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# Visualization of Arenavirus RNA Species in Individual Cells by Single-Molecule Fluorescence In Situ Hybridization Suggests a Model of Cyclical Infection and Clearance during Persistence

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1 Visualization of arenavirus RNA species in individual cells by single-molecule fluorescence in  
2 situ hybridization (smFISH) suggests a model of cyclical infection and clearance during  
3 persistence

4

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- 22 **Running Title:** Cyclical LCMV infection and clearance in persistence
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25 **ABSTRACT**

26 Lymphocytic choriomeningitis mammarenavirus (LCMV) is an enveloped, negative-strand RNA  
27 virus that causes serious disease in humans but establishes an asymptomatic, lifelong infection in  
28 reservoir rodents. Different models have been proposed to describe how arenaviruses regulate  
29 the replication and transcription of their bisegmented, single-stranded RNA genomes,  
30 particularly during persistent infection. However, these models were largely based on viral RNA  
31 profiling data derived from entire populations of cells. To better understand LCMV replication  
32 and transcription at the single-cell level, we established a high-throughput, single-molecule  
33 (sm)FISH image acquisition and analysis pipeline and examined viral RNA species at discrete  
34 time points from viral entry through the late stages of persistent infection *in vitro*. We observed  
35 transcription of viral nucleoprotein and polymerase mRNAs from the incoming S and L segment  
36 genomic RNAs, respectively, within 1 hr of infection, whereas transcription of glycoprotein  
37 mRNA from the S segment antigenome required ~4-6 hr. This confirms the temporal separation  
38 of viral gene expression expected due to the ambisense coding strategy of arenaviruses and also  
39 suggests that antigenomic RNA contained in virions is not transcriptionally active upon entry.  
40 Viral replication and transcription peaked at 36 hours post-infection, followed by a progressive  
41 loss of viral RNAs over the next several days. During persistence, the majority of cells showed  
42 repeating cyclical waves of viral transcription and replication followed by clearance of viral  
43 RNA. Thus, our data support a model of LCMV persistence whereby infected cells can  
44 spontaneously clear infection and become reinfected by viral reservoir cells that remain in the  
45 population.

46

47

48 **IMPORTANCE**

49 Arenaviruses are human pathogens that can establish asymptomatic, life-long infections in their  
50 rodent reservoirs. Several models have been proposed to explain how arenavirus spread is  
51 restricted within host rodents, including the periodic accumulation and loss of replication  
52 competent, but transcriptionally incompetent, viral genomes. A limitation of previous studies  
53 was the inability to enumerate viral RNA species at the single cell level. We developed a high  
54 throughput, single-molecule RNA (sm)FISH assay and used it to quantitate lymphocytic  
55 choriomeningitis mammarenavirus (LCMV) replicative and transcriptional RNA species in  
56 individual cells at distinct time points following infection. Our findings support a model whereby  
57 productively infected cells can clear infection, including viral RNAs and antigen, and later be  
58 reinfected. This information improves our understanding of the timing and possible regulation of  
59 LCMV genome replication and transcription during infection. Importantly, the smFISH assay  
60 and data analysis pipeline developed here is easily adaptable to other RNA viruses.

61

62 **INTRODUCTION**

63 Several members of the arenavirus family are significant threats to human health. Lassa virus  
64 and Junín virus cause hemorrhagic fever syndromes while lymphocytic choriomeningitis virus  
65 (LCMV), the prototypic member of the family, is a well-known cause of severe birth defects and  
66 is highly lethal in immunocompromised individuals (1, 2). A critical imperative to better  
67 understand the key steps of the arenavirus life cycle is made evident by the fact that there are no  
68 FDA-approved vaccines to prevent arenavirus transmission and only a very limited repertoire of

69 antivirals (3, 4). New strategies to prevent and treat arenavirus infections will likely hinge upon  
70 an improved understanding of key phases of the life cycle of these important human pathogens.

71 Arenaviruses are enveloped viruses that have a single-stranded, bisegmented, negative-  
72 sense RNA genome. The ~3.5 kb small (S) and ~7.2 kb large (L) genomic RNA segments each  
73 encode two viral open reading frames in ambisense orientation (Fig. 1A) (1). The nucleoprotein  
74 (NP) and polymerase (L) genes are encoded in typical negative-sense orientation on genomic  
75 RNA while the glycoprotein (GPC) and matrix protein (Z) genes are encoded in pseudo positive-  
76 sense orientation. The canonical sequence of genetic events following the release of arenavirus  
77 genomic RNA into the cytoplasm of a newly infected cell is (i) primary transcription of the NP  
78 and L mRNAs from the viral S and L genomic segments, respectively, followed by (ii) full  
79 length replication of the S and L segment antigenomic RNAs and subsequent transcription of the  
80 GPC and Z mRNAs from the S and L antigenomic RNAs, respectively, and (iv) replication of  
81 additional full-length genomic RNAs from the antigenomic RNA templates (Fig. 1A) (1, 5).

82 While rodent-borne arenaviruses cause severe diseases in humans, they are thought to be  
83 asymptomatic in their sylvatic hosts, where they can establish a persistent, life-long infection (1).  
84 LCMV is carried by the common house mouse and can be transmitted vertically from mother to  
85 pup (6-8). The pups are born infected but never mount an effective immune response to clear the  
86 virus as viral proteins are seen as self-antigens by the pup's developing immune system (6-8) .  
87 Paradoxically, while LCMV can infect most cells in the host rodent, it tightly regulates its spread  
88 and therefore does not overrun its host. Several hypotheses have been proposed for how LCMV  
89 restricts its spread, including through (i) the production of defective interfering (DI) particles (9-  
90 11), which can enter susceptible host cells and make them refractory to productive infection (12,  
91 13) and (ii) the accumulation of transcriptionally-defective genomic and antigenomic RNAs,

92 which limit viral protein expression and infectious virus production (5, 14, 15). It has also been  
93 proposed that LCMV can establish a cyclical, transient pattern of infection such that susceptible  
94 cells are productively infected for a short time before clearing the virus and once again becoming  
95 susceptible to reinfection by neighboring cells that remain productively infected (16-19).

96 A current gap in our knowledge of how arenaviruses restrict their dissemination is that  
97 we lack a detailed understanding of how the events of viral genome replication and transcription  
98 are regulated during the acute and persistent phases of infection. Previous studies examining the  
99 genetic events of arenavirus replication and transcription, including those described above  
100 regarding the accumulation of transcriptionally defective RNAs (5, 14, 15), relied on techniques  
101 such as Northern blot or quantitative RT-PCR. Both are powerful techniques used to examine  
102 RNA. Quantitative RT-PCR is exquisitely sensitive (20), and Northern blot is able to specifically  
103 distinguish between each of the viral RNA species (15, 21-29). However, both techniques  
104 measure RNA at a population level and thus provide population average data. Variability in  
105 RNA expression between individual cells in a heterogeneous population cannot be evaluated  
106 using these approaches. Single-molecule RNA fluorescence in situ hybridization (smFISH) can  
107 bridge this technical gap to allow for detection of RNAs with single-copy sensitivity in  
108 individual cells by fluorescence microscopy (30). In the present study, we designed specific  
109 smFISH probe sets to fluorescently-label different LCMV RNA species (Fig. 1A) and to  
110 quantitatively characterize their expression in single cells at discrete time points throughout the  
111 acute and persistent phases of arenavirus infection in an *in vitro* model. Our studies confirm the  
112 temporal separation of LCMV negative-sense and pseudo positive-sense gene expression and  
113 show a pattern of cyclical loss and reappearance of viral RNA in most cells during persistence in  
114 a cell culture model of infection. Our studies provide insight into the functional genetic

115 composition of infectious virions, the kinetics of transcription and replication in the hours  
116 immediately following initial infection, and support a model of cyclical viral replication and  
117 transcription during persistence. Further, the image acquisition and analysis pipeline developed  
118 here is easily adaptable to other viruses.

119

## 120 RESULTS

121 **Visualization of LCMV RNA species in infected cells.** To visualize LCMV RNAs in  
122 cells by fluorescence microscopy, we designed smFISH probe sets complementary to different  
123 viral RNA species (see overview in Fig. 1A). An important feature of smRNA FISH is the ability  
124 to detect single RNA molecules using multiple, singly-labeled oligonucleotide probes (30). The  
125 probe set binding to a specific target RNA causes single RNAs to appear as bright spots. To  
126 validate our ability to specifically label arenavirus RNAs, we used a cellular mRNA smFISH  
127 probe set specific for the housekeeping gene MDN1 as a control (Fig. 1B) for comparison with a  
128 smFISH probe set designed to target both the viral S genome RNA and GPC mRNA (Fig. 1C).  
129 MDN1 probes detect cytoplasmic mRNAs as well as sites of active transcription in the nucleus  
130 (Fig. 1B). Next, we confirmed that the viral RNA smFISH probe set is highly specific as  
131 fluorescent signal was absent in uninfected cells, but bright spots were detected in LCMV-  
132 infected cells fixed at 24 hpi (Fig. 1C). Moreover, similar to smFISH staining obtained with our  
133 control MDN1, individual smFISH spots were homogeneous in size, shape, and fluorescence  
134 intensity (Fig. 1B and C) consistent with the detection of single RNAs, as shown previously (30,  
135 31). Furthermore, in contrast to the nuclear transcribed MDN1 mRNAs, viral RNAs were largely  
136 excluded from the nucleus, consistent with the cytoplasmic viral life cycle (Fig. 1B to C).



137 **smFISH probes complementary to viral mRNA species provide high signal-to-noise**  
138 **staining.** We designed multiple smFISH probe sets to have specificity for different RNA species  
139 produced during the course of the LCMV life cycle (Fig. 1A). Specifically, these probe sets  
140 target (i) S genome only, (ii) GPC mRNA and S genome, (iii) NP mRNA and S antigenome, or  
141 (iv) L mRNA and L antigenome. When infected cells were stained with probe sets  
142 complementary to “S genome and GPC mRNA” (referred to as “GPC mRNA/S genome” from  
143 this point forward), we noted high quality staining with the GPC mRNA/S genome probes as  
144 evidenced by homogeneity in spot size, shape, and intensity (Fig. 2A) and high signal-to-noise  
145 ratio (Fig. 3). The NP mRNA/S antigenome and L mRNA/L antigenome probe sets yielded  
146 similar high quality staining as evidenced by high signal-to-noise ratios (Fig. 3). However, we  
147 noted lower quality staining with the “S genome only” probes as evidenced by the dimmer  
148 staining (Fig. 2) and low signal-to-noise ratio (Fig. 3). Moreover, the “S genome only” probes  
149 yielded greater non-specific staining in uninfected cells, potentially leading to detection of false-  
150 positive spurious events (Fig. 2C) – perhaps an artifact of the long exposure times and high light  
151 intensity needed to detect this less sensitive probe set binding to its target. Similarly low signal-  
152 to-noise ratios were observed with probe sets specific for “S antigenome only” or “L genome  
153 only” (data not shown). It is possible that the encapsidation of genome and antigenome by viral  
154 nucleoprotein partially occludes smFISH probe hybridization with these target RNA sequences  
155 and thus leads to the lower signal-to-noise ratio observed with these probe sets. Therefore, use of  
156 these probe sets with cells containing small numbers of viral RNAs would be problematic due to  
157 the level of background staining observed (Fig. 2C). However, these probe sets are effective  
158 when paired with cells containing abundant copies of viral genome or antigenome (Fig. 2B and  
159 data not shown) (32), which easily exceeds the quantity of background spots observed in mock-

160 infected control cells (Fig. 2C). Because the probe sets that targeted an mRNA plus either  
161 genome or antigenome provided the highest quality staining and sensitivity, we elected to use  
162 these probe sets to follow the kinetics of viral transcription and replication events in infected  
163 cells.

164 **smFISH spot detection and quantification in individual LCMV-infected cells.** A  
165 primary goal of our study was to globally describe the kinetics of transcription and replication of  
166 the LCMV genome from the early hours following viral entry through the late stages of  
167 persistence. Ideally, we would be able to infect cells at a high multiplicity of infection (MOI) and  
168 take snapshots of a population of synchronously infected cells at time points throughout the  
169 entire course of arenavirus infection. However, we were obliged to infect cells at a low MOI due  
170 to the characteristic high prevalence of DI particles present in LCMV stocks (33). Because only a  
171 small proportion of cells would be productively infected upon viral inoculation, we needed to  
172 image a large population of cells at each time point tested to provide an accurate portrait of the  
173 heterogeneity present in a population of asynchronously infected cells. Thus, it was important for  
174 this study to both image and quantitatively characterize the smFISH staining of viral RNAs in a  
175 high-throughput fashion. To accomplish this goal, we automatically segmented the nuclei using  
176 DAPI and cell outlines using CellMask Green fluorescent staining with CellProfiler software  
177 (34) (Fig. 4A). Next, smFISH-labeled viral RNAs were detected using FISH-quant software (35)  
178 (Fig. 4B). We were able to image two distinct RNA smFISH probe sets labeled with spectrally-  
179 distinct fluorophores in individual cells. This allowed us to characterize relative viral RNA  
180 expression levels and compare localization of different viral RNAs (Figs. 4B to C). We were  
181 able to robustly quantify viral RNAs using FISH-quant across a range of expression levels. We  
182 observed a direct relationship between the number of detected spots and the total fluorescence

183 signal in the smFISH channel up to approximately 1,000 RNAs/cell, after which the number of  
184 detected viral RNAs reached a plateau (Fig. 4D). This represents the point at which smFISH  
185 spots are so dense, that we were no longer able to accurately distinguish closely spaced RNAs.  
186 Examples of a cell displaying moderate levels of viral RNAs where identification of diffraction  
187 limited spots was robust (Fig. 4B) and a cell with very high expression of viral RNAs where  
188 overcrowded spots are unable to be effectively spatially resolved (Fig. 4C) are shown for  
189 reference. Thus, when viral RNA levels are relatively low (less than a thousand copies per cell)  
190 we have high confidence in the accuracy of the quantification provided by FISH-quant.  
191 However, when viral RNA levels are at their peak and RNAs are very dense, quantification is an  
192 underestimate of RNA expression levels and may complicate our ability to assess relative levels  
193 of different RNA species when both are expressed at high levels.

194 **Viral RNA transcription and replication following viral entry.** We next aimed to  
195 monitor the early events of viral genomic transcription and replication immediately following  
196 viral entry. Cells were infected with LCMV at an MOI of 0.1, fixed at multiple time points,  
197 stained for NP mRNA/S antigenome, GPC mRNA/S genome, or L mRNA/L antigenome, and  
198 several hundred cells were imaged and analyzed at each time point. As discussed above in  
199 relation to Fig. 2, our probes sets specific for only genome or antigenome (but not an additional  
200 complementary viral mRNA) have low signal-to-noise ratios and sensitivity when compared to  
201 probe sets that also target a viral mRNA. Importantly, FISH-quant was unable to detect viral  
202 genome or antigenome spots in cells that had been infected with LCMV for less than 8 h (Fig. 2  
203 and data not shown). However, by 8 hpi and later, genome and antigenome spots become  
204 detectable with these probe sets (Fig. 2) (32). Therefore, in this set of experiments, smFISH spots  
205 detected with the NP mRNA/S antigenome, GPC mRNA/S genome, or L mRNA/L antigenome

206 probe sets prior to 8 hpi are presumed to represent only the designated mRNA target in each  
207 case, whereas at 8 hpi and later it is possible to detect a mixture of the targeted RNAs.

208 Representative images of cells infected from 0 to 6 hpi are shown (Fig. 5A to B).  
209 Notably, transcription of the NP mRNA and L mRNA is detected as early as 1 hour following  
210 infection (Fig. 5 and 6A to B) indicating primary transcription of the S and L genomic RNA  
211 occurs soon after entry and uncoating of arenavirus virions. The GPC mRNA, on the other hand,  
212 is first detected at 6 hours following infection (Fig. 5A and 6A and C). This delayed appearance  
213 of the GPC mRNA suggests that transcriptionally competent S antigenomic RNA is not  
214 delivered into cells by incoming virions. Further, it suggests that a 4-6 hour lag is required for  
215 the production of S antigenomic RNA, which serves as the template for transcription of GPC  
216 mRNA (Fig. 1A). This result is in agreement with previous studies that examined arenavirus  
217 mRNA synthesis via Northern blot (5, 26).

218 When examining the subcellular localization of NP mRNA and GPC mRNA or NP  
219 mRNA and L mRNA pairs at 6 hpi or earlier, no overt colocalization between viral mRNAs was  
220 noted (Fig. 5A to B).

221 **Disproportionate transcription of S segment genes early after infection.** For each  
222 probe set used in the experiments shown in Figs. 5 and 6, the number of false-positive viral  
223 RNAs detected in mock-infected cells was used to establish a threshold to classify cells as either  
224 “positive” or “negative” for each of the tested viral RNA species. At 6 hpi (a time point before  
225 virus in the initially infected cells could have completed its life cycle and spread to adjacent,  
226 initially uninfected cells (36-39), we observed that 65-90% of cells were positive for NP mRNA  
227 and 40% were positive for GPC mRNA (Fig. 6C to D). This high frequency of cells containing S  
228 segment-derived transcripts was surprising given the fact we initially infected cells at an MOI of

229 0.1, and thus would have expected approximately only 10% of cells to have been expressing  
230 viral RNAs at this early time point. However, at this same early time point, only 8% of cells  
231 were positive for L segment-derived L mRNA (Fig. 6D), which is consistent with the expected  
232 frequency of viral RNA-positive cells based on the initial MOI. This result may suggest that a  
233 high proportion of viral particles either fail to package the L genome or alternatively deliver a  
234 transcriptionally-defective L genome.

235 **Viral RNA replication and transcription at peak of acute infection.** To profile LCMV  
236 RNAs at the peak of infectious virus release during acute infection, cells were infected with  
237 LCMV at an MOI of 0.01, fixed at various time points between 12 and 96 hpi, stained for NP  
238 mRNA/S antigenome, GPC mRNA/S genome, or L mRNA/L antigenome, and several hundred  
239 cells were imaged and analyzed at each time point. Levels of viral RNAs detected by each probe  
240 set rapidly increased over the first 24 hours of infection (Fig. 7A to B and 8A to B). The  
241 proportion of cells positive for these viral RNAs also rapidly increased over the first 24 hours of  
242 infection such that almost all cells had substantial levels of all viral RNAs (Fig. 8C to D). Peak  
243 viral transcription and replication occurred at 36 hours post infection (Fig. 7A to B and 8A to B).  
244 At this time point, viral smFISH signal was very dense and true levels of viral NP mRNA and  
245 GPC mRNA were likely underestimated due to inability of FISH-quant to accurately count  
246 tightly packed viral RNAs in the cytoplasm of infected cells (Fig. 4B to C, 7A to B, and 8A to  
247 B). Nevertheless, it is clear that RNAs detected by the S segment-specific probe sets greatly  
248 exceeded those detected by the L segment-specific probe set (at least 10-35 fold greater) between  
249 12 and 96 hpi (Fig. 8A to B, Table 1). Following peak viral transcription and replication at 36  
250 hours post-infection, levels of viral RNAs began to decrease (Fig. 7A to B and 8A to B). The  
251 proportion of cells positive for L mRNA/L antigenome expression decreased steadily beginning

252 at 48 hours post-infection (Fig. 8D). In contrast, all cells maintained NP mRNA/S antigenome  
253 and GPC mRNA/S expression over this entire time period (Fig. 8C to D).

254 **Cyclical patterns of infectious virus production and antigen expression during**  
255 **persistent infection.** A key feature of arenavirus infection in cell culture is the cyclical pattern of  
256 infectious virus release observed during the persistent phase of infection (16, 19, 40-42). We  
257 were particularly interested in using smFISH to assess how viral gene expression programs  
258 change during persistence and to examine how this correlated with production of infectious virus  
259 and translation of viral antigens. Toward this goal, cells were infected with LCMV at an MOI of  
260 0.01, supernatants were collected and cells were fixed at multiple time points between 1.5 and 30  
261 days (d)pi. As shown in Fig. 9A, A549 cells persistently infected with LCMV cyclically released  
262 waves of infectious virus over the first 30 days (Fig. 9A). We were curious how much viral  
263 antigen and viral RNA would be expressed in persistently infected cells at time points when cells  
264 were releasing high levels of infectious viral particles but also how much viral material was  
265 expressed at time points when cells released very little infectious virus. Therefore, we performed  
266 IFA to visualize NP or GPC and smFISH to visualize NP mRNA/S antigenome and GPC  
267 mRNA/S in cells at multiple time points that corresponded to high and low points in infectious  
268 viral release (Fig. 9B to C). At 1.5 (d)pi, all cells expressed NP and GPC protein (Fig. 9B to C).  
269 Viral antigen expression mirrored the cycles of infectious virus release as seen in Fig. 9A. Viral  
270 antigen was expressed in fewer cells and/or at lower levels at 8 and 27 (d)pi (time points with  
271 low levels of infectious viral release) compared to 13 and 30 (d)pi (time points with higher levels  
272 of infectious viral release) (Fig. 9B to C). While our goal was to visualize both viral RNA and  
273 viral antigen in infected cells by fluorescence microscopy, the sensitivity of smFISH was greatly  
274 reduced when combined with IFA – as evidenced by higher levels of background and lower

275 signal intensity (Fig. 9B to C). However, taking into account the lower sensitivity of smFISH in  
276 this particular experiment, we did see a correlation between the presence of viral RNA within  
277 cells and the expression of viral proteins (Fig. 9B to C). Thus, these results suggest that cells  
278 infected with LCMV can clear the infection as evidenced by the majority of cells at specific time  
279 points (e.g. 8 and 27 dpi) that lack viral RNA or protein and produce little infectious virus.

280 **Cyclical patterns of genome transcription and replication during persistent**  
281 **infection.** As smFISH sensitivity in our hands is diminished when performed in conjunction with  
282 immunofluorescent staining, we next used smFISH alone to examine the transcription and  
283 replication dynamics of arenavirus genomic RNA during the persistent phase of infection at the  
284 level of individual RNA molecules. Cells were infected with LCMV at an MOI of 0.01, fixed at  
285 multiple time points between 1.5 and 41 days (dpi), stained for NP mRNA/S antigenome, GPC  
286 mRNA/S genome, or L mRNA/L antigenome, and several hundred cells were imaged and  
287 analyzed at each time point. Note that our smFISH assay does not allow us to follow particular  
288 cells over time, but instead to capture the viral RNA signature of individual cells at specific time  
289 points during infection. Following peak RNA transcription and replication at 36 hpi, we  
290 observed decreased levels of NP mRNA/S antigenome, GPC mRNA/S genome, or L mRNA/L  
291 antigenome over the next several days such that at 8 dpi, the majority of cells examined were  
292 negative for all of these viral RNAs (Fig. 10A to F). However, by 13 dpi, the levels of viral  
293 RNAs detected by each probe set were increased and the majority of cells were again positive for  
294 all viral RNAs (Fig. 10C and F). Viral RNA levels again dropped and many cells were no longer  
295 positive for viral RNA by 16 dpi. (Fig. 10C and F). This pattern of viral RNA clearance followed  
296 by increased levels of viral RNA expression and increased frequency of viral RNA expressing  
297 cells in the population repeated in a cyclical fashion multiple times over the first 41 days

298 following infection (Fig. 10). In summary, the sequential loss and reappearance of viral gene  
299 expression observed in these studies may represent a genetic signature of the previously  
300 recognized phenomenon of cyclical production of infectious LCMV virions over time in cell  
301 culture models of persistent infection (16-18).

302 Throughout the time course of persistence examined in this study, NP mRNA/S  
303 antigenome was generally expressed at higher levels than GPC mRNA/S genome (up to 5-fold  
304 higher levels) (Fig. 10B and Table 1). The ratio between levels of NP mRNA/S antigenome and  
305 L mRNA/L antigenome over this time period was more variable. At time points such as 13 and  
306 20 dpi, when most cells in the culture are positively expressing all viral RNA species, NP  
307 mRNA/S antigenome greatly outnumbered L mRNA/L antigenome (~25-fold higher levels) (Fig.  
308 10E and Table 1). However, at other times such as 8, 16, 27, and 34 dpi, when substantial  
309 proportions of cells had lost expression of one or more viral RNAs, the ratio between NP  
310 mRNA/S antigenome and L mRNA/L antigenome in the double-positive cells was greatly  
311 reduced (~2-fold higher levels of NP than L mRNA) (Fig. 10E and Table 1). Notably, the  
312 magnitude of viral RNA expression during persistence never reached the high levels observed at  
313 the peak of acute infection (Fig. 10).

314

## 315 **DISCUSSION**

316 In the current study, we developed a high throughput smFISH assay that allowed us to  
317 visualize single copies of LCMV RNAs in individual cells. Taking advantage of the sensitivity  
318 and quantitative aspect of this assay, we tracked the dynamics of viral replication and  
319 transcription spanning early time points following initial virus entry to late times during



320 persistent infection. We observed that transcription of the negative-sense encoded NP and L  
321 mRNAs precede that of the pseudo positive-sense encoded GPC mRNA, confirming the  
322 temporal separation of gene expression predicted by the ambisense coding strategy of the  
323 arenaviruses and suggesting that antigenomic RNA in virions is not transcriptionally active  
324 following release into a newly infected cell. Our studies demonstrated a hierarchal pattern of  
325 expression among viral RNAs and indicate that many infecting virus particles may lack L  
326 genomic RNA. Finally, over the course of persistent infection, we observed repeated cycles  
327 whereby cells appeared to transition from supporting active viral replication and transcription to  
328 a state where viral RNA is undetectable by smFISH. Collectively, these studies improve our  
329 understanding of the natural history of arenavirus replication and transcription during acute and  
330 persistent infection.

331         The smFISH assay developed here provided us with an opportunity to build upon prior  
332 studies and examine arenavirus genome replication and transcription with greater sensitivity and  
333 detail. Previous studies aimed at elucidating the early events of arenavirus transcription and  
334 genome replication used Northern blot to visualize individual viral RNA species (23, 25, 26).  
335 Analysis of RNA from cells infected with LCMV or the New World arenavirus Pichinde failed  
336 to detect viral RNA from infected cells prior to 9 hpi (23) or 12 hpi (25), respectively. In the  
337 setting of infection with the New World arenavirus Tacaribe, Franze-Fernandez *et al.* detected S  
338 genomic RNA and NP mRNA at 2 hpi and S antigenomic RNA at 4 hpi, while GPC mRNA  
339 appeared several hours following the synthesis of S antigenomic RNA (26). The earliest the viral  
340 L segment has been observed was at 12 hpi (23). In the current study, we are able to detect viral  
341 NP and L mRNAs at 1 hpi. Our data supports previous observations that viral NP mRNA  
342 expression occurs immediately following infection, and that GPC mRNA expression occurs

343 following a lag of several hours (5, 26). By probing single cells, we build upon this prior work  
344 by demonstrating that GPC mRNA expression is not detected, even at low levels, in the first  
345 hours following infection. In light of previous observations that antigenomic L and S segment  
346 RNAs are packaged in viral particles (20, 26), our inability to observe GPC mRNA in cells  
347 immediately following viral entry suggests that S antigenomic RNA packaged in virions is  
348 unable to be transcribed. Further, it suggests that GPC mRNAs are not packaged into viral  
349 particles, as has been suggested for Z mRNA (43).

350 An interesting observation from our study was that, despite infecting cells at an MOI of  
351 0.1, ~65-90% of cells expressed one or more genes encoded on the S genomic RNA segment at 6  
352 hpi. Because it takes ~ 8 h for an infected cell to make new infectious progeny (36-39), we were  
353 surprised to see such a high frequency of cells expressing these viral mRNAs at a time when the  
354 originally-infected cells could not yet have spread virus to additional uninfected cells in the  
355 monolayer. Notably, at this same 6 hpi time point, approximately 8% of cells expressed viral L  
356 mRNAs, which is consistent with the utilized MOI. One possible explanation for this observation  
357 could be that, within the viral stock, there may be a significant population of incomplete viral  
358 particles that possess the S segment but lack the L segment genomic RNA or a functional copy  
359 of this RNA. Indeed, this phenomenon has been observed for influenza A and Rift Valley Fever  
360 virus (44-46). Considering that the genetic basis for how arenavirus DI particles block the  
361 propagation of infectious virus particles is unknown, these results may provide clues for future  
362 studies to define the mechanism at work. Further examination will be necessary to explain the  
363 functional significance of this observation.

364 A hallmark characteristic of LCMV infection is the ability to establish an asymptomatic,  
365 persistent infection in reservoir rodents (47). Further, it is possible to recapitulate key aspects of

366 this persistent infection in cell culture models of infection (5, 40, 41, 48). One notable  
367 characteristic of cell culture models of LCMV infection is the cyclical rise and fall of release of  
368 infectious virus seen during persistence (16, 19, 40-42). Several models have been proposed to  
369 explain how LCMV restricts its spread to establish and maintain a noncytopathic persistent  
370 infection, both *in vitro* and *in vivo*. The first suggests that DI particles, which are produced in  
371 abundance by LCMV, can enter permissive host cells and interfere with the ability of standard  
372 infectious virus particles to successfully infect and complete the viral life cycle (9-13). Hotchin  
373 proposed a second model that he termed cyclical, transient infection. In this model, cells infected  
374 with LCMV are initially productive in making infectious virus particles, but later become  
375 refractory to superinfection and ultimately clear virus (as evidenced by loss of antigen expression  
376 and infectious virus production), only to once again become susceptible to reinfection by the  
377 small number of cells that remain productively infected (16-19). Southern and colleagues  
378 proposed a third model that was based upon the dynamics and genetic identity of viral RNA  
379 species profiled during acute and persistent infection. In particular, they demonstrated by  
380 Northern blot that LCMV RNAs (genome, antigenome, and mRNAs) accumulate to high levels  
381 during persistence, both *in vitro* and *in vivo* (5, 14, 15, 22). Further, they showed that a  
382 proportion of these genomic and antigenomic RNAs, but not mRNAs, contained short deletions  
383 in the untranslated regions at their termini (5, 14, 15, 49). They proposed that these deleted  
384 RNAs were replication competent, but transcriptionally incompetent. This data suggested a  
385 model where, during persistence, viral protein expression and infectious virus production are  
386 inhibited due to the accumulation of high levels of transcriptionally defective genomic and  
387 antigenomic RNAs. Further, because these deleted RNAs were found in virions, it was proposed  
388 that they serve as the molecular basis for DI particle interference. Finally, it was proposed that

389 these deleted RNAs can be repaired by the viral polymerase to initiate bursts of productive  
390 replication/infectious virus production during persistence. Each of these models, whether acting  
391 independently or in combination, would presumably restrict virus spread, allowing the virus to  
392 minimize its impact on host fitness while retaining its ability to propagate and ultimately  
393 maintain itself in nature. A potential caveat to the Southern model comes from the finding that  
394 the 3' and 5' terminal sequences of the LCMV and LASV genomic RNA species were critical  
395 for both the transcription and replication of RNAs in a minigenome reporter assay (50, 51). Thus,  
396 the functional significance of these terminally truncated viral RNAs remains to be further  
397 elucidated.

398         The smFISH approach employed in this study can provide insights to build on this prior  
399 work. First, our study suggests that cells do not maintain high levels of genomic and antigenomic  
400 RNAs throughout persistence. The most likely explanation for the observation of high levels of  
401 genome/antigenome during persistence by Southern and colleagues (5, 14, 15, 22) is that they  
402 measured viral RNAs from a fraction of the total cells within a population at a particular time  
403 point. Second, our findings of large numbers of cells lacking viral RNAs at several persistent  
404 time points (which correlates with loss of infectious virus release and antigen expression)  
405 suggest that the antigen-negative cells observed by Hotchin et al. (16-19) had either cleared  
406 infection or contained undetectable levels of RNA (by smFISH) as opposed to carrying  
407 replication-competent, but transcriptionally-incompetent, terminally-deleted  
408 genomic/antigenomic RNAs (as the Southern model would suggest (5, 14, 15, 49)). Our smFISH  
409 probe sets consisted of pools of 48 short oligo nucleotide probes complementary to different  
410 regions spanning entire viral gene sequences. Therefore, even if genomic segments contained  
411 short terminal deletions, the majority of smFISH probes would bind their targets, and obvious

412 smFISH signal would be present by fluorescence microscopy. Our findings also raise several  
413 new questions that require further study. For example, it will be important to define the  
414 molecular mechanism by which cells clear virus following infection and the specific host  
415 machinery responsible. Additionally, it would be particularly interesting to investigate the  
416 possibility that cycles of reinfection are jump-started from cells maintaining a reservoir of  
417 intracellular infectious virus as has been documented in the literature (52, 53).

418 In summary, we have used fluorescence microscopy to visualize fluorescently-labeled  
419 arenavirus RNA molecules in infected cells. Further, we have described a flexible labeling,  
420 imaging, and image analysis pipeline that could be easily adapted to interrogate the events of  
421 transcription or genomic replication of any RNA virus, particularly where it is critical to image  
422 and quantify RNA levels in hundreds to thousands of cells per experimental condition. We have  
423 taken advantage of this pipeline to examine the transcription and replication kinetics of LCMV  
424 RNAs over the course of infection. In particular, our data support the transient, cyclical infection  
425 model originally proposed by Hotchin (16-19) and suggest that, following a period of productive  
426 infection, cells can clear infection, including viral genetic material and antigen, before becoming  
427 susceptible to reinfection. Further, our data provides some support for the ideas that viral  
428 antigenomic RNA in virions may not be transcriptionally functional upon virus entry and that a  
429 significant fraction of virus particles may lack functional L genomic RNA, an observation that  
430 remains to be fully explored. Developing the ability to label genomic and antigenomic RNAs  
431 with greater sensitivity will be an important next step toward the construction of a quantitative  
432 model of the regulation of viral RNA replication and transcription over time with the goal of  
433 explaining the oscillatory behavior of viral RNA synthesis during persistence. While the cell  
434 culture model of persistent infection has provided interesting observations, it will be particularly

435 important to examine how the genetic events of transcription and replication are regulated in an  
436 *in vivo* model of persistent infection in future studies.

437

## 438 MATERIALS AND METHODS

439 **Cells and Viruses.** A549 (CCL-185) were obtained from American Type Culture  
440 Collection (ATCC, Manassas, VA). A549 cells were cultured in DMEM-F12 (11320-033,  
441 Thermo Fisher), containing 10% fetal bovine serum and 1% Penicillin-Streptomycin (15140-163,  
442 Thermo Fisher). LCMV Armstrong 53b was provided by J. L. Whitton (The Scripps Research  
443 institute, La Jolla, CA). A549 cells were infected with LCMV Arm53b at an MOI of 0.1 (Fig. 5  
444 to 6) or an MOI of 0.01 (Fig. 1, 2, 4, 7, 8, 9, and 10). For experiments examining late, persistent  
445 time points following infections, a T25 tissue culture flask of A549 cells was infected. The flask  
446 of infected cells was trypsinized and cells were plated on glass coverslips 24 hours prior to the  
447 reported time points where cover slips were fixed, stained, and imaged as described below (Fig.  
448 10). Remaining cells were diluted and re-plated in a T25 flask until 24 hours before the next  
449 examined time point where this process was repeated. Standard plaque assay on Vero E6 cells  
450 was used to determine the titer of infectious LCMV in collected supernatants. No cytopathic  
451 effect was observed in persistently infected cultures.

452 **Single molecule RNA-FISH.** Cells were plated on 14 mm round #1 glass coverslips.  
453 Following infection, cells were briefly washed in room temperature DPBS (with Calcium and  
454 Magnesium) (14040133, Thermo Fisher) and fixed in 4% PFA in 1x PBS for 10 minutes at room  
455 temperature. Coverslips were washed twice in room temperature PBS and fixed again at -20° C  
456 with 70% ethanol for at least two hours. Coverslips were washed twice with 2x SSC (AM9770,

457 Thermo Fisher) and washed once with 2x SSC and 10% Formamide (BP227, Fisher Scientific).  
458 smFISH probes to different viral RNA species (Fig. 1A) were designed using the Stellaris Probe  
459 Designer at <https://www.biosearchtech.com/> (Table S1). Unlabeled smFISH probes had a 3'  
460 modified base with an amine functional group. Pools of 48 individual smFISH probes to a  
461 particular target RNA were combined at equimolar ratios and were covalently labeled with Cy3  
462 (PA23001, GE Healthcare), AlexaFluor 568 (A20003, Thermo Fisher), or Cy5 (PA25001, GE  
463 Healthcare) as previously described (54). Coverslips were placed face down on a 100 ul drop of  
464 hybridization mix containing 75 ng of smFISH probe dissolved in hybridization buffer composed  
465 of 10% dextran sulfate (D8906, Sigma-Aldrich), 2x SSC, and 10% Formamide. Hybridization  
466 occurred in a humidified chamber at 37° C overnight. Coverslips were washed twice in 2x SSC,  
467 10% formamide at 37° C for 30 minutes. Coverslips were then washed once in 1x PBS. For  
468 cellular segmentation, cells were stained with HCS CellMask™ Green stain (H32714, Thermo  
469 Fisher) diluted at 50 ng/ml in PBS for 5 minutes at room temperature (note: this is significantly  
470 more dilute than recommended in the product information, but we found it necessary to prevent  
471 overstaining cells and thus to prevent spectral bleed through into the AlexaFluor 568  
472 fluorescence channel). Nuclei were stained with 4', 6-diamidino-2-phenylindole hydrochloride  
473 30 (DAPI) (D9542, Sigma-Aldrich) at 1 µg/ml in PBS for 5 minutes at room temperature. Cells  
474 were washed a final time in PBS, briefly washed in water, dried and mounted with ProLong Gold  
475 Antifade Reagent (P36934, Thermo Fisher).

476 Combined immunofluorescence and smFISH staining protocols were performed as  
477 previously described (55). LCMV nucleoprotein (NP) was labeled with antibody 1-1.3 and  
478 glycoprotein (GPC) was labeled with antibody 33.6 (both from M. Buchmeier, University of

479 California Irvine). Primary antibodies were visualized with a secondary Alexa Fluor 488-  
480 conjugated goat anti-mouse IgG (H+L) (A-11029, Thermo Scientific).

481 **Image Acquisition.** Wide-field fluorescent Z-stacks were acquired using a Nikon Ti  
482 Eclipse microscope with a  $60 \times 1.4$  NA objective. Samples were illuminated with an LED light-  
483 source (Lumencor Spectra X light engine) with appropriate filter sets and images were captured  
484 with a Hamamatsu Orca flash 4.0 LT sCMOS camera. Z-stacks were captured at 300 nm  
485 increments, and the microscope was controlled by Nikon NIS Elements software. Captured ND2  
486 images were converted to Tiffs using the open source Bio-formats tool kit  
487 (<http://www.openmicroscopy.org/>) (56).

488 **Image Segmentation and Analysis.** DAPI and CellMask Green Z-stacks were projected  
489 using a focus-based projection method as previously described (57). Projected DAPI images  
490 were used for automatic nuclear segmentation in CellProfiler (Broad Institute) (34) and served as  
491 the seed for automatic secondary cellular segmentation using the projected CellMask Green  
492 images (Fig. 4A). Statistics including average pixel intensity within the regions defined by  
493 primary and secondary segmentation were extracted from maximum intensity projections of  
494 smFISH Z-stacks using CellProfiler (Fig. 4B to D).

495 Single smFISH labeled RNAs were detected and localized in 3D using FISH-quant (35).  
496 Briefly, smFISH Z-stacks were filtered using the “Dual Gaussian Filter” and spots were detected  
497 using the “Local Maximum” method. As a large number of acquired images required analysis,  
498 images were analyzed in “Batch Mode” with settings determined to give low rates of false  
499 positive detections. The signal-to-noise ratio of different smFISH probe sets was determined as  
500 the average signal amplitude of identified smFISH spots in an individual cell divided by the



501 standard deviation of the fluorescent signal in a region of the same cell where smFISH spots  
502 were absent (Fig 3).

503 Box and whisker plots were created using the ggplot2 package in R. The box represents  
504 the interquartile range of the data with the center line representing the median. Individual dots  
505 represent cells that are more than 1.5 times the interquartile range away from the median of the  
506 data.

507

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525

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678

679 **FIGURE LEGENDS**

680 FIG 1 LCMV RNA species can be specifically visualized using multiple, singly-labeled  
681 oligonucleotide smFISH probes.

682 (A) Overview of the scheme used by arenaviruses to transcribe and replicate their single-  
683 stranded, ambisense, bisegmented genome. smFISH probes that recognize the S segment  
684 genomic RNA are shown in gray, probes that recognize the S segment genome and GPC mRNA  
685 are shown in red, probes that recognize the S segment antigenome and NP mRNA are shown in  
686 green, and probes that recognize the L segment antigenome and L mRNA are shown in pink.  
687 smFISH probe sets consist of pools of 48 individual 20mer oligonucleotides each labeled with a  
688 single fluorophore at their 3' terminus. (B) Uninfected cells were stained with a control smFISH  
689 probe set specific to the cellular mRNA MDN1 labeled with Cy3. (C) Cells were either infected  
690 with LCMV at an MOI of 0.01 or, as a control, remained uninfected (mock). Cells were fixed at  
691 24 hpi and stained with a Cy5-labeled smFISH probe set specific for S segment genomic RNA  
692 and GPC mRNA. Boxed regions of the cell are magnified and shown in columns labeled  
693 "Zoom". Green arrows indicate example smFISH stained spots most likely representing single  
694 labeled RNAs. Nuclear (hatched line) and cytoplasmic (solid line) boundaries are shown in blue.  
695 The same intensity levels for a particular probe set were applied to all images of mock- and  
696 LCMV-infected cells to permit comparisons. Scale bars are 10  $\mu$ m.

697

698 FIG 2 smFISH probe sets recognizing viral mRNA species exhibit high signal-to-noise staining.

699 Mock- or LCMV-infected cells (24 hr pi) were simultaneously stained with smFISH probe sets  
700 specific for either GPC mRNA and S genome (Cy5; green) or S genome only (AlexaFluor 568;  
701 red). Representative LCMV infected cells with moderate (A) or high (B) levels of viral RNA as  
702 well as a representative mock-infected cell (C) are displayed. Multiple Z stacks were acquired  
703 spanning the thickness of the cell and max intensity projections are displayed. Boxed regions of  
704 the cell are magnified and shown in rows labeled “Zoom”. Nuclear (hatched line) and  
705 cytoplasmic (solid line) boundaries are shown in blue. (A to C) The same intensity levels for a  
706 particular probe set were applied to all images of mock- and LCMV-infected cells to permit  
707 comparisons. Scale bars are 10  $\mu$ m.

708

709 FIG 3. smFISH probe sets recognizing viral mRNA species exhibit high signal-to-noise staining  
710 Signal-to-noise ratio of different smFISH probe sets labeled with the indicated fluorophores.  
711 Signal-to-noise ratio was calculated as average amplitude of detected smFISH spots divided by  
712 the standard deviation of signal in a region of the cell with no detected spots. The signal-to-noise  
713 ratio of 20 cells per smFISH probe set labeled with the indicated fluorophore was calculated, and  
714 the mean and standard deviation are graphed.

715

716 FIG 4 Automated detection and quantitation of LCMV RNAs labeled with spectrally distinct  
717 fluorophores.

718 (A) Cell nuclei and cytoplasms were automatically segmented using focus-based projections of  
719 DAPI (nuclei) or CellMask Green (cytoplasm) Z stacks acquired through the thickness of the  
720 cell. Note that pixel intensities of the CellMask Green projection displayed here have been log

721 transformed to aid visualization. Nuclear (hatched line) and cytoplasmic (solid line) boundaries  
722 are shown in white. The scale bar is 10  $\mu\text{m}$ .

723 (B and C) Maximum intensity projections of LCMV-infected cells were fixed 24 hpi and stained  
724 with smFISH probe sets to the NP mRNA/S antigenome (Cy5; green) and GPC mRNA/S  
725 genome (A568; red). The boxed region of each cell is magnified and shown in the row labeled  
726 “Zoom”. Cells were segmented based on DAPI and CellMask Green staining (see panel A) and  
727 spots were detected and localized in 3D using FISH-quant. Individually detected RNAs are  
728 circled in green (NP mRNA/S antigenome) or red (GPC mRNA/S genome). The “Spots only”  
729 column shows only the position of detected spots in relation to the cell boundaries defined by  
730 segmentation. Nuclear (hatched line) and cytoplasmic (solid line) boundaries are shown in blue.  
731 The same intensity levels for a particular probe set were applied to both images of LCMV-  
732 infected cells to permit comparisons. The scale bar is 10  $\mu\text{m}$ .

733 (D) Scatter plot shows the relationship between the fluorescence intensity in the smFISH channel  
734 in the maximum intensity projection of smFISH images and the number of smFISH spots  
735 detected by FISH-quant for LCMV-infected cells fixed 24 hpi and stained with the Cy5-labeled  
736 smFISH probes specific for NP mRNA/S antigenome.

737

738 FIG 5 Transcription of NP and L genes is detectable soon following infection while GPC  
739 transcription exclusively occurs after a several hour lag.

740 Cells were infected with LCMV at an MOI of 0.1, fixed at various times following infection, and  
741 stained for NP mRNA (green) using a Cy5-labeled NP mRNA/S antigenome probe set, GPC  
742 mRNA (red) using an A568-labeled GPC mRNA/S genome probe set (A) or NP mRNA (green)

743 using an A568-labeled NP mRNA/S antigenome probe set and L mRNA (magenta) using a  
744 Quasar 670-labeled L mRNA/L antigenome probe set (B). Note that for the time points shown  
745 (less than 8 hpi), genomic and antigenomic RNAs are not detectable by smFISH probe sets with  
746 exclusive specificity for these RNAs (data not shown). Therefore, spots detected in this figure  
747 are presumed to represent only the mRNAs, but not the genome or antigenome, targeted by each  
748 respective probe set. Nuclear (hatched line) and cytoplasmic (solid line) boundaries as  
749 determined by Cell Profiler are shown in blue. Identified spots are outline by circles that are  
750 green for NP mRNA, red for GPC mRNA, and magenta for L mRNA. (A and B) The same  
751 intensity levels for a particular probe set were applied to all images of mock- and LCMV-  
752 infected cells across the time course to permit comparisons. Representative maximum intensity  
753 projections from 1 of 2 independent experiments are shown. Scale bar is 10  $\mu$ m.

754

755 FIG 6 Transcription of NP and L genes is detectable immediately upon infection while GPC  
756 transcription exclusively occurs after a several hour lag.

757 Related to Fig. 5. (A and B) Boxplots represent the number of viral RNAs detected in cells at  
758 early time points following infection with LCMV (see Figure 5). (C and D) Stacked bar graphs  
759 show the proportion of cells expressing RNAs detected by one, both, or neither viral RNA  
760 smFISH probe set. Between 620 and 1316 cells were examined at each time point. In each case  
761 RNAs identified by specific probe sets are designated by color (green for NP mRNA/S  
762 antigenome probes, red for GPC mRNA/S genome probes, and magenta for L mRNA/L  
763 antigenome probes). Note that for time points prior to 8 hpi, genomic and antigenomic RNAs are  
764 not detectable by smFISH probe sets with exclusive specificity for these RNAs (data not shown).  
765 Therefore, spots detected before 8 hpi are presumed to represent only the mRNAs, but not the

766 genome or antigenome, recognized by each respective probe set. Spots detected at 8 hpi or later  
767 are presumed to be a mixture of all RNAs recognized by a particular probe set (e.g. mRNA and  
768 genome or antigenome).

769

770 FIG 7 Peak viral RNA replication and transcription occurs 36 hpi and is slowly lost from  
771 infected cells over the following days.

772 Cells were infected with LCMV at an MOI of 0.01, fixed at various times following infection,  
773 and stained using smFISH probe sets specific for NP mRNA/S antigenome (Cy5; green) and  
774 GPC mRNA/S genome (A568; red) (A) or NP mRNA/S antigenome (A568; green) and L  
775 mRNA/L antigenome (Quasar 670; magenta) (B). (A and B) Representative maximum intensity  
776 projections of fields of infected cells at various time points from 1 of 2 independent experiments  
777 are shown. Each probe set is shown in its own row to highlight the difference in levels to which  
778 these RNAs accumulate. The same intensity levels for a particular probe set were applied to all  
779 images of mock- and LCMV-infected cells across the time course to permit comparisons. Scale  
780 bars = 10  $\mu$ m.

781

782 FIG 8 Peak viral RNA replication and transcription occurs 36 hpi and is slowly lost from  
783 infected cells over the following days.

784 Related to Fig. 7. (A and B) Boxplots represent the number of mRNAs detected in cells at time  
785 points during the peak period of LCMV infection. (C and D) Stacked bar graph shows the  
786 proportion of cells expressing RNAs detected by one, both, or neither viral smFISH probe set.  
787 Between 480 and 1659 cells were examined at each time point. RNAs identified by specific

788 probe sets are designated by color (green for NP mRNA/S antigenome probes, red for GPC  
789 mRNA/S genome probes, and magenta for L mRNA/L antigenome probes).

790

791 FIG 9 Cyclic periods of infectious virus particle release and antigen expression during  
792 persistence.

793 Cells were infected with LCMV at an MOI of 0.01. (A) Supernatants from infected cells were  
794 collected at indicated time points post infection, titered, and the data are presented as mean  
795 PFU/ml  $\pm$  SD from 3 independent experiments. (B to C) Cells were fixed at the indicated time  
796 points following infection and stained using smFISH probe sets specific for NP mRNA/S  
797 antigenome (A568; red), GPC mRNA/S genome (Cy5; magenta), and NP protein (Alexa 488;  
798 green) (B) or GPC protein (Alexa 488; green) (C). A single Z slice of a representative field of  
799 infected cells at various time points is shown. Each antibody or probe set is shown in its own row  
800 and the same intensity levels were applied to all images of mock- and LCMV-infected cells  
801 across the time course to permit comparisons. Scale bar is 10  $\mu$ m.

802

803 FIG 10 Cyclic periods of viral RNA production and viral RNA loss occur during persistence.

804 Cells were infected with LCMV at an MOI of 0.01, fixed at the indicated time points following  
805 infection, and stained using smFISH probe sets specific for NP mRNA/S antigenome (Cy5;  
806 green) and GPC mRNA/S genome (A568; red) (A) or NP mRNA/S antigenome (A568; green)  
807 and L mRNA/L antigenome (Quasar 670; magenta) (D). (A and D) Representative maximum  
808 intensity projections of fields of infected cells at various time points from 1 of 2 independent  
809 experiments are shown. Each probe set is shown in its own row to highlight the difference in

810 levels to which these RNAs accumulate. The same intensity levels for a particular probe set were  
811 applied to all images of mock- and LCMV-infected cells across the time course to permit  
812 comparisons. Scale bar is 10  $\mu\text{m}$ . (B and E) Line graphs show the average number of the  
813 indicated viral RNAs detected in cells at time points during the persistent phase of LCMV  
814 infection. (C and F) Stacked bar graph shows the proportion of cells expressing RNAs detected  
815 by one, both, or neither viral smFISH probe set. Between 316 and 1218 cells were examined at  
816 each time point.

817 **Table 1: Ratio in the expression levels of viral mRNAs in individual infected cells.**

	Time points	Ratio NP mRNA and S antigenome /GPC mRNA and S genome ( $\pm$ SD)	Ratio NP mRNA and S antigenome /L mRNA and L antigenome ( $\pm$ SD)
Early time points (hpi) MOI = 0.1	0.5	ND	ND
	1	ND	ND
	2	ND	ND
	3	ND	ND
	4 <sup>a</sup>	4.3 $\pm$ 3.4	ND
	6 <sup>a</sup>	6.0 $\pm$ 4.0	14.7 $\pm$ 9.5
	8	4.9 $\pm$ 4.1	14.8 $\pm$ 12.1
	10	5.6 $\pm$ 5.4	12.5 $\pm$ 9.7
	12	5.0 $\pm$ 4.9	10.8 $\pm$ 9.5
Peak time points (hpi) MOI = 0.01	12	4.6 $\pm$ 4.1	8.8 $\pm$ 6.7
	24	3.5 $\pm$ 3.4	14.8 $\pm$ 7.2
	36	2.3 $\pm$ 1.9	18.0 $\pm$ 12.8
	48	2.2 $\pm$ 1.2	28.7 $\pm$ 14.8
	60	2.9 $\pm$ 1.2	34.1 $\pm$ 13.3
	72	2.9 $\pm$ 1.1	30.1 $\pm$ 16.2
	96	4.0 $\pm$ 1.9	24.5 $\pm$ 13.4
Persistent time (dpi) MOI = 0.01	1.5	2.3 $\pm$ 1.6	10.2 $\pm$ 12.3
	4	4.3 $\pm$ 2.0	10.5 $\pm$ 10.4
	6	3.9 $\pm$ 2.4	4.9 $\pm$ 6.8
	8	1.0 $\pm$ 0.7	2.0 $\pm$ 3.0
	13	4.4 $\pm$ 2.8	24.1 $\pm$ 23.9
	16	1.8 $\pm$ 1.7	2.4 $\pm$ 3.5
	20	6.2 $\pm$ 3.7	28.1 $\pm$ 23.5
	23	3.2 $\pm$ 2.0	5.8 $\pm$ 7.3
	27	4.9 $\pm$ 4.2	2.7 $\pm$ 4.5
	30	4.0 $\pm$ 2.3	9.1 $\pm$ 8.9
	34	0.7 $\pm$ 1.2	2.2 $\pm$ 3.4
37	4.5 $\pm$ 3.8	6.1 $\pm$ 9.1	
41	1.0 $\pm$ 1.3	7.3 $\pm$ 9.0	

818 <sup>a</sup>Note that for time points prior to 8 hpi, genomic and antigenomic RNAs are not detectable by  
819 smFISH probe sets with exclusive specificity for these RNAs (data not shown). Therefore, spots  
820 detected before 8 hpi are presumed to represent only the mRNAs, but not the genome or  
821 antigenome, recognized by each respective probe set. Spots detect at 8 hpi or later are presumed



822 to be a mixture of all RNAs recognized by a particular probe set (e.g. mRNA and genome or  
823 antigenome).  
824





















