplemental Fig. 3).

230

Bichet et al. 2020



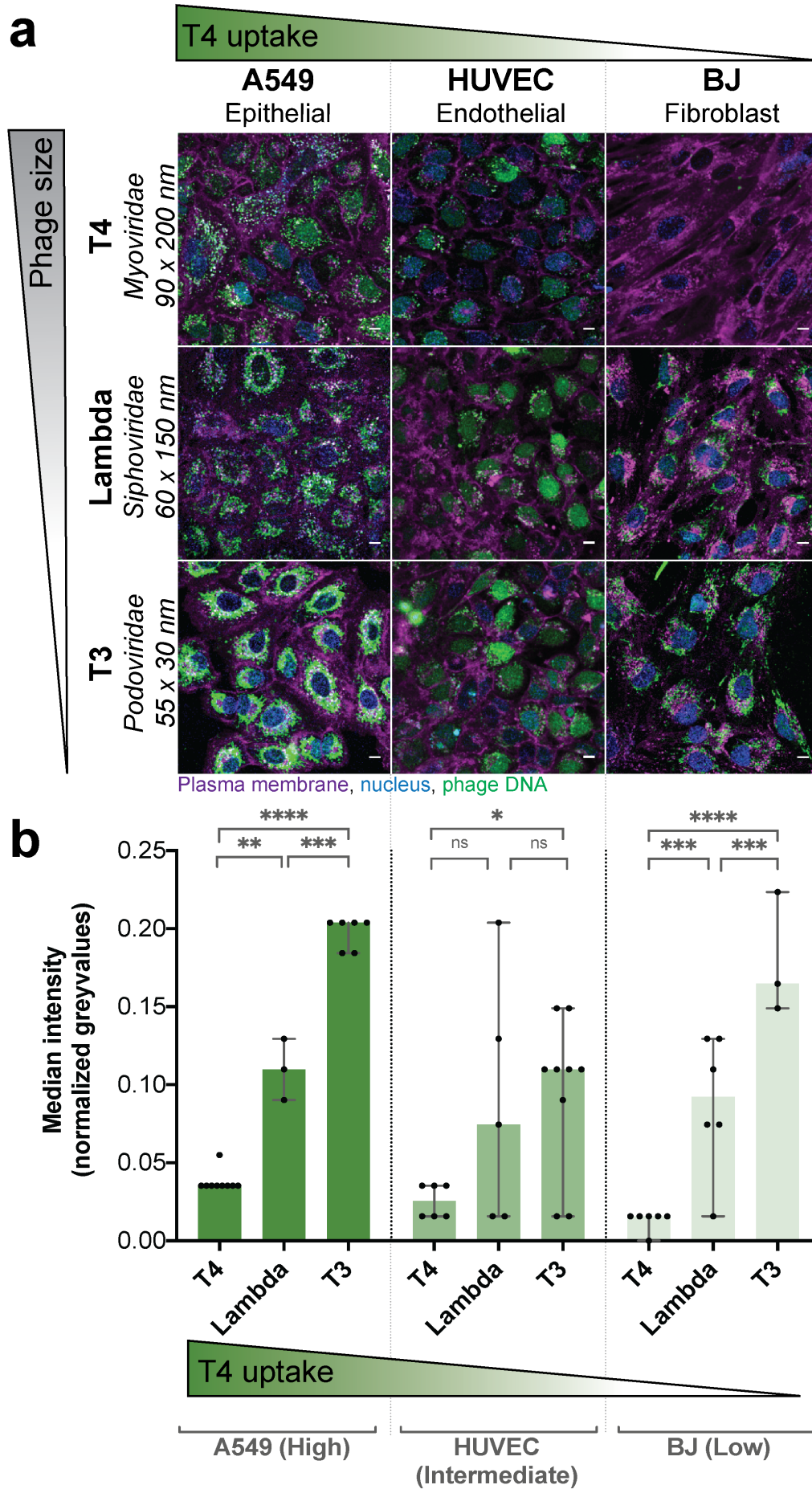
231
232

233 **Figure 3. Uptake of phages under fluid flow and shear stress.** T4 phage was applied to
234 HUVECs within a microfluidic channel of a microfluidic device under a shear stress of 0.1
235 dyne/cm² for two or 18 hours with images collected via real-time microscopy images. **(a)**
236 Schematic of the microchannel showing the flow from one port of the channel to the other side
237 of the channel where the waste was collected. **(b)** Cells were stained with nucleus stain (blue),
238 plasma membrane stain (magenta) and T4 phages labelled with DNA-complexing stain (green).
239 Control cells without phages i). Cells were incubated under a constant flow of phages at a rate
240 of 8 μ L/min equivalent to a shear stress of 0.1 dyne/cm² for either ii) two or iii) 18 hours. Scale
241 bar: 10 μ m.

Bichet et al. 2020

242 **Phage size affects intracellular uptake.** Next, we looked at the effect of phage size on the
243 rate of cellular uptake. We chose three *Escherichia coli* infecting phages; T4 phage from the
244 *Myoviridae* family, measuring 90 nm wide and 200 nm long with a contractile tail; Lambda
245 phage from the *Siphoviridae* family, measuring 60 nm wide and 150 nm long with a non-
246 contractile tail; and T3 phage from the *Podoviridae* family, measuring 55 nm wide and 30 nm
247 long with a small non-contractile tail. We tested these phages against three cell lines
248 representative of high, intermediate, and low rates of uptake (Fig. 1c); A549 cells with a high
249 rate of uptake, HUVECs with an intermediate rate, and BJ cells with a low rate. We incubated
250 phages with the cell layers for two hours, acquiring images every two minutes (Supplemental
251 movies SM1-2, 7, 10-15), with the final time point represented in Fig. 4a. The first row of
252 images shows clear differences in T4 phage uptake between the three cell lines (Fig. 4a).
253 However, when we applied the smaller sized Lambda and T3 phages to the three cells lines,
254 we saw a large increase in the uptake of both phages compared with T4. This was particularly
255 evident in the BJ cell line, which had effectively no T4 phage uptake over a two-hour period
256 but nonetheless, demonstrated increased uptake of the smaller sized Lambda and T3 phage.
257 We quantified phage uptake across phage size and cell type using a pipeline built with
258 CellProfiler software (60) (see methods) to analyse the median grayvalue intensity in the cell
259 region (median over all pixels in FOV marked as cells) as a proxy for fluorescence intensity of
260 phage (Figure 4). For T3 phage, the smallest phage of the three tested, we observed the highest
261 rate of uptake across the three cell lines (ANOVA one way, $F(2, 45) = 71,32$; $P < 0.0001$).
262 This was especially evident for the BJ cell line where the median intensity of the phage
263 fluorescence signal increased from a median of 0.01 normalised grey value with T4 phage (\pm
264 0.006 SD; FOV $n = 6$; CV = 49%) up to 0.09 with Lambda (± 0.04 SD; FOV $n = 6$; CV =
265 49%), and finally to 0.16 with T3 phage (± 0.04 SD; FOV $n = 3$; CV = 22%) (Fig. 4b). Based
266 on our microscopy results, we suggest that smaller sized phages broadly increase the rate of
267 cellular uptake, and that these effects were more pronounced in our intermediate and low
268 uptake cell lines.
269

Bichet et al. 2020



Bichet et al. 2020

271 **Figure 4. Cellular uptake of differing phage particle sizes.** Real-time microscopy images
272 showing differential uptake of phages based on particle size. One representative cell line from
273 each of the three high, intermediate, and low uptake groups were picked. **(a)** Images were
274 acquired in real-time. Cells were stained with Hoechst 33342 nucleus stain (blue), CellMask
275 plasma membrane stain (magenta) and T4 phages labelled with SYBR-Gold DNA-complexing
276 stain (green). Three cell lines used; A549, HUVEC and BJ, with the three phages; T4, Lambda,
277 and T3. Green gradient shows the qualitative increase in T4 phage uptake shown in Figure 1C.
278 Grey gradient shows qualitative decrease in sizes of phages T4 (90×200 nm), Lambda ($60 \times$
279 150 nm), and T3 (55×30 nm). Scale bar = $10 \mu\text{m}$. The image shown is the last image at two-
280 hour acquisition. **(b)** Fluorescence intensity of the “phage object” area in normalized grey
281 values quantified from the CellProfiler software of the phage channel fluorescence at the two-
282 hour time point (A549 - T4 $n = 9$; A549 - Lambda $n = 3$; A549 - T3 $n = 6$; HUVEC - T4 $n = 6$;
283 HUVEC - Lambda $n = 3$; HUVEC - T3 $n = 6$; BJ - T4 $n = 6$; BJ - Lambda $n = 6$; BJ - T3 $n =$
284 3). Scatter plots show medians over all pixels in FOV marked as cells; error bars represent 95%
285 confidence intervals; each point represents one FOV. P-values calculated from a one-way
286 ANOVA ($P < 0.0001$: ****; $P < 0.05$: *; ns: non-significant).
287

Bichet et al. 2020

288 **Phages rapidly adhere to eukaryotic cells, resulting in inactivation and uptake.** We
289 demonstrate that phages have different rates of cellular uptake depending on both cell type and
290 the size of phages. Yet whether these phages remained functional, or if they were inactivated
291 by the cellular uptake and trafficking pathways remains unclear. To answer this, we quantified
292 the number intracellular phages using two methods; classic plaque formation unit (PFU) assays
293 and Droplet Digital PCR (ddPCR). Briefly, PFUs allowed us to quantify the number of active
294 or functional phages present within the cells, while ddPCR quantified the absolute number of
295 phage DNA genome copies present in the sample. We evaluated the accuracy of the two
296 techniques with a dilution series of our initial phage sample from 10^9 to 10^2 phages per ml
297 using PFU and ddPCR (Supplemental Fig. 4). Subtracting active phages (PFU) from phage
298 DNA genome copies (ddPCR) enabled us to quantify the proportion of phages inactivated or
299 damaged during cellular uptake and trafficking, as these damaged phages are no longer able to
300 infect their bacterial host and therefore will only be detected by ddPCR.

301

302 In order to differentiate between intracellular phages and those adhered to the cell surface, we
303 incubated cells with T4 phages ($\sim 10^9$ per ml) for two different periods of time: 30 seconds,
304 which is too short for phage internalisation; and 18 hours, to maximise the number of
305 internalised phages. After incubation at each time point, cells were extensively washed to
306 remove non-adherent phages, lysed, and phages quantified by both PFU and ddPCR (Fig. 5a).
307 We compared the same representative cell types previously used for high, intermediate, and
308 low uptake cell lines (Fig. 5b). For all three cell lines, we saw between 90 and 2200 active
309 phages (PFU) per ml adhered to the cells within 30 second treatment, with a median of 625
310 phages/ml (± 420 SD; $n = 6$; CV = 60.1%), 325 phages/ml (± 300 SD; $n = 6$; CV = 76.6%) and
311 250 phages/ml (± 850 SD; $n = 6$; CV = 114%) for A549, HUVEC and BJ cells, respectively,
312 suggesting a small, yet persistent number of phages rapidly adhere to the cell layers. After 18
313 hours of incubation, we saw a large increase in the number of functional phages associated
314 with the cells, with between 1.1×10^4 to 3.1×10^6 phages per ml accumulating within the three
315 cell lines. Looking across the different cell lines, we see the highest accumulation of phages in
316 the intermediate uptake cells, with a median of 5×10^5 phages/ml ($\pm 6.4 \times 10^5$ SD; $n = 9$; CV
317 = 85.7%) followed by the high and low uptake with medians respectively of 1.2×10^5
318 phages/ml ($\pm 8.4 \times 10^4$ SD; $n = 9$; CV = 65.7%) and 5×10^4 phages/ml ($\pm 1.4 \times 10^6$ SD; $n = 9$;
319 CV = 144.3%), although there were no significant difference between the three cell lines ($P >$
320 0.1, ANOVA one way). We note that the BJ cell line, which showed the lowest rate of uptake

Bichet et al. 2020

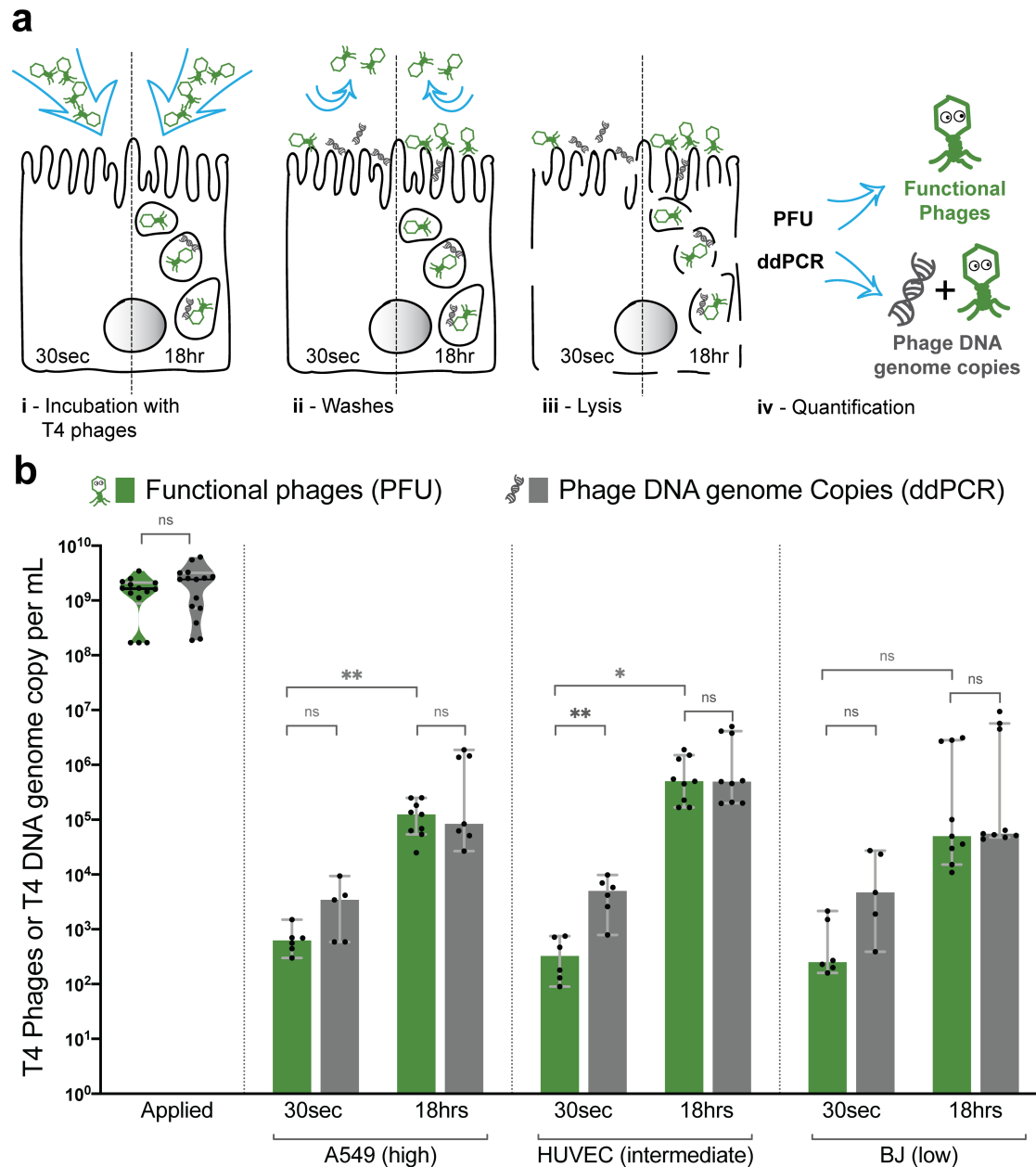
321 observed via microscopy (Figure 1), still accumulated active phages over prolonged periods of
322 time with non-significant differences of active phages at 18 hours observed with two other cell
323 lines (unpaired t-test, two tailed, $P > 0.08$ with A549 and $P > 0.6$ with HUVEC).

324

325 Surprisingly, when looking at ddPCR results, we see an increase in phage DNA associated with
326 the 30 second treatment in all cell lines, with between 3.9×10^2 and 2.7×10^4 DNA genome
327 copies per ml persisting. When quantifying the inactivated phages at 30 second treatment,
328 which was calculated as the difference in DNA genome copies and PFU, we observe between
329 7×10^1 and 2.7×10^4 inactivated phages per ml. These results suggest that; 1) phages rapidly
330 adhere to eukaryotic cell layers, with a portion of these phages being inactivated, 2) longer
331 incubation time allows for adhered phages to be internalised and accumulate inside of the cells,
332 and 3) that the majority of internalised phage particles remain active and stably persist within
333 the cells for up to 18 hours.

334

Bichet et al. 2020

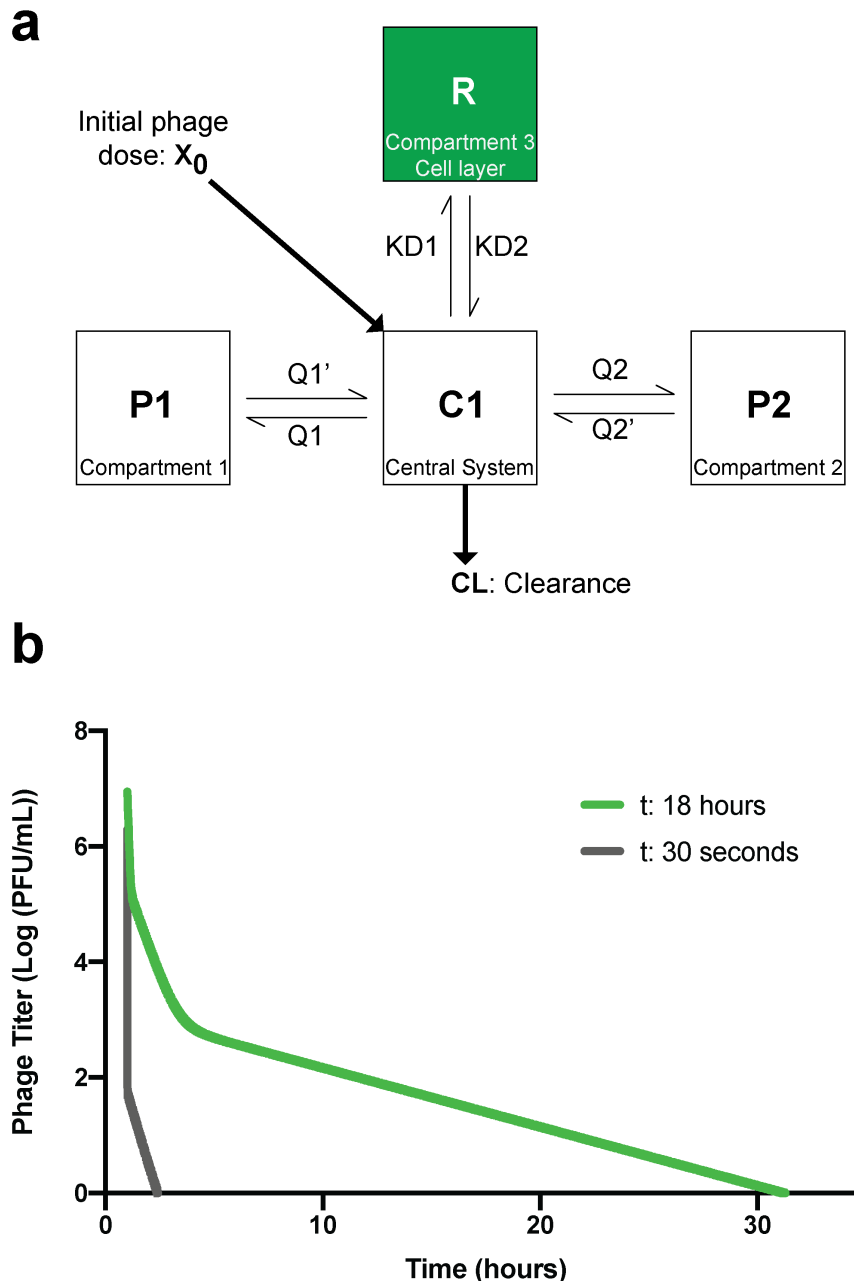


335
336

337 **Figure 5. Quantifying adherence and internalisation of phages and their activity.** Applied
338 T4 phages were quantified using both traditional plaque assays (PFUs) and Digital Droplet
339 PCR (ddPCR), across three different cell-lines: A549, HUVEC and BJ, representative of the
340 high, intermediate, or low phage uptake. Phages were applied to cells for either 30 seconds as
341 an adherence control, or for 18 hours to saturate phage uptake. (a) Schematic representation of
342 the experiment showing steps taken to obtain the samples with two incubation times: 30
343 seconds or 18 hours. Active or functional phages are represented in green and phage DNA or
344 inactive phages are represented in grey; (i) Incubation of phages with cells, (ii) Extensive
345 washing of cells to remove non-adherent phages, (iii) Chemical and mechanical cell lysis, and
346 (iv) Quantification of the active and total phages via PFU and ddPCR. (b) Active T4 phages in
347 green quantified by PFU and in grey the total number of T4 phage DNA genome copies per
348 sample quantified by ddPCR, including both active and inactive phages. Scatter plots show
349 medians of phages or DNA genome copies per mL; error bars represent 95% confidence
350 intervals; each point represents one sample. P-values calculated from an unpaired t-test
351 between each pair (P < 0.001: ***; P < 0.01: **; P < 0.05: *; ns: non-significant).

Bichet et al. 2020

352 **Phage inactivation and internalisation influences pharmacokinetics.** A key underexplored
353 aspect of phage pharmacokinetics (PK) are the non-specific interactions between phages and
354 the epithelial and endothelial cell layers of the body. To address this, we integrated our
355 experimental data (Fig. 5) with an established PK model for phage administration in a rats (38).
356 The model was established from a single-dose PK study performed in healthy rats, following
357 an intravenous bolus of phages. The phage disposition in blood was well characterised using a
358 standard three-compartment PK model (38). In order to evaluate the impact of phage
359 inactivation and internalisation by eukaryotic cells on phage distribution, a fourth-compartment
360 was added to the existing model, representing the epi- and endothelial cell layers (Fig. 6a).
361 Deterministic simulations were subsequently performed with the previously estimated PK
362 parameters (38) and the first-order inactivation constant estimated from our *in vitro* data
363 (ddPCR). Inactivation rate constant was calculated using both the 30 seconds- and 18 hours-
364 derived constants, as a representative of both the rapid and prolonged phage accumulation by
365 cell layers (Fig. 6b, supplemental data SD2, SD3 and table 5). Deterministic simulations were
366 performed at an IV bolus dose of 10^9 phages. With the inactivation rate constant calculated
367 using 30 second data, complete removal of phages was noted as short as three hours. This is
368 consistent with the 30 second *in vitro* data, in which rapid inactivation was observed.
369 Comparatively, using the inactivation rate constant calculated using the 18 hours data, an initial
370 rapid decay of circulating phages, followed by a longer tail and phage persistence in the blood
371 was observed for up to 32 hours. These two models represent two extremes of rapid and
372 prolonged phage accumulation by cell layers, highlighting the potential impact of these cellular
373 mechanisms on phage disposition. Further studies characterising both the affinity of phage–
374 eukaryotic interactions and their influence on PK are needed for better clinical translation.
375



376
377

378 **Figure 6. Pharmacokinetic (PK) model of inactivation and internalisation of phages by**
 379 **eukaryotic cell layers.** Extrapolation of *in vitro* results were fit to a rat model of phage decay
 380 at an initial dose of 10^9 phages. **(a)** Schematic of the standard 3-compartments model with an
 381 additional inactivation compartment. X_0 , the initial dose of phages and CL phage total body
 382 clearance. C1, the central compartment representing the central system (*i.e.*, the blood). P1 and
 383 P2 compartments represent various organs or tissues participating in phage decay. R, the new
 384 compartment representing *in vitro* cell layers as a major sink of phages. Q1 and Q2 are
 385 intercompartmental clearance one and two, respectively. KD1 is the first order inactivation
 386 constant and KD2 is the first order reactivation constant (assumed to be zero in our model). **(b)**
 387 Graph representing the decay of phages per central volume of distribution over time calculated
 388 with *in vitro* data using an R model with an integration step of 0.001. First order for the 30
 389 seconds graph is of 1511 1/h/rat and for the 18 hours the first order is of 0.44 1/h/rat calculated
 390 from the geometric mean of ddPCR data (calculation in supplemental table 5).

391 **Discussion**

392 Phage therapy is one of the more promising solutions in the fight against antibiotic resistance
393 (25). However, the effective use of phages as a treatment against multi-drug resistant bacterial
394 pathogens remains a major challenge to successfully and reproducibly implement. Our
395 understanding of the interactions between bacteriophages and eukaryotic cells may have
396 important implications and impacts for the success of phage therapy. In this article, we
397 investigate the interactions between phages and *in vitro* tissue culture cell layers. We
398 demonstrate that cell type plays a major role in the uptake process of phages, with individual
399 cells taking up phages at different rates, even amongst the same cell types (e.g., epithelial,
400 endothelial, etc.). Uptake rates were validated under shear stress conditions using a
401 microfluidic device that mimics the shear stress to which endothelial cells are exposed to *in*
402 *vivo* (61,62), and shown to be comparable with static conditions. Using live cell imaging we
403 show that phages accumulate within cells over time and that smaller phages are internalised at
404 higher rates than larger phages. Phages were found to rapidly adhere to eukaryotic cells, with
405 adherent phages being internalised by macropinocytosis over the following 18 hours of
406 incubation, with functional phages accumulating and stably persisting within the cells. Finally,
407 we incorporate our results into an established pharmacokinetic (PK) model (38), demonstrating
408 the potential impact of phage accumulation by epithelial and endothelial cell layers, which
409 represent an unaccounted sink of phages within the body.

410

411 The mechanism of non-specific phage uptake by *in vitro* tissue culture cell lines was previously
412 demonstrated to occur via macropinocytosis (8). Cell types have been reported to have differing
413 basal rates of macropinocytosis depending on their function and location within the body (24),
414 thereby influencing their rate of phage uptake. The macropinosome plays further roles in the
415 presentation of antigens for pattern recognition receptors located in other organelles, and in the
416 activation of innate immune responses (63). The fate of phages within the macropinosome is
417 still unknown. It is clear phages accumulate within the macropinosomes over time, but whether
418 they are subsequently degraded by the endosomal/lysosomal system, or recycled and fused
419 back with the plasma membrane remains to be investigated (24). Here we propose that, within
420 seconds, phages rapidly adhere to the eukaryotic cell membrane. Secondly, this adherence
421 leads to an internalisation of phages via non-specific macropinocytosis. Thirdly, this allows for
422 the steady accumulation of phages within the macropinosomes and cell over time. Further
423 research is required to probe the specific mechanisms of phage adherence, uptake, and the
424 cellular mechanisms that govern the trafficking of phages within cells (64,65).

Bichet et al. 2020

425

426 Using three different phages, from different families, with different sizes, we were able to show
427 an effect of phage size on the rate of uptake across three different cell lines. Using real-time
428 microscopy, we demonstrate that the smaller sized phage particles had increased rates of
429 uptake, especially in low uptake cell lines. One hypothesis for the increased uptake of smaller
430 sized phages, may simply be the result of an increased number of particles capable of
431 interacting with actin-mediated ruffles associated with of macropinosome formation, thereby
432 leading to a higher number of phages engulfed within each macropinosome (24,66). It is also
433 possible that there is a difference in ruffle size across cell lines leading to a predisposition of
434 some cells to uptake smaller or bigger size phages. A similar relationship between particle size
435 and uptake has been made with nanoparticles, where it was observed that 50 nm size
436 nanoparticles had high rates of uptake, which coincidentally are of similar size as our T3 phages
437 (67–69). It was also shown that the cell type, as well as the particles shape influenced uptake,
438 with disc-shaped particles having higher rates of uptake compared with elongated rod shapes.
439 Again, our T3 phage are coincidentally similarly shaped to the disc-shaped particles (70,71),
440 indicating that the shape of phages may play a role in their uptake; a factor that we cannot rule
441 out from our study. It is intriguing to speculate that much of the research into nanoparticle
442 delivery has converged upon parameters analogous with biology's own naturally-occurring
443 nanoparticles; the bacteriophages.

444

445 We observed, that following the rapid adsorption of phages with *in vitro* cell culture layers, a
446 high proportion of phages were inactivated (Fig. 5b); a phenomenon which has not been
447 previously reported. Our first hypothesis to explain this rapid inactivation is that upon
448 interaction with cell surface features, phages are mechanically triggered to eject their genomes,
449 thereby leading to inactivation (72). This would implicate an increase of phage DNA at the cell
450 surface, which we observed via ddPCR results, along with a concomitant decrease in functional
451 phage particles. Another hypothesis is that the transient and non-specific interactions between
452 the phages and the cell surface features, including glycoproteins, glycolipids, and mucins (73),
453 may physically block or impede the phage such that it is unable to access its host bacterial
454 receptor for plaque quantification, thereby only being detected via ddPCR. Finally, this
455 inactivation could be the result of the degradation of the phage capsid due to enzymes,
456 secretions, or cellular products. Though our negative controls show this is unlikely, as there
457 was no effect of spent cell culture media on phage infectivity (Supplemental Fig. 5). At this
458 stage whilst we cannot conclude the specific mechanisms inducing this rapid loss of phage

Bichet et al. 2020

459 viability, our results clearly suggest that this rapid inactivation and adsorption to the cell layer
460 may represent a major sink for circulating phages in the body.

461

462 During phage therapy, the cells, organs, and systems of the body play an important role in the
463 efficacy of treatment due to their effect on the sequestration of active phages and limiting
464 accessibility to the site of infection (30,31). It has been proposed that the mononuclear
465 phagocyte system (MPS) is primarily responsible for the filtering and removal of the phages
466 during phage therapy, with the liver and spleen considered the main organs responsible for
467 filtering circulating phages (43,74,75). Recent case studies of phage pharmacokinetics (PK)
468 following intravenous (IV) administration have shown rapid clearance in both humans and
469 animal models, with over > 99% of phages applied removed from circulation within the first
470 few hours (38,76). In an extensive literature review, Dabrowska 2019 (30) noted that the phage
471 titres in the blood immediately following intravenous injection (1-5 minutes) were markedly
472 less than expected hypothetical dilutions. Even when accounting for phage dilutions in the
473 blood or total body volume, phage titres only reached between 0.02% and 0.4% of their
474 predicted titres. This suggests that there is significant and rapid uptake of phages by the organs
475 and cells of the MPS, or alternatively, the rapid adherence and inactivation of phages to the
476 epi- and endothelial cells lining the circulatory system. All the cell lines used in this study;
477 endothelial, epithelial, macrophages or fibroblast, may be in contact with phages at any time
478 during therapy. Our results demonstrate that phages rapidly adhered to and are subsequently
479 internalised by these cells (Fig. 5b). The model we developed in this article (Fig. 6), whilst
480 preliminary, illustrated the potential impact that cell layers play in the inactivation of phages
481 following their delivery to a patient. We suggest that the cell layers of the body are a major
482 sink for phages and that these interactions have unrecognised impacts on phage PK and
483 therapy. If this mechanism is true, then phage most likely display non-linear PK (in addition to
484 non-linear clearance). These results have unrecognised consequences on phage dosing
485 regimens during treatment, implying higher doses and more frequent administrations of phages
486 to patients may be needed in order to reach an optimal phage dose to fight the infection.

487

488 With these new findings on the role of eukaryotic cells in the uptake and inactivation of phages
489 during phage therapy, we hope to help in the design and engineering of treatment for patients
490 and to improve the clinical outcome of phage therapy.

491 **Materials and methods**

492 **Bacterial stocks and phage stocks.** The bacterial strains used in this study,
493 *Escherichia coli* B strain HER 1024 and *E. coli* B strain W3350, were cultured in lysogeny
494 broth (LB) media (10 g tryptone, 5 g yeast extract, 10 g NaCl, in 1 litre of distilled water
495 [dH₂O]) at 37 °C shaking overnight and used to propagate and titre phages T4, T3 and lambda
496 supplemented with 10 mM CaCl₂ and MgSO₄. Phages T4, T3 and lambda were cleaned and
497 purified using the Phage on Tap protocol (PoT) (45) and titred up to a concentration of
498 approximately 10¹⁰ phages per ml. After purification, phages were stored in a final solution of
499 SM Buffer (2.0 g MgSO₄·7H₂O, 5.8 g NaCl, 50 ml of 1M Tris-HCl pH 7.4, dissolve in 1 litre
500 of dH₂O) at 4 °C.

501 **Endotoxin removal.** For each of the phage samples, endotoxin removal protocol was
502 followed from the Phage on Tap (PoT) protocol (45). Phages lysates were cleaned four times
503 with octanol to remove endotoxins from the lysate.

504 **Cell line stocks.** Seven cell lines were used in this study, all grown at 37 °C and 5%
505 CO₂ and supplemented with 1% penicillin-streptomycin (Life Technologies Australia Pty. Ltd)
506 A549 cells were grown in Ham's F-12K (Kaighn's) (also called F12-K) (Life Technologies
507 Australia Pty. Ltd) media with 10% Fetal Bovine Serum (FBS) (Life Technologies Australia
508 Pty. Ltd), HUVECs were grown in F12-K media with 20% FBS, HeLa and HT-29 cells were
509 both grown in Dulbecco's Modified Eagle Medium (DMEM) (Life Technologies Australia Pty.
510 Ltd) supplemented with 10% FBS, MDCK-I cells were grown in Modified Eagle Medium
511 (MEM) (Life Technologies Australia Pty. Ltd) with 10% FBS, BJ cells were grown in DMEM
512 media with 10% FBS and 1% sodium pyruvate (Sigma-Aldrich, Australia) and finally the
513 suspension of THP-1 cells were maintained in Roswell Park Memorial Institute (RPMI) 1640
514 media (Life Technologies Australia Pty. Ltd) with 10% FBS. For differentiation, phorbol 12-
515 myristate 13-acetate (PMA) (Sigma-Aldrich, Australia) was added to a final concentration of
516 25 mM and incubated for 48 hours. After incubation PMA supplemented media was removed
517 and cells were further grown in PMA free media for 24 hours to obtain differentiated
518 macrophages. These differentiated cells were stable for up to one week.

519 **Confocal microscopy.** For confocal microscopy experiment, cells were seeded in an
520 IBIDI μ-Slide 8-well glass-bottom slide (DKSH Australia Pty. Ltd) and grown to 80-90%
521 confluency for acquisition. Cells were treated for 20 min with the respective culture media for
522 each cell line with 5% Hoechst 33342 stain, excitation/emission 361/497 nm (Life
523 Technologies Australia Pty. Ltd) and 1% CellMask deep red plasma membrane stain,
524 excitation/emission 649/666 nm (Life Technologies Australia Pty. Ltd). After incubation cells

Bichet et al. 2020

525 were washed three times with Dulbecco's phosphate-buffered saline (DPBS) 1× and then left
526 in Hank's Balanced Salt Solution (HBSS) with 1% FBS until acquisition. Purified phages were
527 labelled with 1% SYBR-Gold, excitation/emission 495/537 nm (Life Technologies Australia
528 Pty. Ltd) for one hour in the dark at 4 °C, followed by three washes with HBSS in Amicon-
529 Ultra4 centrifugal unit 100-kDa membrane (Merck Pty. Ltd) to remove excess of stain. The
530 washed phages were resuspended in a final volume of 1 ml in HBSS media. From a 10⁹ phage
531 per ml solution, we added 200 µl per well to the cells under the microscope right before the
532 start of the acquisition. Cells were imaged with HC PL APO 63x/1.40 Oil CS2 oil immersion
533 objective by Leica SP8 confocal microscope on inverted stand with a hybrid detector (HyD) in
534 real time. Excitation used for Hoechst 33342 (blue), SYBR-Gold and CellMask deep red was
535 405, 488 and 638 nm; corresponding emission was recorder at 412-462, 508-545 and 648-694
536 nm detection ranges respectively. HyD detector was used in sequential mode to detect the
537 phages, it increases the sensitivity of detection by acquiring the same image multiple times and
538 accumulating the fluorescence signal. All live cell imaging experiments were completed in
539 triplicate (three fields of view per session). One image was acquired every two minutes for two
540 hours. Each field of view was hand-picked depending on the cell confluency and success of
541 staining. Videos were created through post-processing using the FIJI software version 2.0.0-
542 rc-68/1.52f (77). First, the three channels acquired were merged and processed with a Gaussian
543 Blur filter of 0.8. Second, each channel brightness and contrast were enhanced for printing
544 quality. Finally, the time and scale were added to the final movie saved in 12 fps.

545 **Quantification of phages in live cell imaging.** For each live cell experiment, we
546 quantified cells that contained intracellular green fluorescence as indicative of SYBR-Gold
547 labelled phages. Live cell images were acquired every ten minutes were quantified by manual
548 counting the total number of cells in the field of view and the number of cells with intracellular
549 phages to calculate the percentage of cells containing intracellular phages. Results were plotted
550 using the GraphPad Prism version 8.4.2 for macOS GraphPad Software, San Diego, California
551 USA, www.graphpad.com, to show uptake of phages over time.

552 **Clustering analysis.** Univariate clustering was performed using the dynamic
553 programming algorithm in the R package Ckmeans.1d.dp (46).

554 **Flow conditions in microfluidic chip.** A chip mold with 500 µm wide, 350 µm high
555 and 1.3 cm long channels was designed using SolidWorks® 2017. The moulds were 3D-printed
556 using Object Eden360V (Stratasys, USA) with a manufacturer-provided polymer FC720 and
557 surface-salinized in a vacuum desiccator overnight with 20 µl Trichloro(1*H*,1*H*,2*H*,2*H*-

Bichet et al. 2020

558 perfluorooctyl)silane (Sigma-Aldrich, USA). The microfluidic chips were manufactured via
559 soft-lithography, by casting a 10:1 mixture of Sylgard 184 PDMS and its curing agent (Dowsil,
560 USA) respectively, onto the moulds and were cured at 90 °C until completely solidified (~2
561 hours). The chips were then cut with a surgical knife, gently peeled off, trimmed and their inlet
562 and outlet were punched with 2 mm biopsy punchers (ProSciTech, Australia). Subsequently,
563 the chips were washed in pentane and acetone to remove residual uncured PDMS. Atmospheric
564 plasma generated at 0.65 Torr with high radio frequency was used to bond the PDMS chip to
565 a glass cover slip No. 1.5H (0.170 mm ± 0.005 mm thickness) optimized for confocal
566 microscopy (Marienfeld), for 20 seconds. The microchannel of the assembled microfluidic
567 device were washed with ethanol (80% v/v)-sterilised, UV-sterilised and pre-treated with 1:50
568 MaxGel™ ECM (Sigma-Aldrich) in cold F12-K media for two hours at 37 °C and 5% CO₂.
569 The microchannel was then washed with F12-K media to remove residual ECM. Schematic
570 and picture of the microdevice is included in supplemental Fig. 2. The channel was seeded with
571 10 µl of HUVECs at a concentration of 5 × 10⁵ cells/ml, which were carefully pipetted through
572 the in port. The seeded chip was incubated statically for 12 hours to allow cell attachment at
573 37 °C and 5% CO₂. This was followed by perfusing the attached cells with complete media for
574 24 hours at 0.66 µl/min flow rate to establish a confluent cell layer. The cell layer was then
575 perfused with cell culture media supplemented with 20% of FBS for another 24 hr at 8 µl/min
576 to acclimate the cells to the shear stress. Perfusion was mediated by a single-channel syringe
577 pump (New Era Pump Systems, USA) using a 10 ml 21 gauge needled-syringe fitted to Teflon
578 tubes of 1/16" inner diameter and 1/32" outer diameter (Cole-Palmer, USA) that were
579 previously sterilised using ethanol (80% v/v)-sterilised, DPBS and UVs. HUVECs were then
580 stained with nucleus stain, Hoechst 33342 (blue), plasma membrane stain, CellMask (magenta)
581 under static conditions for 20 min. T4 phages labelled with DNA-complexing stain, SYBR-
582 Gold (green) were then added to the chip under 8 µl/min flow rate for either two or 18 hours.
583 After incubation under flow with the phages, the in and out port of the chips were sealed using
584 sterilized-binder paper clips and the chip placed under the microscope. The images were
585 acquired with HC PL APO 63x/1.40 Oil CS2 oil immersion objective on an inverted Leica SP8
586 confocal microscope. A hybrid detector (HyD) was used to visualise phage DNA
587 (excitation/emission 495/537 nm), other channels were acquired with conventional PMT
588 detectors for CellMask (excitation/emission 649/666 nm) and for Hoescht 33342
589 (excitation/emission 361/497 nm).

590 **Image analysis with CellProfiler.** To quantify the fluorescence intensity of SYBR-
591 Gold labelled phages (495 nm wavelength), we used a pipeline created in CellProfiler (60) (see
592 the pipeline used in supplemental data, SD1), allowing us to measure the pixel grey values as
593 a proxy for fluorescence intensity across the image. First, we segmented regions covered by
594 nuclei by applying the IdentifyPrimaryObjects module to the Hoechst channel image. Second,
595 we defined expanded regions around the nuclei for cytoplasmic measurements using the
596 IdentifySecondaryModule with the parameter Distance-N set to 200. Third, we masked out
597 nuclei regions in the of the nuclei SYBR (phages) channel. This is to exclude fluorescence
598 coming from the cell nuclei due to the leaking of the SYBR dye from the phage capsid to the
599 cell nuclei, which would lead to false positive quantification. Finally, the grey values image
600 intensity in the masked SYBR channel and additional parameters of the secondary objects were
601 measured (Supplemental data SD1). Only a single time point at two hours at each field of view
602 was used for the analysis. The number of images analysed for each condition varied, as manual
603 quality control was applied to exclude out of focus and non-analysable fields of view.

604 **Intracellular phages.** For the intracellular phages experiment, cells were plated in T25
605 cm² flasks until they reached confluency. For the 18 hours experiment, phages were applied in
606 volumes of 3 ml of media with 10⁹ phages/ml per flask and incubated overnight at 37 °C and
607 5% CO₂. The control flasks were incubated with 3 ml of phage-free media. After the 18 hours
608 incubation, control flasks were incubated with the same phage dilution for 30 seconds. The
609 initial dilution for each flask was collected for quantification. Cells were washed three times
610 with 5 ml of 1 × DPBS to remove non-adherent phages. Next, one ml of 0.5% trypsin was
611 added per flask and incubated at 37 °C and 5% CO₂ for a few minutes, once cells detached, the
612 cells were resuspended in 5 ml of 1 × DPBS and spun at 1500 rpm for three minutes and washed
613 three times with 5 ml 1 × DPBS to remove any non-adherent phages. After the washes, cells
614 were resuspended in 1 ml of lysis buffer (0.5 M EDTA and 1 M Tris at pH 7.5, complete with
615 dH₂O and adjust pH to 8) and left at room temperature for 20 min. After incubation the cells
616 are passed through a 30 G syringe three times to ensure complete cell lysis. The lysis was
617 confirmed by looking at the sample under a microscope.

618 **ddPCR setup.** Digital Droplet Polymerase Chain Reaction (ddPCR) was performed
619 following manufacturer's instructions (Bio-Rad, Australia). A 20 µl reaction was assembled
620 with primers, probe, ddPCR Supermix for probe (Bio-Rad, Australia) and sample. The primer
621 and probe sequence and PCR parameters are shown in supplemental Table 2 - 4. ddPCR
622 reaction mix was then loaded into eight channel disposable droplet generator cartridge (Bio-

Bichet et al. 2020

623 Rad, Australia). 70 µl of droplet generation oil was added to each channel and placed in the
624 Bio-Rad QX200 droplet generator. The droplets were transferred into the deep well 96 well
625 plate (Bio-Rad, Australia), using a multichannel pipette. The plate was then sealed using Bio-
626 Rad plate sealer and then placed in a conventional thermocycler and the PCR product was
627 amplified (supplemental Table 4). After amplification, the plate was loaded into the droplet
628 reader (Bio-Rad, Australia) to quantify the fluorescent droplets. Analysis of the data was
629 performed using the Poisson distribution with QuantaLife software (Bio-Rad, Australia).

630 **PFU quantification.** The Plaque Forming Unit (PFU) assay was performed using LB
631 agar plates where a thin layer of soft LB agar was mixed with one ml of host bacterial culture
632 and the desired dilution of phages was poured on the agar plate. The plate was incubated over-
633 night at 37 °C before counting the number of plaques formed on the bacterial lawn. The results
634 were calculated in PFU. To obtain the number of inactive phages we subtracted PFU numbers
635 (active phages) from the ddPCR numbers (total number of phages).

636 **Pharmacokinetics Model.** A previously developed PK model in healthy rats was
637 utilized to evaluate the impact of phage inactivation on *in vivo* phage disposition (38). An
638 additional compartment was incorporated to describe the inactivation and reactivation of
639 phages by the epi- and endothelial cells. The rates of inactivation and reactivation was
640 described by first-order rate constant, KD, and was assumed to be constant over time. The
641 differential equations for phage disposition and inactivation were represented by:

$$642 \frac{dA_1(t)}{dt} = -(CL_T + Q_1 + Q_2) \frac{A_1(t)}{V_1} + Q_1 \frac{A_2(t)}{V_2} + Q_2 \frac{A_3(t)}{V_3} - KD_1 A_1(t) + KD_2 A_4(t)$$

$$643 \frac{dA_2(t)}{dt} = Q_1 \frac{A_1(t)}{V_1} - Q_1 \frac{A_2(t)}{V_2}$$

$$644 \frac{dA_3(t)}{dt} = Q_2 \frac{A_1(t)}{V_1} - Q_2 \frac{A_3(t)}{V_3}$$

$$645 \frac{dA_4(t)}{dt} = KD_1 A_1(t) - KD_2 A_4(t)$$

$$646 CL_T = \frac{V_{max}}{K_m + \frac{A_1}{V_1}}$$

647 where

648 Q_1 = inter-compartmental clearance 1 (mL/h/Rat).

649 Q_2 = inter-compartmental clearance 2 (mL/h/Rat).

650 V_1 = Volume of distribution of the central compartment (mL/Rat).

651 V_2 = Volume of distribution of the peripheral compartment 1 (mL/Rat).

652 V_3 = Volume of distribution of the peripheral compartment 2 (mL/Rat).

Bichet et al. 2020

653 k_m = Phage titre that produces 50% of the maximal elimination rate of the system (PFU/mL/Rat).

654 V_{max} = Maximum elimination rate (PFU/h/Rat).

655 KD_1 = Inactivation rate constant (1/h).

656 KD_2 = Reactivation rate constant (1/h).

657 Deterministic was performed using model-predicted median PK parameters in rats without
658 inter-individual variability and random unexplained variability (Supplementary table 5).

659 Inactivation rate constant was determined using the ddPCR results as described in

660 Supplementary table 5. First order for the 30 seconds graph is of 1415 1/h/rat and for the 18

661 hours the first order is of 0.358 1/h/rat calculated from the ddPCR data. Reactivation rate

662 constant was fixed to 0. Deterministic simulations were performed in R using mrgsolve

663 (version 0.10.4) (38,78).

664

Bichet et al. 2020

665

666 **Authors participation**

667 Conceptualization: MCB, JJB; Methodology: MCB, WHC, WR, AO, LAF, CAH, YWL;
668 Formal Analysis: MCB, JJB; Investigation: MCB; Resources: JJB; Writing – Original Draft
669 Preparation: MCB, RP, JJB; Writing – Review and Editing: all authors contributed;
670 Supervision and Funding Acquisition: JJB

671

672 **Funding**

673 Marion C. Bichet was supported by Monash Graduate Scholarship (MGS). This work,
674 including the efforts of Jeremy J. Barr, was funded by the Australian Research Council DECRA
675 Fellowship (DE170100525), National Health and Medical Research Council (NHMRC:
676 1156588), and the Perpetual Trustees Australia award (2018HIG00007).

677

678 **Acknowledgements**

679 We thank the following labs for kindly providing the cell lines; Hudson Institute of Medical
680 Research and the Oncogenic Signalling Lab for providing the A549 cell line; the Nucleic Acids
681 and Innate Immunity Research Group for providing the HT29 and BJ cell lines; the University
682 of Melbourne and the Obstetrics, Nutrition and Endocrinology Group for providing the
683 HUVECs; Monash University and the Moseley Laboratory for providing the HeLa cell line.
684 We thank the following facilities for kindly providing equipment and guidance; Monash Micro
685 Imaging facility for help with microscopy acquisition, Monash School of Engineering for
686 providing support in the fabrication of microfluidic devices, and Department of Biochemistry
687 & Molecular Biology (Monash Biomedicine Discovery Institute) Imaging Facility for
688 providing access to the ddPCR equipment. This work was performed in part at the Melbourne
689 Centre for Nanofabrication (MCN) in the Victorian Node of the Australian National
690 Fabrication Facility (ANFF).

691

692

693 **References:**

694

- 695 1. Rohwer F, Segall AM. In retrospect: A century of phage lessons. *Nature*.
696 2015;528(7580):46–8.
- 697 2. Hatfull GF. Dark Matter of the Biosphere: the Amazing World of Bacteriophage
698 Diversity. *J Virol*. 2015 Aug;89(16):8107–10.
- 699 3. Rohwer F. Global phage diversity. *Cell Press*. 2003;113:141.
- 700 4. Clokie MRJ, Millard AD, Letarov A V, Heaphy S. Phages in nature. *Bacteriophage*.
701 2011;1(1):31–45.
- 702 5. Breitbart M, Hewson I, Felts B, Mahaffy JM, Nulton J, Salamon P, et al. Metagenomic
703 Analyses of an Uncultured Viral Community from Human Feces. *J Bacteriol*.
704 2003;185(20):6220–3.
- 705 6. Manrique P, Bolduc B, Walk ST, Van Der Oost J, De Vos WM, Young MJ. Healthy
706 human gut phageome. *Proc Natl Acad Sci*. 2016;113(37):10400–5.
- 707 7. Shkoporov AN, Hill C. Bacteriophages of the Human Gut: The “Known Unknown” of
708 the Microbiome. *Cell Host and Microbe*. 2019;Elsevier 25:195–209.
- 709 8. Nguyen S, Baker K, Padman BS, Patwa R, Dunstan RA, Weston TA, et al.
710 Bacteriophage transcytosis provides a mechanism to cross epithelial cell layers. *MBio*.
711 2017;8(6).
- 712 9. Sender R, Fuchs S, Milo R. Are We Really Vastly Outnumbered? Revisiting the Ratio
713 of Bacterial to Host Cells in Humans. *Cell*. 2016; Cell Press;164:337–40.
- 714 10. Barr JJ. A bacteriophages journey through the human body. *Immunol Rev*.
715 2017;279(1):106–22.
- 716 11. Górski A, Wazna E, Dąbrowska BW, Dąbrowska K, Świtała-Jeleń K, Międzybrodzki
717 R. Bacteriophage translocation. *FEMS Immunol Med Microbiol*. 2006;46(3):313–9.
- 718 12. Ghose C, Ly M, Schwanemann LK, Shin JH, Atab K, Barr JJ, et al. The Virome of
719 Cerebrospinal Fluid: Viruses Where We Once Thought There Were None. *Front*
720 *Microbiol*. 2019 Sep;10.
- 721 13. Międzybrodzki R, Kłak M, Jonczyk-Matysiak E, Bubak B, Wójcik A, Kaszowska M,
722 et al. Means to facilitate the overcoming of gastric juice barrier by a therapeutic
723 staphylococcal bacteriophage A5/80. *Front Microbiol*. 2017 Mar;8:1–11.
- 724 14. Geier MR, Trigg ME, Merril CR. Fate of bacteriophage lambda in Non-immune germ-

Bichet et al. 2020

- 725 free mice. *Nature*. 1973;246(5430):221–3.
- 726 15. Huh H, Wong S, St. Jean J, Slavcev R. Bacteriophage interactions with mammalian
727 tissue: Therapeutic applications. *Adv Drug Deliv Rev*. 2019;145:4–17.
- 728 16. Dabrowska K, Switała-Jelen K, Opolski A, Weber-Dabrowska B, Gorski A. A review:
729 Bacteriophage penetration in vertebrates. *J Appl Microbiol*. 2005;98(1):7–13.
- 730 17. Dor-On E, Solomon B. Targeting glioblastoma via intranasal administration of Ff
731 bacteriophages. *Front Microbiol*. 2015;6.
- 732 18. Srivastava AS, Chauhan DP, Carrier E. In utero detection of T7 phage after systemic
733 administration to pregnant mice. *BioTechniques*. 2004; 37.
- 734 19. Gorski A, Dabrowska K, Switała-Jele K, Nowaczyk M, Weber-Dabrowska B,
735 Boratynski J, et al. New insights into the possible role of bacteriophages in host
736 defense and disease. *Med Immunol*. 2003 Feb;2:2.
- 737 20. Karimi M, Mirshekari H, Moosavi Basri SM, Bahrami S, Moghoofei M, Hamblin MR.
738 Bacteriophages and phage-inspired nanocarriers for targeted delivery of therapeutic
739 cargos. *Adv Drug Deliv Rev*. 2016;106:45–62.
- 740 21. Handley SA, Thackray LB, Zhao G, Presti R, Miller AD, Droit L, et al. Pathogenic
741 simian immunodeficiency virus infection is associated with expansion of the enteric
742 virome. *Cell*. 2012;151(2):253–66.
- 743 22. Lehti TA, Pajunen MI, Skog MS, Finne J. Internalization of a polysialic acid-binding
744 *Escherichia coli* bacteriophage into eukaryotic neuroblastoma cells. *Nat Commun*.
745 2017;8(1).
- 746 23. Tao P, Mahalingam M, Marasa BS, Zhang Z, Chopra AK, Rao VB. In vitro and in
747 vivo delivery of genes and proteins using the bacteriophage T4 DNA packaging
748 machine. *Proc Natl Acad Sci*. 2013 Apr;110(15):5846–51.
- 749 24. Kerr MC, Teasdale RD. Defining Macropinocytosis. *Traffic*. 2009 Apr;10(4):364–71.
- 750 25. Gordillo Altamirano FL, Barr JJ. Phage Therapy in the Postantibiotic Era. *Clin*
751 *Microbiol Rev*. 2019;32(2):1–25.
- 752 26. Merril CR, Scholl D, Adhya SL. The prospect for bacteriophage therapy in Western
753 medicine. *Nat Rev Drug Discov*. 2003 Jun;2(6):489–97.
- 754 27. Kutter E, De Vos D, Gvasalia G, Alavidze Z, Gogokhia L, Kuhl S, et al. Phage
755 Therapy in Clinical Practice: Treatment of Human Infections. *Curr Pharm Biotechnol*.
756 2010 Feb;11(1):69–86.
- 757 28. Abedon ST, Kuhl SJ, Blasdel BG, Kutter EM. Phage treatment of human infections.

- 758 Bacteriophage. 2011;1(2):66–85.
- 759 29. Matsuzaki S, Uchiyama J. Phage Pharmacokinetics: Relationship with Administration
760 Route. In: Phage Therapy: A Practical Approach. Springer International Publishing.
761 2019;43–57.
- 762 30. Dąbrowska K. Phage therapy: What factors shape phage pharmacokinetics and
763 bioavailability? Systematic and critical review. 2019;Med Res Rev 39(5):2000–25.
- 764 31. Dąbrowska K, Abedon ST. Pharmacologically Aware Phage Therapy:
765 Pharmacodynamic and Pharmacokinetic Obstacles to Phage Antibacterial Action in
766 Animal and Human Bodies. Microbiol Mol Biol Rev. 2019;83(4):1–25.
- 767 32. Payne RJH, Jansen VAA. Understanding bacteriophage therapy as a density-dependent
768 kinetic process. J Theor Biol. 2001 Jan;208(1):37–48.
- 769 33. Payne RJH, Jansen VAA. Phage therapy: The peculiar kinetics of self-replicating
770 pharmaceuticals. Clinical Pharmacology and Therapeutics. 2000;68:225–30.
- 771 34. Hoffmann M. Animal Experiments on Mucosal Passage and Absorption Viraemia of
772 T3 Phages after Oral, Trachéal and Rectal Administration. Zentralblatt fur Bakteriologie
773 Parasitenkunde, Infekt und Hyg. 1965;198(4):371–90.
- 774 35. Hildebrand GJ, Wolochow H. Translocation of Bacteriophage Across the Intestinal
775 Wall of the Rat. Exp Biol Med. 1962 Jan;109(1):183–5.
- 776 36. Keller R, Engley FB. Fate of Bacteriophage Particles Introduced into Mice by Various
777 Routes. Exp Biol Med. 1958 Jul;98(3):577–80.
- 778 37. Sweere JM, Belleghem JD Van, Ishak H, Bach MS, Popescu M, Sunkari V, et al. of
779 bacterial infection. 2019 Mar;9691.
- 780 38. Lin Y-W, Kyung Chang RY, Rao GG, Jermain B, Han M-L, Zhao J, et al.
781 Pharmacokinetics/pharmacodynamics of antipseudomonal bacteriophage therapy in
782 rats: A Proof-of-Concept study. Clin Microbiol Infect. 2020 May.
- 783 39. Doub JB, Ng VY, Johnson AJ, Slomka M, Fackler J, Horne B, et al. Salvage
784 Bacteriophage Therapy for a Chronic MRSA Prosthetic Joint Infection. Antibiotics.
785 2020;9:241.
- 786 40. Hodyra-Stefaniak K, Lahutta K, Majewska J, Kaźmierczak Z, Lecion D, Harhala M, et
787 al. Bacteriophages engineered to display foreign peptides may become short-
788 circulating phages. Microb Biotechnol. 2019 Jul;12(4):730–41.
- 789 41. Carroll-Portillo A, Lin HC. Bacteriophage and the innate immune system: Access and
790 signaling. Microorganisms. 2019;7(12):1–11.

Bichet et al. 2020

- 791 42. Van Belleghem JD, Dąbrowska K, Vaneechoutte M, Barr JJ, Bollyky PL. Interactions
792 between bacteriophage, bacteria, and the mammalian immune system. *Viruses*. 2019
793 Jan;11(1).
- 794 43. Hodyra-Stefaniak K, Miernikiewicz P, Drapała J, Drab M, Jonczyk-Matysiak E,
795 Lecion D, et al. Mammalian Host-Versus-Phage immune response determines phage
796 fate in vivo. *Sci Rep*. 2015 Oct;5(1):1–13.
- 797 44. Sweere JM, Van Belleghem JD, Ishak H, Bach MS, Popescu M, Sunkari V, et al.
798 Bacteriophage trigger antiviral immunity and prevent clearance of bacterial infection.
799 *Science*. 2019 Mar;363(6434).
- 800 45. Bonilla N, Rojas MI, Netto Flores Cruz G, Hung S-H, Rohwer F, Barr JJ. Phage on
801 tap—a quick and efficient protocol for the preparation of bacteriophage laboratory
802 stocks. *PeerJ*. 2016;4:e2261.
- 803 46. Wang H, Song M. Ckmeans.1d.dp: Optimal k-means clustering in one dimension by
804 dynamic programming. *R J*. 2011;3(2):29–33.
- 805 47. Kim HJ, Li H, Collins JJ, Ingber DE. Contributions of microbiome and mechanical
806 deformation to intestinal bacterial overgrowth and inflammation in a human gut-on-a-
807 chip. *Proc Natl Acad Sci*. 2016;113(1):e7–15.
- 808 48. Kim HJ, Huh D, Hamilton G, Ingber DE. Human Gut-on-a-Chip inhabited by
809 microbial flora that experiences intestinal peristalsis-like motions and flow. *R Soc*
810 *Chem*. 2012;12:2165–74.
- 811 49. Navabi N, McGuckin MA, Linden SK. Gastrointestinal Cell Lines Form Polarized
812 Epithelia with an Adherent Mucus Layer when Cultured in Semi-Wet Interfaces with
813 Mechanical Stimulation. *PLoS One*. 2013;8(7).
- 814 50. Kim L, Toh YC, Voldman J, Yu H. A practical guide to microfluidic perfusion culture
815 of adherent mammalian cells. *Lab Chip*. 2007;7(6):681–94.
- 816 51. Thuenauer R, Rodriguez-Boulan E, Rümer W. Microfluidic approaches for epithelial
817 cell layer culture and characterisation. *Analyst*. 2014;139(13):3206–18.
- 818 52. Son Y. Determination of shear viscosity and shear rate from pressure drop and flow
819 rate relationship in a rectangular channel. *Polymer (Guildf)*. 2007 Jan;48(2):632–7.
- 820 53. Chin WH, Barr JJ. Phage research in ‘organ-on-chip’ devices. *Microbiol Aust*. 2019
821 Mar.
- 822 54. Barr JJ, Auro R, Sam-Soon N, Kassegne S, Peters G, Bonilla N, et al. Subdiffusive
823 motion of bacteriophage in mucosal surfaces increases the frequency of bacterial

- 824 encounters. *Proc Natl Acad Sci.* 2015;112(44):13675–80.
- 825 55. Yum K, Hong SG, Healy KE, Lee LP. Physiologically relevant organs on chips.
826 *Biotechnol J.* 2014 Jan;9(1):16–27.
- 827 56. Wang C, Lu H, Alexander Schwartz M. A novel in vitro flow system for changing
828 flow direction on endothelial cells. *J Biomech.* 2012;45(7):1212–8.
- 829 57. Abaci HE, Shen YI, Tan S, Gerecht S. Recapitulating physiological and pathological
830 shear stress and oxygen to model vasculature in health and disease. *Sci Rep.* 2014
831 May;4(1):1–9.
- 832 58. Park JY, White JB, Walker N, Kuo CH, Cha W, Meyerhoff ME, et al. Responses of
833 endothelial cells to extremely slow flows. *Biomicrofluidics.* 2011 Jun;5(2):022211.
- 834 59. Davies PF, Spaan JA, Krams R. Shear stress biology of the endothelium. *Ann Biomed*
835 *Eng.* 2005 Dec;33:1714–8.
- 836 60. McQuin C, Goodman A, Chernyshev V, Kamensky L, Cimini BA, Karhohs KW, et
837 al. CellProfiler 3.0: Next-generation image processing for biology. *PLoS Biol.* 2018;
- 838 61. Hosta-Rigau L, Städler B. Shear stress and its effect on the interaction of myoblast
839 cells with nanosized drug delivery vehicles. *Mol Pharm.* 2013 Jul;10(7):2707–12.
- 840 62. Han J, Zern BJ, Shuvaev V V., Davies PF, Muro S, Muzykantov V. Acute and chronic
841 shear stress differently regulate endothelial internalization of nanocarriers targeted to
842 platelet-endothelial cell adhesion molecule-1. *ACS Nano.* 2012 Oct;6(10):8824–36.
- 843 63. Canton J. Macropinocytosis: New Insights Into Its Underappreciated Role in Innate
844 Immune Cell Surveillance. *Front Immunol.* 2018;9:2286.
- 845 64. Talman L, Agmon E, Peirce SM, Covert MW. Multiscale models of infection. Vol. 11,
846 *Current Opinion in Biomedical Engineering.* 2019;Elsevier 11:102–8.
- 847 65. Bodner K, Melkonian AL, Barth AIM, Kudo T, Tanouchi Y, Covert MW. Engineered
848 Fluorescent *E. coli* Lysogens Allow Live-Cell Imaging of Functional Prophage
849 Induction Triggered inside Macrophages. *Cell Syst.* 2020;10(3):254-264.
- 850 66. Swanson JA, Watts C. Macropinocytosis. *Trends in Cell Biology.* 1995;Elsevier
851 *Current Trends* 5:424–8.
- 852 67. Lu F, Wu S-H, Hung Y, Mou C-Y. Size Effect on Cell Uptake in Well-Suspended,
853 Uniform Mesoporous Silica Nanoparticles. *Small.* 2009; 5(12):1408–13.
- 854 68. Yin Win K, Feng SS. Effects of particle size and surface coating on cellular uptake of
855 polymeric nanoparticles for oral delivery of anticancer drugs. *Biomaterials.* 2005;
856 26(15):2713–22.

Bichet et al. 2020

- 857 69. Zhu J, Liao L, Zhu L, Zhang P, Guo K, Kong J, et al. Size-dependent cellular uptake
858 efficiency, mechanism, and cytotoxicity of silica nanoparticles toward HeLa cells.
859 *Talanta*. 2013;107:408–15.
- 860 70. Agarwal R, Singh V, Journey P, Shi L, Sreenivasan S V., Roy K. Mammalian cells
861 preferentially internalize hydrogel nanodiscs over nanorods and use shape-specific
862 uptake mechanisms. *Proc Natl Acad Sci*. 2013 Oct;110(43):17247–52.
- 863 71. Hsiao I-L, Gramatke AM, Joksimovic R, Sokolowski M, Gradzielski M, Haase A. Size
864 and Cell Type Dependent Uptake of Silica Nanoparticles. *J Nanomed Nanotechnol*.
865 2014;05(06).
- 866 72. Kellermayer MSZ, Vörös Z, Csík G, Herényi L. Forced phage uncorking: viral DNA
867 ejection triggered by a mechanically sensitive switch. *Nanoscale*. 2018;10:1898.
- 868 73. Barr JJ, Auro R, Furlan M, Whiteson KL, Erb ML, Pogliano J, et al. Bacteriophage
869 adhering to mucus provide a non-host-derived immunity. *Proc Natl Acad Sci*.
870 2013;110(26):10771–6.
- 871 74. Geier MR, Trigg ME, Merrill CR. Fate of bacteriophage lambda in Non-immune germ-
872 free mice. *Nature*. 1973;246(5430):221–3.
- 873 75. Merrill CR, Biswas B, Carlton R, Jensen NC, Creed GJ, Zullo S, et al. Long-circulating
874 bacteriophage as antibacterial agents. *Proc Natl Acad Sci*. 1996 Apr;93(8):3188–92.
- 875 76. Schooley RT, Biswas B, Gill JJ, Hernandez-Morales A, Lancaster J, Lessor L, et al.
876 Development and use of personalized bacteriophage-based therapeutic cocktails to
877 treat a patient with a disseminated resistant *Acinetobacter baumannii* infection.
878 *Antimicrob Agents Chemother*. 2017;61(10):1–14.
- 879 77. Schindelin J, Arganda-Carreras I, Frise E, Kaynig V, Longair M, Pietzsch T, et al. Fiji:
880 an open-source platform for biological-image analysis. *Nat Methods*. 2012;9(7).
- 881 78. Bunn A, Korpela M. A dendrochronology program library in R (dplR).
882 *Dendrochronologia*. 2018 Jul;26(2):115-24.
- 883