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1 **Kinematics of intracellular chlamydiae provide evidence for**
2 **contact-dependent development**

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16

17 **Running title:** Kinematics of intracellular chlamydiae
18

19 **Abstract**

20

21 A crucial process of chlamydial development involves differentiation of the replicative reticulate
22 body (RB) into the infectious elementary body (EB). We present experimental evidence to
23 provide support for a contact-dependent hypothesis for explaining the trigger involved in
24 differentiation. We recorded live-imaging of *C. trachomatis*-infected McCoy cells at key times
25 during development and tracked the temporo-spatial trajectories of individual chlamydial
26 particles. We found that movement of the particles is related to development. Early-to-mid
27 developmental stages involved slight wobbling of RBs. The average speed of particles increased
28 sharply at 24 hpi (after the estimated onset of RB-to-EB differentiation). We also investigated a
29 penicillin-supplemented culture containing EBs, RBs, and aberrantly enlarged stressed
30 chlamydiae. Near-immobile enlarged particles are consistent with their continued tethering to the
31 chlamydial inclusion membrane (CIM). We found a significantly negative, non-linear association
32 between speed and size/type of particles, providing further support for the hypothesis that
33 particles become untethered near the onset of RB-to-EB differentiation. This study establishes
34 the relationship between the motion properties of the chlamydiae and developmental stages,
35 whereby wobbling RBs gradually lose contact with the CIM and RB detachment from the CIM is
36 coincidental with the onset of late differentiation.

37

38

39 **Introduction**

40

41 Members of the *Chlamydiaceae* are ubiquitous bacterial pathogens in humans and animals.
42 While many primary chlamydial infections are asymptomatic or of limited severity, severe
43 disease and the most serious sequelae are thought to be associated with chronic or persistent
44 infection or repeat infections that may occur over years or decades. In the laboratory, cultured
45 eukaryotic cells such as HEp-2 or McCoy cells and various animal models are used as suitable
46 model systems for primary chlamydial infection. Under these optimized conditions, chlamydiae
47 undergo a typical developmental cycle, which is highly conserved across the genus. Initial
48 internalization of the infectious chlamydial elementary body (EB) particle occurs within the first
49 two hours, shortly followed by differentiation of the EB into the chlamydial replicative form, the
50 reticulate body (RB). RBs are thought to multiply exponentially, replicating their DNA every 2-3
51 hours for approximately 6-10 generations. At 16+ hours post-infection (hpi), an unknown signal
52 provokes the onset of RB to EB differentiation, whereby individual RBs engage in a cellular
53 condensation process, progressing through a poorly-defined intermediate body (IB) form, and
54 ending with the metabolically-inactive, but highly infectious EB, thereby closing the
55 developmental “cycle”. Unlike the initial differentiation step which can be reasonably well
56 synchronized, the late differentiation step is always asynchronous. Indeed, few EBs can be
57 observed in relatively young inclusions while significant numbers of RBs can often be seen in
58 late inclusions (for *C. trachomatis*, these stages occur at approximately 20 and 48 hpi,
59 respectively). Another perennial observation is that RBs are often observed in association with
60 the chlamydial inclusion membrane (CIM) (21, 22, 27, 37), the membrane of the parasitophorous
61 vacuole that contains the chlamydiae and is derived from the host cell plasma membrane (14).

62 This is further supported by various imaging methods including cryo-electron microscopy (14),
63 indirect immunofluorescence, confocal electron microscopy and Nomarski differential
64 interference contrast imaging (10, 15, 16, 31).

65
66 Models for persistent or chronic infection have also been established both *in vitro* and in animal
67 models. Under conditions that induce a classical stress response in many bacteria, such as
68 exposure to IFN- γ (3) or penicillin (23, 34), infection with phage (18), or deprivation of iron (30)
69 or amino acids (9), chlamydial RBs undergo a dramatic morphological change to non-dividing,
70 aberrantly enlarged RBs (termed maxiRBs or mRBs), that will not differentiate into EBs (3, 23,
71 30, 34). Coincidental to the morphological change, expression of stress response genes is up-
72 regulated (e.g. hsp60), while expression of genes thought to be involved in late differentiation
73 (e.g. *omcB*) is blocked (5, 6, 13). Because mRBs may be kept in culture for several weeks
74 (except for phage-induced stress) and removal of the stressor “unlocks” development and allows
75 resumption of late differentiation to EBs, the stress response of the chlamydiae is thought to
76 represent a suitable *in vitro* model for persistent infection (1, 2, 4, 25).

77
78 We have previously proposed a model for chlamydial development that reconciles many of the
79 observations outlined above (26, 35). Two essential features of the model are RBs replicating in
80 type III secretion (T3S)-mediated contact with the CIM, and disruption of T3S activity through
81 physical detachment from the CIM being associated with RB to EB differentiation. This so-
82 called “contact-dependent” model has several important theoretical implications. First, an RB
83 that actively translocates T3S effector proteins through the CIM (i.e. an RB tethered to the CIM)
84 should not differentiate into an EB. Second and correlated to the first, an RB whose T3S system

85 remains active for extended periods of time, by definition, should represent a persisting
86 chlamydial cell. Biomathematical simulations predict two situations under which detachment of
87 RBs from the CIM is physically restricted: the case of normal size RBs in a small, tight
88 inclusion, and that of abnormally large RB(s) in normal size inclusions (17, 35). The biological
89 relevance of these simulations lies in the frequent occurrence of multiple small or lobar
90 inclusions, e.g. for *C. pneumoniae* and *C. caviae*, in a single infected cell, and the observation of
91 stress-induced mRBs, respectively. In either case, because of imposed spatial constraints,
92 disruption of contact-induced T3S activity through physical RB detachment becomes a statistical
93 rarity as the RB/mRB size approaches that of the inclusion that contains it. Remarkably,
94 therefore, the observed *in vitro* persistence is not only a prediction of the biomathematical model
95 but an implication of it as well.

96

97 We now present experimental evidence using innovative real-time light microscopy that provides
98 [some](#) support to the contact-dependent hypothesis in its most fundamental aspects. We captured
99 images of *C. trachomatis*-infected McCoy cells at key times during development using a
100 Richardson RTM3 microscope optimized for live cell imaging in extreme dark field (28). Live
101 images were obtained with a high resolution color analogue output video camera and recorded
102 with Volocity software (Improvision, Coventry, UK). Taped imaging sequences were captured in
103 Final Cut Pro and converted to Quicktime movies. We then analyzed these movies to obtain the
104 spatial time-dependent trajectories of the movement of individual chlamydial particles in each
105 infected cell, allowing kinematic calculations of the displacement and speed of individual
106 particles. ~~Results of these experiments provide new evidence in support of the T3S mediated~~
107 ~~contact dependent hypothesis for chlamydial development.~~

108

109 **Materials and Methods**

110

111 **Live microscopy in the RTM3**

112 We used the Richardson RTM-3 microscope that allows capture of very high resolution (>50
113 nm) images under real time conditions, facilitated by design improvements including an
114 extremely light-tight and ultraclean design, removal of stray wavelengths (UV, near IR), reduced
115 vibration, and images being captured via an electronic detector rather than the human eye (29).
116 An extreme-dark condenser and infinity-corrected 100x oil objective (1.4 NA), and images
117 captured to tape through the 3 chip-CCD camera gave a resolution of NTSC 640x480 pixels.
118 Live imaging was simultaneously viewed on a high resolution broadcast monitor (tape capture)
119 and on a MacIntosh G4 (still images or time-lapse capture with Volocity software, Improvion,
120 Coventry, UK).

121

122 Living infected and mock-infected McCoy cells were imaged at various times post-infection.
123 Semi-confluent McCoy monolayers seeded in 2-chambered slides (one chamber uninfected)
124 were infected with *C. trachomatis* serovar K/UW-31 with an MOI of ~1. Alternatively, cells in
125 one chamber were exposed to penicillin G at 24 hpi. Times were selected to distinguish early
126 through mature inclusions. Imaging [of one representative inclusion at each developmental time](#)
127 was captured on DVCPPro tape (30 frames/sec, 30 mB/frame) as well as individual images
128 captured using Volocity software (Improvion, Inc.). Tape segments were converted to Quick
129 Time movies with Final Cut Pro. Duplicate slides were prepared at specific developmental times

130 and fixed for IFA or immunoperoxidase staining for direct comparison with images of unfixed
131 cells.

132

133 **Tracking of chlamydiae**

134 We chose a section of each movie of 10-100 seconds duration in which focus was optimal
135 (inspection by eye). [One movie was recorded for each time point and tracked particles were](#)
136 [confined to a single inclusion in the recorded movie.](#) Each frame was extracted to a separate
137 Tagged Image File format (using Blaze Media Pro, version 7.0). A computer-coded loop, in
138 Matlab 7.3.0 (R2007b) with Image Processing Toolbox, imported each frame in succession and
139 the frames were converted from RBG format to inverted grayscale images. Particles in each
140 frame were identified according to the level of white intensity relative to the background
141 intensity of color in neighboring pixels, after a process of calibration of the algorithm's color
142 intensity threshold level for particle detection. Each particle location, in Cartesian pixel
143 coordinates, was determined based on the color intensity in all neighboring pixels. Visual
144 inspection of the algorithm output of particle locations on the images of each frame confirmed
145 accurate identification of particles. Once the particles were located for each frame, a list of
146 scrambled particle locations at consecutive frames was sorted and particles were matched
147 between frames (this computationally intensive procedure was based on the maximum plausible
148 displacement in any time interval a particle could move, taken to be 15 pixels per 1/30 second
149 time interval between frames, and accounted for possible appearing and vanishing of particles in
150 and out of focus between frames). This provided a complete description of each particle's two-
151 dimensional time-dependent trajectory. [Not all particles in each inclusion could be analyzed](#)
152 [because it was not possible to identify unique particles that were highly clustered near others.](#)

153 [particularly in regions in which cell debris and other material obscured the view. All particles](#)
154 [that could be clearly identified within the optimal resolution attainable and could be tracked over](#)
155 [at least five frames \(0.17 seconds\) were included in the analysis; particles that were identified as](#)
156 [moving in and out of the plane of focus were included. Particles were tracked for as long as](#)
157 [possible, up to the duration of the movie section analyzed \(10-100 seconds\). The numbers of](#)
158 [particles included at each time point are indicated in Table 1.](#) Elementary kinematic properties
159 (displacement and speed) were then calculated.

160

161

162 **Results**

163

164 **Movement of chlamydial particles is related to development**

165 In Figure 1, we display still images of *C. trachomatis*-infected McCoy cells at times
166 representative of stages of chlamydial development (corresponding video recordings are
167 provided in Supplemental Material, Videos #1-12). Although visualization of nascent inclusions
168 is not possible using this methodology, newly internalized (or surface-attached) EBs were clearly
169 identified by comparison with uninfected cells (compare uninfected and 0 hpi cells in Figure 1).
170 At later developmental stages, the inclusion became increasingly distinguishable owing to the
171 higher light refraction of densely packed inclusions and to the differential velocity of chlamydiae
172 within the inclusion compared to cytosolic contents. EBs/IBs and RBs were recognizably
173 distinct, respectively as densely centered particles of diameter ranging from 0.2 to 0.3 μm and as
174 hollow centered particles ranging from 0.6 to 1.0 μm . The slight difference between these
175 measurements and accepted sizes (0.25 μm and 0.8-1.0 μm , respectively) most likely owes to the

176 diffuse boundaries between EBs/IBs and IBs/RBs and/or to the dark field illumination method
177 used for observation. In some instances, single particles were highly mobile in the plane of
178 observation; but in other cases, movement was unobservable. The degree of particle movement
179 differed widely: at one extreme, RBs were primarily immobile with only occasional episodes of
180 wobbling movement; at the other extreme, most EBs were rapidly mobile. In between these two
181 extremes were intermediate-size particles assumed to be in the process of differentiation to EBs.
182 We quantified these observations through analysis of the recorded real-time video of *C.*
183 *trachomatis*-infected McCoy cells. For each movie, we tracked the trajectory of individual
184 chlamydial particles over time (see Figs. 2a, b for examples). The average speed of each particle
185 over the time of tracking was determined. We found that the average particle speed changed over
186 the time-course of the developmental cycle (Fig. 3a, Table 1), consistent with the observations of
187 high EB mobility and in-place RB wobbling.

188

189 **Wobbling of RBs in-place increases during exponential growth**

190 During the early to mid stages of development, there are only RBs (8-16 hpi) or a mixture of
191 RBs and IBs (16-20 hpi). We calculated that the average speed of RBs was 0.93 $\mu\text{m}/\text{sec}$ (mean
192 over all particles, standard error 0.11 $\mu\text{m}/\text{sec}$) at 8 hpi, 1.10 $\mu\text{m}/\text{sec}$ (0.07 $\mu\text{m}/\text{sec}$, SE) at 14 hpi,
193 and 2.74 $\mu\text{m}/\text{sec}$ (0.37 $\mu\text{m}/\text{sec}$, SE) at 16 hpi (Fig. 3a, Table 1). The maximum speed of the
194 fastest particle observed at these times was 8.69 $\mu\text{m}/\text{sec}$ at 16 hpi. The magnitudes of these
195 speeds are very small relative to the size of the RB particles and in comparison with the speed of
196 the small EBs (see Table 1). Moreover, the maximum displacement (maximum distance between
197 two points of a particle's trajectory) traveled by RBs up to 20 hpi was below the distance
198 corresponding to the RB radius (average displacement of RBs was 0.18 μm and the maximum

199 measured displacement over all particles before 20 hpi was 0.59 μm), reflecting the in-place
200 wobbling of RBs. Interestingly, the (average and maximum) speed of RB particles increased
201 with time (although the size of particles did not change).

202

203 **The average speed of RB movement increases sharply at mid-cycle, then decreases**

204 The average speed of particles increased with time until approximately 24 hpi with a sharp
205 upward trend between 20 and 24 hpi for approximately half of the particles analyzed (Fig. 3a).

206 Accrued velocity did not correlate with a significant change in particle size as size was randomly
207 distributed across all particles tested at 24 hpi (Fig. 3b; $p=0.2160$, Mann-Whitney test based on
208 categorizing all particles tracked at 24 hpi according to whether their average speed was greater
209 than or less than the median particle's speed (7.23 $\mu\text{m}/\text{sec}$)). At 24 hpi the mean average speed of
210 particles was 9.02 $\mu\text{m}/\text{sec}$ and the maximum average speed observed was 28.83 $\mu\text{m}/\text{sec}$.

211 Moreover, chlamydial particles at 24 hpi not only displayed accrued average speed, but they also
212 | traveled distances within the inclusion several times greater than the RB radius ([Table 1](#)),
213 | consistent with the presumed un-tethering of these particles from the CIM between 20 and 24
214 | hpi. Time 24 hpi coincides with the time at which a substantial proportion of RBs are undergoing
215 | differentiation, and there is still sufficient physical space within the inclusion to allow maximal
216 | movement. After this point in the developmental cycle, the average speed per particle decreases
217 | (Fig. 3a, Table 1). The number of chlamydial particles, and proportion that have become IBs or
218 | EBs, increases with time; consequently the space available for their movement decreases (Fig.
219 | 1).

220

221 **Penicillin-induced persistent forms are static**

222 To further explore the influence of particle size on particle motion, we investigated the
223 movement properties of stress-induced mRBs that are known to “persist” under *in vitro* culture
224 conditions. In this experiment, *C. trachomatis*-infected McCoy cells were exposed to 100U/ml
225 penicillin G at 24 hpi and the real-time movement was recorded a further 24 hours later (see
226 Supplemental Material for video recording). Under these conditions, inclusions contained a
227 mixture of particles of sizes consistent with EBs, RBs, large penicillin-induced mRBs, as well as
228 a variety of intermediate-size forms (see Fig. 4a). We quantified the size and average speed of
229 chlamydial particles of each type (Fig. 4b; Supplemental Material, Figs. 5-6, Video #13).

230 [Particles of the size of mRBs had slower speeds \(0.81 \$\mu\text{m}/\text{sec}\$ median, 0.75-0.88 \$\mu\text{m}/\text{sec}\$](#)
231 [interquartile range\) than RBs/IBs \(1.44 \$\mu\text{m}/\text{sec}\$, 1.15-2.05 \$\mu\text{m}/\text{sec}\$ IQR\), which were slower than](#)
232 [EBs \(4.12 \$\mu\text{m}/\text{sec}\$, 3.48-4.72 \$\mu\text{m}/\text{sec}\$ IQR\); Figure 4b.](#) While there is clearly a significantly
233 negative, non-linear association between speed and particle size, a rise in the average speed of
234 RB particles (or IBs in the process of differentiation) [relative to mRBs](#) and an even more
235 substantial rise for EBs are observed. [The association between speed and particle size is apparent](#)
236 [and is statistically significant, however, it should be noted that the sample size around the cutoff](#)
237 [between EBs and RBs is relatively small \(Fig. 4b\).](#) The near immobility of mRBs is consistent
238 with their [possible](#) continued tethering to the inclusion membrane. [We also compared the speeds](#)
239 [of particles consistent with the size of EBs \(Figure 4b\) with particles in the normal development](#)
240 [experiments at 49 hours post infection, so that it is at a similar time post infection and the](#)
241 [majority of particles in the normal development experiment were EBs for reasonable](#)
242 [comparison. We found that speeds of EBs in the penicillin-treated culture were not statistically](#)
243 [different from those in the untreated culture \(\$p=0.4624\$, Mann-Whitney test\) however the treated](#)
244 [culture had slightly lower average speeds \(median 4.1 \$\mu\text{m}/\text{sec}\$ compared with 4.4 \$\mu\text{m}/\text{sec}\$ \).](#)

245

246 **Discussion**

247

248 We have previously proposed a hypothesis for the development of intracellular chlamydiae based
249 on a combination of electron microscopic and other observations (26) and further developed the
250 hypothesis using biomathematical modeling (35). Tenets of the so-called contact-dependent
251 hypothesis of chlamydial development are that (i) as RBs, chlamydiae grow strictly in contact
252 with the plasma membrane-derived CIM, (ii) contact with the CIM is mediated by surface
253 projections hypothesized to correspond to T3S injectisomes, (iii) disruption of T3S activity
254 through physical detachment from the CIM is associated with the onset of late differentiation.
255 The implied biological significance of the hypothesis is that maintained contact with the CIM
256 permits continued delivery of chlamydial T3S effectors into the host cell cytosol and subsequent
257 subversion of cellular processes to benefit chlamydial growth and that disruption of contact
258 through physical detachment interrupts T3S effector translocation, thus rendering the host cell
259 less hospitable for chlamydial growth. Because contact of chlamydial particles with the CIM, or
260 loss thereof, has direct implications on the ability of chlamydiae to move inside the inclusion, we
261 sought to quantify the movement (distance traveled and velocity) of individual chlamydial
262 particles at different times along development. For this analysis, we used a Richardson RTM3
263 light microscope (28, 29) that is optimized for high resolution white-light microscopy and live
264 recording (30 frames per second) of *Chlamydia*-infected cells in real time. Limitations of the
265 quantification of movement include (i) the movies are a cross-sectional two-dimensional slice of
266 the infected cells and so particles that move in and out of focus are difficult to track. We have
267 made allowances for this in our image processing computer algorithms but if fast moving

268 particles move far when out of focus, then identification and matching of particles is prevented;
269 (ii) during late stages of development the inclusion lumen is densely packed with EBs and ~~the~~
270 spatial constraints are likely to prevent unhindered movement. The high density at late stages
271 also makes it difficult to distinguish some particles between frames. To alleviate this problem we
272 only included particles for which we could clearly identify and match particles between frames;
273 (iii) we could not record the same cell for each stage of the developmental cycle included in our
274 analysis. We recorded representative cells in the culture at each time point. We acknowledge that
275 there would be some asynchrony of infection or development, and may be some differences at
276 times where the same phenotypes are seen.

277

278 The results of our analysis reveal a close relationship between key developmental stages and
279 motion properties of the chlamydiae. While “movement” of chlamydial bodies has been noted
280 previously (22), this is the first detailed study using advanced microscopic techniques to (a)
281 confirm that chlamydiae definitely do undergo movement and (b) link this movement to specific
282 stages of development. The magnitude and speed of RB wobbling from early to mid stages of
283 development (Fig. 3a, 8-20 hpi) steadily increases with time, suggesting that individual RBs
284 gradually lose contact with the CIM allowing for increased movement, and reciprocally
285 supporting their hypothesized tethering to the CIM via T3S injectisomes. This is consistent with
286 a decreasing number of surface projections along development as observed by Matsumoto in *C.*
287 *psittaci* (20). Between 20 and 24 hpi, a remarkable gain in movement is observed for
288 approximately half of the particles in an inclusion, such that average velocities of individual
289 particles are ~4 times greater at 24 hpi than at 20 hpi (Table 1). Coincidentally the distances
290 traveled by individual particles are substantially increased, ~~strongly supporting the notion that~~

291 | ~~these particles have broken free from the CIM~~. These results are consistent with the contact-
292 dependent hypothesis where untethering of individual particles is predicted to be asynchronous
293 during late differentiation.
294

295 Naturally, larger-sized particles will move slower than smaller particles when acted upon by the
296 same force. It is, therefore, predictable that the decreasing size of the chlamydial particle (from
297 the 0.8-1.0 μm RB to the 0.25 μm EB) will affect the degree of movement during late
298 differentiation. However, we found that the fastest particles in the developmental cycle (at 24
299 hpi) were of the size of RB particles (Fig. 3b). Given the very marked change observed from
300 essentially no movement or slight in-place wobbling of RBs to very fast motion of newly
301 detached RBs, IBs or EBs, we conclude that the large gain of velocity observed between 20 and
302 24 hpi is not accounted for significantly by the change in the size of the chlamydial particle as it
303 undergoes late differentiation. A steady decrease in speed was also observed between 24 and 49
304 hpi, i.e. during late differentiation. We speculate that particles gradually lose velocity as they
305 bounce off each other and off the CIM in the increasingly crowded inclusion lumen.

306 Biomathematical simulations of the contact-dependent hypothesis predict that both the
307 multiplicity of inclusions within a single infected cell and the size of the chlamydial particle
308 relative to that of the inclusion are determining factors in the outcome of an infection (17, 35). In
309 multiple, smaller inclusions within an infected cell, the size differential between the inclusion
310 and that of a replicating RB it contains may become small enough such that loss of T3S-
311 mediated contact between the RB and the CIM becomes improbable. Following the same logic,
312 the likelihood of an extremely large RB becoming untethered from the CIM diminishes with
313 | increasing RB size. We ~~tested~~explored this part of the hypothesis using *C. trachomatis* grown in

314 the presence of penicillin. Penicillin-exposed chlamydial cultures are known to produce
315 aberrantly enlarged mRBs and provide a model for chlamydial persistence, a hallmark of
316 chlamydial chronic infection and disease in humans (1, 2, 4, 25). In our experiments, cultures
317 were supplemented with penicillin G at 24 hpi and observed at 48 hpi, allowing for inclusions to
318 contain a mixture of persistent mRBs as well as normal RBs, IBs and EBs. We found that EBs in
319 inclusions exposed to penicillin moved at maximal speeds similar to those observed at 49 hpi in
320 normal cultures (Fig. 4b, Table 1). Particles of the size of RBs were observed to wobble in-place,
321 while mRBs were either completely static or wobbled slightly in-place. This is, consistent with
322 these particles being more extensively tethered to the underlying CIM and. ~~This would suggest~~
323 that mRBs ~~do may~~ not become un-tethered from the CIM, therefore, do not enter late
324 differentiation, and *de facto* persist. Hence, the motion properties of chlamydial particles within
325 persistent inclusions are ~~entirely~~ consistent with the results of biomathematical simulations that
326 predict that mRBs persist *in vitro* owing to their continued tethering to the CIM. Although, this
327 hypothesis ultimately requires experimental verification, it is consistent with gene expression
328 studies that have shown both at the transcriptional and protein levels that expression of the T3S
329 injectisome genes is not significantly affected during persistent growth (5, 24).

330

331 The type III secretion system is thought to be central to the virulence of many bacterial
332 pathogens including *Chlamydia* spp. (11, 19, 26). However, there is no consensus as to whether a
333 functional T3SS exists during the intracellular developmental cycle, and if so, whether tethering
334 of each single RB to the inclusion membrane via TTSS injectisomes is necessary for chlamydial
335 development. A role of T3S in chlamydial pathogenesis is supported by virulence-related
336 properties of several chlamydial T3S-translocated effectors (7, 8, 33). Our study, however,

337 ~~suggests that chlamydial T3S activity—and conversely its disruption through loss of RB contact~~
338 ~~with the CIM—is a determinant of chlamydial intracellular development. This is further~~
339 ~~supported by Other~~ studies ~~that have indicated the coupling of~~ T3S expression or T3S activity
340 with development (12, 32, 36). An attractive hypothesis emerging from these converging results
341 is that T3S-mediated translocation of early-mid cycle effector(s) to the infected cell cytosol
342 maintains the host in a state optimized for chlamydial exponential growth and that disruption of
343 this state through interruption of T3S translocation may “alert” chlamydiae within the inclusion
344 to initiate late differentiation and subsequent progress of the infection. Stress-induced inhibition
345 of this process would then represent a survival mechanism of the chlamydiae whereby a
346 sustained level of T3S translocation activity maintains viability of fewer chlamydiae but for
347 extended periods of time. Although our study does not directly address T3S activity and its
348 potential role in development, our findings are consistent with the contact-dependent hypothesis.
349 Further experiments beyond the scope of this study would be required to investigate the potential
350 role of the T3S system on chlamydial development. Our novel approach of using real-time light
351 microscopy and kinetic ~~analysis~~ modeling with *Chlamydia* has described chlamydial movement
352 in a way that has never been done previously. It has elucidated properties ~~shed important light on~~
353 ~~what we speculate are key events~~ in the regulation of the unique developmental cycle of this
354 medically important pathogen.

355

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364

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482 **Figure Legends**

483

484 **Figure 1:** Representative still image frame from each time point recorded by our RTM live
485 imaging during normal development. Cells are unstained and viewed under RTM image contrast.
486 Times 40 and 44 hpi are shown in DIC mode. Each bar represents the scale of 10 μm .

487

488 **Figure 2a:** The trajectory over 1.6 seconds of the movement of the center of one representative
489 chlamydial particle at 14 hpi in an unstained infected McCoy cell (100x original magnification).
490 The center of this particle moved a distance of 1.28 μm in 1.6 seconds (i.e., with an average
491 speed of 0.8 $\mu\text{m}/\text{sec}$).

492

493 **Figure 2b:** Tracking of 9 individual particles indicating: elapsed time, distance travelled,
494 maximum velocity, and average velocity for the case of normal development (without the
495 addition of penicillin) at 14 hpi.

496

497 **Figure 3a:** Average speed of chlamydial particles at each time point in the developmental cycle.

498

499 **Figure 3b:** Univariate scatterplot: particles tracked at 24 hpi were separated into 2 groups based
500 on whether the average particle speed was greater or less than the median average speed, and the
501 radii of chlamydial particles in the fast versus slow groups are compared. The p-value refers to a
502 Mann-Whitney statistical test.

503

504 **Figure 4a:** Still image from live video imaging of a *C. trachomatis*-infected McCoy cell (48 hpi)
505 exposed to penicillin (100U/ml added at 24 hpi). Cells are unstained and viewed under RTM
506 image contrast (100x original magnification). Sample EBs, RBs and mRBs are indicated.

507

508 **Figure 4b:** Average velocity of chlamydial particles versus particle radius in an inclusion of *C.*
509 *trachomatis*-infected McCoy cell (48 hpi) exposed to penicillin (24 hpi). [Spearman correlation](#)
510 [coefficient between velocity and particle size was determined \(\$r\$, with p-value\).](#)

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