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Wilson, D. P. and Whittum-Hudson, J. A. and Timms, P. and Bavoil, P. M. (2009) *Kinematics of intracellular chlamydiae provide evidence for contact-dependent development.* Journal of Bacteriology, 191(18). pp. 5734-5742.

Kinematics of intracellular chlamydiae provide evidence for contact-dependent development

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Running title: Kinematics of intracellular chlamydiae

19 **<u>Abstract</u>**

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.

Introduction

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

3

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

4

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

Materials and Methods

110

Live microscopy in the RTM3

We used the Richardson RTM-3 microscope that allows capture of very high resolution (>50

nm) images under real time conditions, facilitated by design improvements including an

extremely light-tight and ultraclean design, removal of stray wavelengths (UV, near IR), reduced

vibration, and images being captured via an electronic detector rather than the human eye (29).

An extreme-dark condenser and infinity-corrected 100x oil objective (1.4 NA), and images

captured to tape through the 3 chip-CCD camera gave a resolution of NTSC 640x480 pixels.

Live imaging was simultaneously viewed on a high resolution broadcast monitor (tape capture)

and on a MacIntosh G4 (still images or time-lapse capture with Volocity software, Improvision,

Coventry, UK).

121

Living infected and mock-infected McCoy cells were imaged at various times post-infection.

Semi-confluent McCoy monolayers seeded in 2-chambered slides (one chamber uninfected)

were infected with *C. trachomatis* serovar K/UW-31with an MOI of ~1. Alternatively, cells in

one chamber were exposed to penicillin G at 24 hpi. Times were selected to distinguish early

through mature inclusions. Imaging of one representative inclusion at each developmental time

was captured on DVCPro tape (30 frames/sec, 30 mB/frame) as well as individual images

captured using Volocity software (Improvision, Inc.). Tape segments were converted to Quick

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,

7

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

8

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 μm/sec (mean

192 over all particles, standard error 0.11 μm/sec) at 8 hpi, 1.10 μm/sec (0.07 μm/sec, SE) at 14 hpi,

193 and 2.74 μm/sec (0.37 μm/sec, SE) at 16 hpi (Fig. 3a, Table 1). The maximum speed of the

194 fastest particle observed at these times was 8.69 μm/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

9

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 μm/sec)). At 24 hpi the mean average speed of

210 particles was 9.02 μm/sec and the maximum average speed observed was 28.83 μm/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**

10

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 µm/sec median, 0.75-0.88 µm/sec

231   interquartile range) than RBs/I Bs (1.44 µm/sec, 1.15-2.05 µm/sec IQR), which were slower than

232   EBs (4.12 µm/sec, 3.48-4.72 µm/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 µm/sec compared with 4.4 µm/sec).

11

245

<u>Discussion</u>

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

12

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 <u>are likely to</u> 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

14

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 suggests

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 coupleding 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 analysismodeling with *Chlamydia* has described chlamydial movement

352 in a way that has never been done previously. It has elucidated propertiesshed 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

356 **Acknowledgements**

364

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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 μm/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.

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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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