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Bacterial Competition Assays

--Manuscript Draft--

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Abstract:	In the environment, bacteria do not live alone but are in contact with other bacterial cells. Interactions between bacteria play a critical but underestimated role in nature and pathogenesis. The outcome of these associations depends on collaboration or competition behaviors between the cells, Recently, assays have been developed to test competition occurring between bacteria. Here, we first describe a macroscopic assay on agar plate by mixing predator and fluorescent prey strains. The level of fluorescence recovery after incubation, and the number of viable prey cells remaining allows to quantify the result of the competition. The second assay allows to image bacterial competition by following the fate of fluorescent-tagged predator and prey cells on an agar pad placed under a fluorescence microscope.

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Editorial Board

Journal of Visualized Experiments

Dear Rachelle, dear JoVE editorial staff,

We are submitting a manuscript describing assays recently developed to test competition between bacteria and entitled "bacterial competition assays" by Yannick R. Brunet, Erwan Gueguen, Jean-Raphaël Fantino, Leon Espinosa, Adrien Ducret, Tâm Mignot and Eric Cascales to be considered for publication in the Journal of Visualized Experiments. Our manuscript is submitted with an author-produced video.

The possibility of publishing these protocols in JoVE has been discussed with Dr. Rachelle Baker a few months ago. We think it is timely as we recently published two articles reporting data using these techniques (Gueguen *et al.*, Applied and Environmental Microbiology (2013) and Brunet *et al.*, Cell Reports (2013)) and an increasing number of studies are currently published on the topic of bacterial competition as two anti-bacterial mechanisms, the Type VI secretion system and the contact-dependent inhibition, are currently garnering attention and are considered as high topics of research in microbiology.

We have suggested a number of potential referees, all working in the field of bacterial competition. Most of them have recently published articles using similar protocols and their feedbacks will be interesting to us. We will fax the filled Author Licence Agreement tomorrow.

We look forward to hearing from you.

Sincerely,

Eric

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KEYWORDS: Microbial communities, bacterial competition, fluorescence microscopy, bacterial secretion, protein transport, cell-cell contact

SHORT ABSTRACT: In the environment, bacteria do not live alone but collaborate or compete with other bacterial cells. We describe here two assays allowing to test and quantify the outcome of interactions between two different bacterial strains.

LONG ABSTRACT: In the environment, bacteria do not live alone but are in contact with other bacterial cells. Interactions between bacteria play a critical but underestimated role in nature and pathogenesis. The outcome of these associations depends on collaboration or competition behaviors between the cells, Recently, assays have been developed to test competition occurring between bacteria. Here, we first describe a macroscopic assay on agar plate by mixing predator and fluorescent prey strains. The level of fluorescence recovery after incubation, and the number of viable prey cells remaining allows to quantify the result of the competition. The second assay allows to image bacterial competition by following the fate of fluorescent-tagged predator and prey cells on an agar pad placed under a fluorescence microscope.

INTRODUCTION:

It is known since a long time that bacteria can affect plants, animals and men. The toxins and their dedicated delivery systems have been identified and characterized in the last decades. However the concept that bacteria can compete and affect the growth of other bacterial species has been underestimated. In the environment, bacteria do not live alone. They are in contact with many other species and have to collaborate or compete to access nutrients or to

colonize more efficiently the ecological niche. Different mechanisms to outcompete non-sibling bacteria have been identified, ranging from diffusible toxic chemical compounds to small peptides, large proteins or dedicated macromolecular complexes: (i) antibiotics [1], (ii) lantibiotics, colicins and related bacteriocins, that are bacterial toxins released by producing cells and targeting closely related strains [2], (iii) contact-dependent inhibition (CDI), outer membrane proteins related to Type V secretion systems [3] and (iv) the Type VI secretion system (T6SS), a macromolecular system acting as a micro-syringe to deliver anti-bacterial toxins into target cells [4,5]. Interestingly, antibiotics and colicins are diffusible molecules while CDI and T6SS depend on close contact between predator and prey cells. The study of these different anti-bacterial systems needs to develop protocols to rapidly test whether a bacterial strain – or mutants – can affect the growth of other bacteria and to identify the determinants responsible for this phenomenon. Here, we provide protocols recently used in several studies to quantitatively measure the outcome of a competition between bacterial species. First we describe a macroscopic assay allowing to test the susceptibility of a fluorescent-tagged prey cell to potential predators, using mixed colonies on agar plates. A second approach allows to image the bacterial competition using time-lapse fluorescence microscopy. These two assays can be used to quantify the competition between bacteria by measuring a competitive index. Both assays have been developed in different laboratories in the recent years and we refer the readers to the original publications [6-20].

PROCEDURE:

1. Macroscopic assay to monitor inter-bacterial competition

1.1. Preparation of the prey and predator strains (Days 1 and 2)

Day 1.

1. Transform the prey (or recipient) strain (*E. coli* W3110 in the movie) with the pUA66-*rrnB* vector that confers a strong GFP+ phenotype (*gfpmut2* gene fused to the ribosomal *rrnB* promoter [21]).
2. Spread the transformants on a Luria broth (LB) agar plate supplemented with kanamycin (50 µg/ml, i.e Kan^{50}). Incubate the plate overnight at 37 °C.
3. Streak the predator strain from a frozen stock (*C. rodentium* ICC168 in the movie) onto a fresh LB agar plate. Incubate the plate overnight at 37 °C.

Day 2.

4. Check the strong GFP^+ phenotype of W3110/pUA66-*rrnb* colonies using a LI-COR Odyssey Fc imager. Set up the excitation channel at 600 nm with an acquisition time of 40-50 sec. Use W3110/pUA66 (GFP^- phenotype) as a negative control.

1.2. Visualization of inter-bacterial growth competition with LI-COR Odyssey Fc imager (Days 2 and 3).

1. Using a sterile toothpick, inoculate two 14-ml polypropylene tubes with 3 ml of LB with an individual colony of the prey (W3110/pUA66-*rrnb*) and of the predator (*C. rodentium*).
2. Grow the cells at 37°C with agitation. Measure OD_{600nm}. When the optical densities reach 0.5, harvest 1-ml of each culture. Wash cells to remove the antibiotics by centrifugation at 3000 × *g* for 5 min, and resuspend in 1 ml of LB medium without antibiotic.
3. In a sterile 1.5 ml tube, mix predator and prey cells to a 4:1 ratio: add 400 µl of the predator suspension to 100 µl of the prey suspension. Different ratios should be first tested to determine the best conditions to observe killing of the prey. As controls, mixtures with *C. rodentium* and non-fluorescent W3110 and between W3110 and W3110 pUA66-*rrnB* are also made.
4. Spot 25 µl of the mixture in triplicate onto a pre-warmed dry agar plate. The engineered *C. rodentium* strain we used in this assay expresses its CTS1 T6SS after induction with IPTG and arabinose. We thus supplemented LB agar with 2% arabinose and 500 µM IPTG.
5. Incubate the plates overnight for 14 hours at 30°C (various incubation times and temperatures should be tested in pilot experiments to define the best conditions).

Day 3.

6. Place the plates into a LI-COR Odyssey Fc imager. Set up the excitation channel at 600 nm with an acquisition time of 40-50 sec. Save the images.

1.3. Quantification of the fluorescence level with a M200 TECAN microplate reader (Day 3).

1. With a sterile toothpick or loop, collect the cells grown in the spots and resuspend in 1 ml of LB. Mix by vortexing. Alternatively, the spot can be cut off and cells resuspended into LB. Read the absorbance at 600 nm and normalize to an OD_{600nm} of 0.5.
2. Transfer 150 µl into wells of a black 96-well plate. Place the plate into a TECAN microplate reader.
3. Read the absorbance at 600 nm and fluorescence (excitation: 485 nm; emission: 530 nm).
4. Save the data (in an Microsoft Excel table). Subtract the value of a blank sample (LB without cells) to the values of fluorescence and OD_{600nm}. Calculate the relative fluorescence by dividing the intensity of fluorescence by the OD_{600nm}. This ratio is given in arbitrary units (A.U.)

1.4. Enumeration of viable prey and predator cells (Days 3 and 4).

1. Make serial 10^{-1} to 10^{-7} dilutions of the normalized bacterial suspensions (part 1.3.1.) in LB medium without antibiotics (in triplicates).
2. Vortex and spread 100 µl of each dilution onto two plates: one supplemented with Kan^{50} to select viable W3110 *E. coli* prey cells and one supplemented with nalidixic acid (20 µg/ml, *i.e.* Nal^{20}) to select *C. rodentium* predator cells (the strain we used is Nal resistant)
3. Incubate the plates overnight at 37°C.

Day 4.

4. Enumerate colonies and report the number of viable prey cells or calculate the competitive index ([prey cfu/predator cfu] after incubation / [prey cfu/predator cfu] at time zero).

2. Fluorescence microscopy to monitor inter-bacterial competition

2.1. Preparation of the prey and predator strains (Days 1 and 2)

Day 1.

1. To facilitate live imaging of bacterial competition, predator and prey should produce distinct fluorescent markers. Transform the predator strain (entero-aggregative *E. coli* 17-2 in the movie) with the pUA66-*rrnB* vector [21]. Transform the prey strain (*E. coli* K-12 strain W3110 in the movie) with the pFPV-mCherry vector (*mCherry* gene under the control of the strong ribosomal *rpsM* promoter [22]).
2. Spread the transformants on a LB agar plate supplemented with kanamycin for the predator and on LB agar plate supplemented with ampicillin for the prey (100 µg/ml, *i.e.* Amp^{100}). Incubate the plates overnight at 37 °C.

Day 2.

3. Check the GFP^+ and $mCherry^+$ phenotypes of transformed predator and prey cells respectively using a LI-COR Odyssey Fc imager.
4. Grow the fluorescent predator and prey cells overnight in LB supplemented with antibiotics at 37°C with aeration.

2.2. Preparation of the samples and fluorescence microscopy (Day 3)

Day 3.

1. Subculture the two strains (1 :100 dilution) into the appropriate medium. In the movie, bacteria have been subcultured into Minimal Eagle Medium (MEM), a medium previously shown to allow maximal expression of the EAEC *sci-2* Type VI secretion gene cluster [23]. T6SS gene cluster are usually tightly controlled and therefore the choice of the medium is critical [24]. Grow bacterial cultures at 37°C with agitation until $OD_{600nm} \approx 1.5$ is reached.
2. Prepare agar pads by dissolving agarose into 5 ml of phosphate buffered saline (PBS) (final concentration 1.5 %). After melting, cast two thin agarose pads. We use a homemade pad caster (5-mm deep, 10-mm large and 50-mm long trench in Plexiglas).
3. Pellet 2.0 OD_{600nm} units of each of the bacterial cultures in a 1.5-ml microcentrifuge tube at 6,000 × *g* for 5 min. at room temperature. Discard the supernatant and immediately resuspend the pellet into 35µl of PBS (57 OD_{600nm} units per ml).
4. To follow inter-bacteria competition predator and prey are mixed. As a negative control, the prey is mixed with a T6SS-defective derivative of the predator strain. Gently mix the predator and the prey with a 10:1 ratio in a new 1.5-ml microcentrifuge tube.

5. Spot 2 µl of the mixtures onto a 24 x 50 mm cover-slip and cover with a thin agarose pad. At this stage, the aim is to obtain a monolayered bacterial lawn in which predator and prey cells are in close contact. The spots must be placed close to each other to follow the two mixed-populations during the same experiment.
6. Prior to microscopy acquisition, incubate the montage 3 hours at 37°C in a Petri dish to prevent drying.
7. Place the montage under the fluorescent microscope. Define 5 focused independent fields for each of the two mixed populations.
8. Collect phase contrast, GFP and mCherry images regularly using an exposure time of 5 ms for phase contrast and 8 ms for GFP and mCherry fluorescences (minimal exposure time to minimize photobleaching and phototoxicity). The fluorescence microscope used in the movie is equipped with the Perfect Focus System (PFS) that automatically maintains focus during the course of the experiment.
9. Reconstruct the movie by merging GFP- and mCherry-channel time-lapse images using the ImageJ software (Rasband, W.S., ImageJ, U. S. National Institutes of Health, Bethesda, Maryland, USA, http://imagej.nih.gov/ij/, 1997-2012) or an appropriate program. Slight movements of the whole fields during the course of the experiment can be corrected by using the StackReg plugin in ImageJ. In order to number the cell death events, independent mCherry bacterial objects (prey cells) should be identified and derivated by substracting these objects from the n+1 to n slides of the stack.

REPRESENTATIVE RESULTS AND FIGURES:

Figure 1 shows a representative example of a macroscopic growth competition assays. (A) and (B) Growth competition assay using a fluorescent *E. coli* strain as prey and different strains of *C. rodentium* (WT and its T6SS- derivative as predators). (A) Graph: The relative fluorescence recovered from the bacterial spot shown below is expressed in arbitrary units (A.U.) and is the mean of fluorescence levels obtained from three independent experiments (each measured in triplicate). Bacterial Spot: Fluorescent images of the competition assays (obtained with a LI-COR Odyssey imager). (B) Number of recovered *E. coli* cells (survival expressed in colony forming units [cfu]). The triangles indicate values obtained from three independent assays (in triplicate) and the average is indicated by the bar.

Figure 2 shows a representative example of a fluorescence microscopy growth competition assay. (A) Time-lapse recording of a bacterial lawn constituted of predator GFP^+ cells (green) and prey mCherry+ cells (red). One image every 1.5 hours is shown. Prey cell death events (prey

cells disappearing in the next image) are marked with white arrows. (B) Number of prey cell death events relative to the initial number of prey cells is plotted versus time. Black line, wild-type EAEC as predator; red line, Δ*tssE2* (a strain mutated in an essential component of the EAEC *sci-2* T6SS) as predator.

DISCUSSION:

In this manuscript and the accompanying video we provide protocols to test competition between bacterial strains. These protocols have been recently used in our laboratory to demonstrate the function of the EAEC and *C. rodentium* Type VI secretion systems as macromolecular systems required to target and to kill other bacteria [18, 20].

Variations on a theme. Similar protocols were previously developed in laboratories working in bacterial competition [6, 8, 9, 11-17]. The protocols showed here can be easily modified, such incubating the mixed culture directly on agar plates or on a filter placed on agar plates. As the assays can be used to test the predatory role of different bacteria for which mechanisms or regulatory networks involved for competition are different, the assays require to be set-up depending of the growth needs of the studied microorganisms (medium and temperature) or by varying the contact time between and/or the ratio of predator and prey cells. One important and limiting point is however to perform the assays using predator and prey cells sharing similar growth rates and behaviors in the conditions used.

For the macroscopic assay described here, we have detailed a protocol using a fluorescent reporter. However, different reporters might be used, depending of the availability of prey cells or of a LI-COR Odyssey imager. For example, a prey expressing the *lacZ* gene instead of a fluorescently labeled prey can be used, such as recently reported by Lossi et al. [25]. In this case, the predatory effect can be visualized on agar plates supplemented with 5-bromo-4-chloro-3-indolyl β-D-galactopyranoside (BCIG, or X-Gal) and quantified by measuring the β-galactosidase activity on the ortho-nitrophenyl β-galactoside (ONPG) substrate using the Miller protocol [26].

How to report data? For the macroscopic assay, different formulas have been used: percentage of surviving cells, competitive index (CI). The CI is probably the most accurate as it takes into account the number of predators and the prey/predator ratio at the beginning of the experiment: calculate the ratio of prey on predator at time 0 and after the incubation. CI is the ratio after incubation divided by the ratio at time 0. For the fluorescence microscopy assay, images should be analyzed using softwares that perform single-cell tracking such as the algorithms developed by ImageJ or MatLab.

Additional applications. In addition to test competition between bacteria, the macroscopic assay might be used to identify molecular determinants that control the outcome of the competition. Recently, LeRoux et al have used a genome-wide transposon screen to

identify proteins that control prey cells susceptibility to a T6SS-mediated attack [19]. Susceptible *lacZ+ Pseudomonas aeruginosa* prey cells were mutagenized and individual mutants were mixed with predators and spotted on plates supplemented with the chromogenic substrate X-Gal. Resistant prey cells were identified by the positive *lacZ* phenotype observable by the blue color of the spot.

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DISCLOSURES

The authors declare that they have no competing financial interests.

REAGENTS:

Name of the Reagent	Company	Comments (optional)
MEM	Gibco	Minimal Eagle Medium
Luria Broth	Gibco	
Antibiotics	Sigma Aldrich	

EQUIPMENT:

Material Name	Company	Comments (optional)
Black 96-well plates, clear bottom	Greiner	
Odyssey Imager	LI-COR	
Microplate reader	TECAN	
Fluorescence microscope TE-	Nikon	Automated, inverted

2000-E		epifluorescence microscope
Perfect Focus System	Nikon	Automatically maintains focus
CoolSNAP HQ2 Camera	Roger Scientific	
GFP Filter Set 49002	Chroma	
mCherry Filter Set 49008	Chroma	
Metamorph Software	Molecular Device	
ImageJ Software	U. S. National Institute of Health	

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*Figure 1
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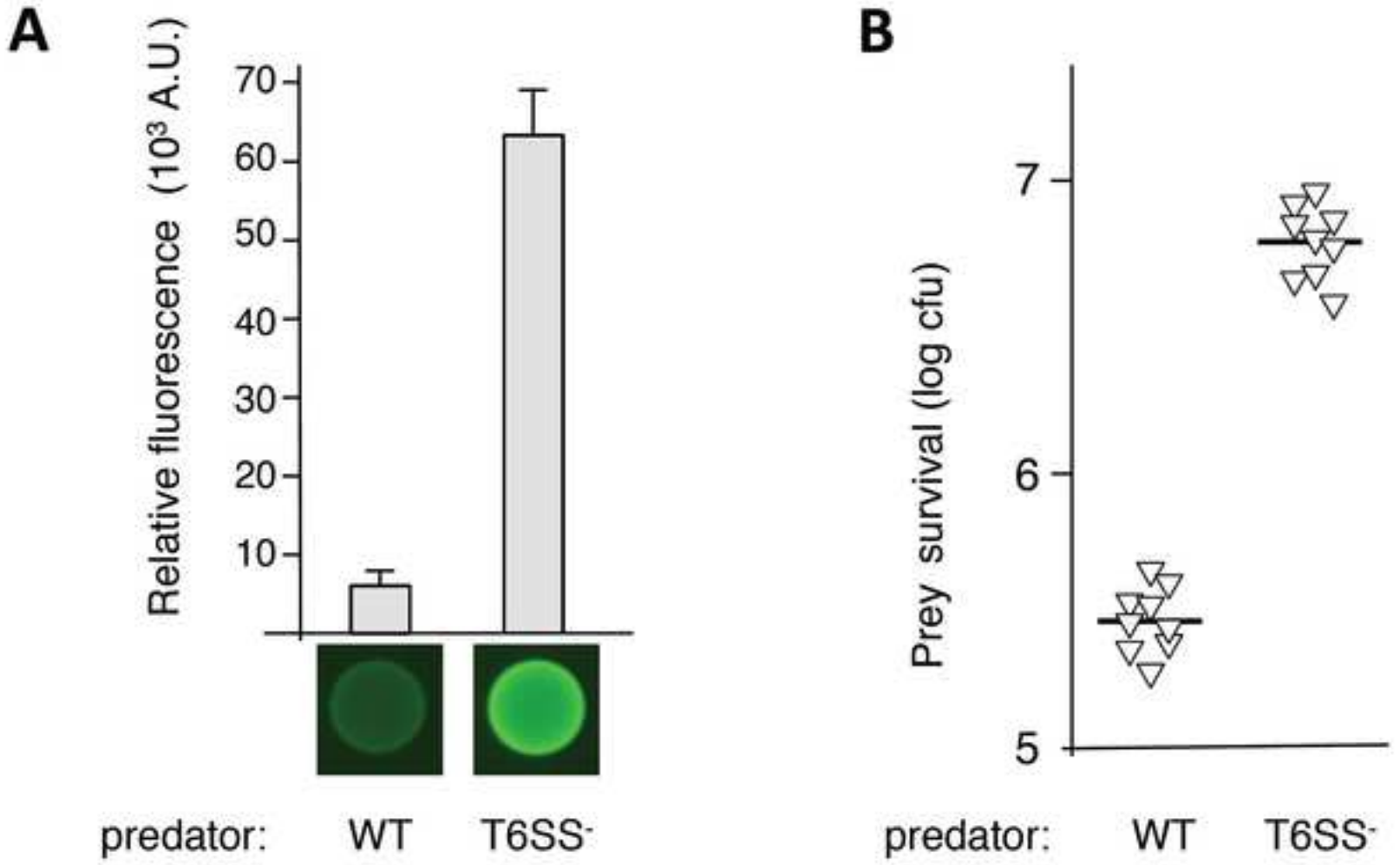

*Figure 2
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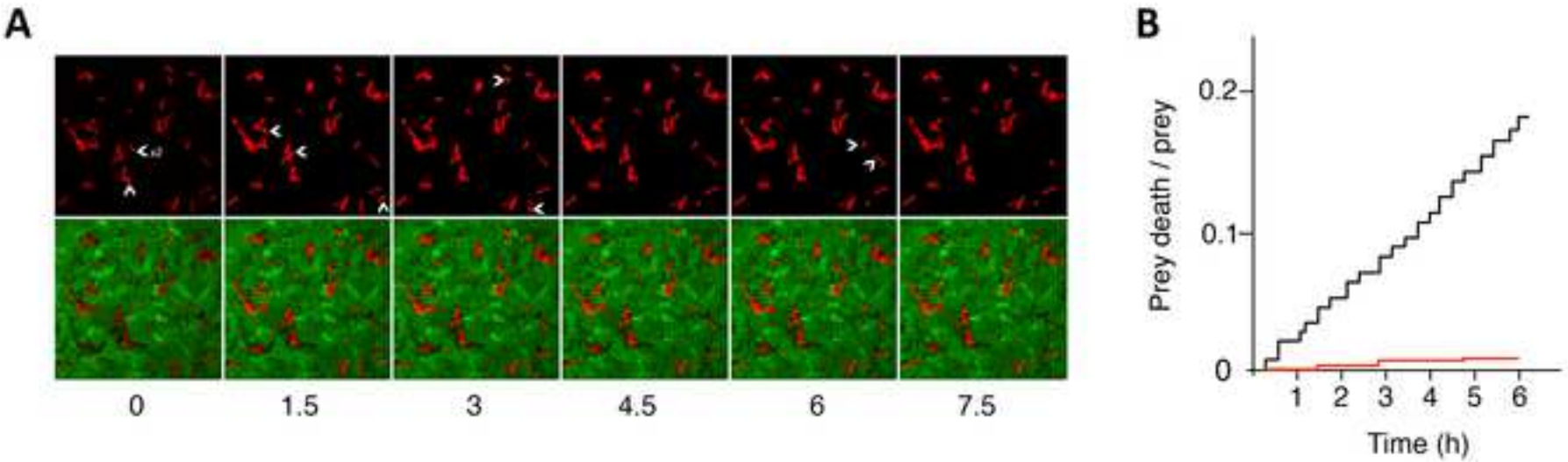

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*Table of Reagents/ Materials Used
Click here to download Table of Reagents/ Materials Used: Brunet-Gueguen_Tables.docx

REAGENTS:

Name of the Reagent	Company	Comments (optional)
MEM	Gibco	Minimal Eagle Medium
Luria Broth	Gibco	
Antibiotics	Sigma Aldrich	

EQUIPMENT:

Material Name	Company	Comments (optional)
Black 96-well plates, clear bottom	Greiner	
Odyssey Imager	LI-COR	
Microplate reader	TECAN	
Fluorescence microscope TE-2000-E	Nikon	Automated, inverted epifluorescence microscope
Perfect Focus System	Nikon	Automatically maintains focus
CoolSNAP HQ2 Camera	Roger Scientific	
GFP Filter Set 49002	Chroma	
mCherry Filter Set 49008	Chroma	
Metamorph Software	Molecular Device	
ImageJ Software	U. S. National Institute of Health	

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