Journal of Visualized Experiments

Quantitative examination of antibiotic susceptibility of Neisseria gonorrhoeae aggregates using a Bactiter assay and Live/Dead Baclight stain

--Manuscript Draft--

Article Type:	Invited Methods Article - JoVE Produced Video
Manuscript Number:	JoVE58978R3
Full Title:	Quantitative examination of antibiotic susceptibility of Neisseria gonorrhoeae aggregates using a Bactiter assay and Live/Dead Baclight stain
Keywords:	gonorrhea; Antibiotic resistance; decreased susceptibility; Biofilm; aggregation; quantification; Visualization
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Question	**Response**
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TITLE:

Quantitative Examination of Antibiotic Susceptibility of *Neisseria gonorrhoeae* Aggregates Using ATP-utilization Commercial Assays and Live/Dead Staining

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KEYWORDS:

gonorrhea, antibiotic resistance, decreased susceptibility, biofilm, aggregation, quantification, visualization

SUMMARY:

A simple ATP-measuring assay and live/dead staining method were used to quantify and visualize *Neisseria gonorrhoeae* survival after treatment with ceftriaxone. This protocol can be extended to examine the antimicrobial effects of any antibiotic and can be used to define the minimal inhibitory concentration of antibiotics in bacterial biofilms.

ABSTRACT:

The emergence of antibiotic resistant *Neisseria gonorrhoeae* (GC) is a worldwide health threat and highlights the need to identify individuals who fail treatment. This Gram-negative bacterium causes gonorrhea exclusively in humans. During infection, it is able to form aggregates and/or biofilms. The minimum inhibitory concentration (MIC) test is used for to determine susceptibility to antibiotics and to define appropriate treatment. However, the mechanism of the eradication in vivo and its relationship to laboratory results are not known. A method that examines how GC aggregation affects antibiotic susceptibility and shows the relationship between aggregate size and antibiotic susceptibility was developed. When GC aggregate, they are more resistant to antibiotic killing, with bacteria in the center surviving ceftriaxone treatment better than those in the periphery. The data indicate that *N. gonorrhoeae* aggregation can reduce its susceptibility to ceftriaxone, which is not reflected using the standard agar plate-based MIC methods. The

method used in this study will allow researchers to test bacterial susceptibility under clinically relevant conditions.

INTRODUCTION:

Gonorrhea is a common sexually transmitted infection (STI)[1]. *Neisseria gonorrhoeae* (GC), a Gram-negative diplococcal bacterium, is the causative agent of this disease. Symptoms of genital infection can result in pain during urination, generalized genital pain, and urethral discharge. Infection is often asymptomatic[2-5], and this allows for extended colonization. These untreated infections are a major health concern, as they have the potential to facilitate transmission of the organism and this can lead to complications such as pelvic inflammatory disease (PID) and disseminated gonococcal infection (DGI)[6]. Antibiotic-resistant gonorrhea is a major public health crisis and an increasing socioeconomic burden[7]. Reduced susceptibility to cephalosporins has resulted in treatment regimen change from a single antibiotic to dual therapy, which combines azithromycin or doxycycline with ceftriaxone[8]. The increased failure of ceftriaxone and azithromycin[9,10], in combination with asymptomatic infections, highlights the need for understanding gonorrhea treatment failures.

The minimum inhibitory concentration (MIC) test, including agar dilution and disc diffusion tests, has been used as the standard medical test for identifying resistance to an antibiotic. Nevertheless, it is unclear if the MIC test reflects bacterial antibiotic resistance in vivo. The formation of bacterial biofilms contributes to the survival of bacteria in the presence of bactericidal concentrations of antibiotic: the MIC testing is unable to detect this effect[11]. Because GC can form biofilms on mucosal surfaces[12], we hypothesize that antibiotic susceptibility within aggregates would be different from that seen in individual GC. Additionally, studies have shown that three phase variable surface molecules, Pili, opacity-associated protein (Opa), and lipooligosaccharides (LOS), that regulate inter-bacterium interactions, lead to different sized aggregates[13-15]. The contribution of these components to antibiotic resistance has not been examined due to the lack of proper methods.

Currently, there are several methods to measure biofilm eradication. The most widely used quantitative method is by measuring the changes in biomass using crystal violet staining[16]. However, the method requires significant experimental manipulation, which can potentially generate errors in experiment repeats[17]. The live/dead staining method used here allows visualization of live and dead bacteria and their distribution within the biofilm. However, the biofilm structure can pose as a physical barrier that reduces dye penetration. Therefore, to quantify live/dead bacteria within a group, the staining is limited to small biofilms or its precursor- microcolonies or aggregations. Other methods, including the agar dilution and disc diffusion tests, are not able to measure the effects of aggregation. To examine GC susceptibility within aggregation after antibiotic exposure, an ideal method would need to have both a quantitative assay that can measure live bacteria and visualize their distribution.

The procedure described here combines an ATP-utilization measurement and a live/dead staining assay to quantitatively and visually examine GC susceptibility within aggregates in the presence of antibiotics.

PROTOCOL:

1. General maintenance of GC strains

1.1 Streak *N. gonorrhoeae* strains on GCK agar with 1% Kellogg supplements[18] (**Table 1**, **Table 2**) from freezer stocks and incubate 37 °C with 5% CO_2 for 16-18 h. Use MS11 expressing phase-variable Opa (MS11Opa+), no Opa (MS11ΔOpa), or a truncated LOS (MS11ΔLgtE).

1.2 Carefully pick pili negative (colony without dark edge) or positive (colony with dark edge) colonies from each strain based on colony morphology[19] using a dissecting light microscope and streak onto a new GCK plate.

1.3 Incubate at 37 °C with 5% CO_2 for 16–18 h before use.

2. Viability quantification of GC aggregations

2.1 Collect GC using a sterile applicator. Swab GC from the plate and re-suspend GC in pre-warmed broth(GCP, **Table 3**) supplemented with 4.2% $NaHCO_3$ and 1% Kellogg solutions[18]. Use spectrophotometry at a wavelength of 650 nm (an OD_{650} of 1 = ~1 x 109 CFU/mL) to determine the concentration of suspended bacteria.

2.2 Adjust the concentration of GC to ~1 x 10^8 CFU/mL.

2.3 Add 99 µL of adjusted GC suspension into wells of a 96-well plate.

2.4 Incubate the plate for 6 h at 37 °C with 5% CO_2 to allow the bacteria to aggregate.

2.5 Add 1 µL of serial diluted ceftriaxone (1000, 100, 50, 25, 12.5, 6.2, 3.1, 1.5, 0.8, 0.4, 0.2 µg/mL) into each well. Leave some wells untreated to serve as controls.

2.6 Incubate the plate for 24 h at 37 °C with 5% CO_2.

2.7 Sonicate the suspension 3 times in each well for 5 s at 144 W and 20 kHz.

2.8 Add 100 µL of commercially available ATP utilization glow reagent into each well, pipette up-and down for 3 times, and incubate for 15 min at 37 °C with 5% CO_2.

2.9 Carefully transfer 150 µL of mixture from each well into a new well in a 96-well black microplate and avoid introducing bubbles.

2.10 Measure the absorbance of each well at 560 nm using the plate reader.

2.11 Calculate the survival rate by the ratio of the reading obtained after serial ceftriaxone treatment to the reading from untreated wells.

3. Fluorescence microscopic analysis of Live/Dead of GC aggregates

3.1 Collect GC using a sterile applicator. Swab GC from the plate and re-suspend GC in pre-warmed GCP media plus 1% Kellogg supplements.

3.2 Determine the number of bacteria by spectrophotometry at a wavelength of 650 nm and adjust the concentration of GC to ~1 x 10^7 CFU/mL.

3.3 Add 198 µL of GC suspension into in 8-well coverslip-bottom chambers.

3.4 Incubate the chamber for 6 h at 37 °C with 5% CO_2 to allow aggregation formation.

3.5 Add 2 µL of ceftriaxone (100 µg/mL or various dilutions) into each well within each aggregation condition. Incubate for the desired time at 37 °C with 5% CO_2.

3.6 Add 0.6 µL of live/dead staining solution mixture into each well and incubate for 20 min at 37 °C with 5% CO_2.

3.7 Acquire Z-series images using a confocal microscope (an equivalent microscope can be used).

3.8 Analyze the images using ImageJ software for measurement of the size of GC aggregates and the fluorescence intensity ratio (FIR) of live-to-dead staining in each aggregate.

4. Image analysis

4.1 Estimation of the size of bacterial aggregates.

4.1.1 Open ImageJ and open an image by dragging a raw image file to the ImageJ menu bar.

4.1.2 Click **Freehand Lines** in the ImageJ menu bar and circle the area of each aggregation in the image.

4.1.3 Click **Analyze | Measure** in the ImageJ menu bar.

4.1.4 Obtain the number in a new window under the **Area** column.

4.2 Quantification of live-to-dead ratio of aggregations

4.2.1 Open ImageJ and open an image by dragging a raw image file to the ImageJ menu bar.

4.2.2 Click **Analyze** | **Set Measurements** in the ImageJ menu bar.

4.2.3 Check the integrated density in the pop-up window and click **OK**.

4.2.4 Click **Image | Color | Channels Tool** in the menu bar and select **Color**.

4.2.5 Check **Channel 1** as the fluorescence for live bacteria staining.

4.2.6 Click **Freehand Lines** in the ImageJ menu bar and circle the area of each aggregation.

4.2.7 Click **Analyze | Measure** in the ImageJ menu bar.

4.2.8 Obtain the number under **IntDen** column.

4.2.9 Check **Channel 2** as the fluorescence for dead bacterial staining. Repeat steps 4.2.6-4.2.8.

4.2.10 Obtain the live-to-dead ratio by dividing number from step 4.2.8 by number from step 4.2.9.

4.3 Statistical analysis

4.3.1 Open GraphPad Prism and enter the numbers obtained from ImageJ to the desired column.

4.3.2 Click **Analyze** and select **t tests** under **Column analyses**.

4.3.3 Check the desired column for comparison and click **OK**.

4.3.4 Obtain the P-Value under **Analysis** window.

4.3.5 Select the **Linear Regression** under **XY analyses** from step 4.3.3

4.3.6 Obtain the **R-square** and the **P-Value** under the Analysis window.

REPRESENTATIVE RESULTS:

Two methods were employed: an ATP utilization assay and a live/dead staining assay. The results can either be combined or individually used for examining bacterial survival within aggregates after antibiotic treatment. The ATP utilization assay has been shown to measure accurately viable bacteria in *S. aureus* biofilms[20,21]. Here, MS11Opa+Pil+ strain was used to examine the role of GC aggregation in antibiotic susceptibility. Non-aggregated MS11Opa+Pil+, aggregated MS11Opa+Pil+, or aggregated and then disrupted by sonication MS11Opa+Pil+ were treated with serial dilutions of ceftriaxone and the ATP level measured (**Figure 1A**). In comparing the percent survival with and without antibiotic treatment, pre-aggregated GC had significantly higher survival than non-aggregated or aggregation-disrupted GC with equal at or above 0.015 μg/mL

of ceftriaxone (MIC from agar dilution test (**Table 4**)). MS11Opa+Pil-, MS11ΔOpa or MS11ΔLgtE, which have the same agar dilution MIC (**Table 4**), but form smaller aggregates, was examined and compared to MS11Opa+Pil+ (**Figure 1B**). MS11Opa+Pil+, forming larger aggregates, had the higher ATP level with ceftriaxone treatment than the mutant strains.

Live/dead staining has been used in several biofilm/aggregation-related studies[22,23]. To determine the effect of aggregation, pre-aggregated MS11Opa+Pil+ was treated with or without ceftriaxone and imaged. This allows both visualization (which can be quantified) and the distribution of live and dead GC after antibiotic treatment (**Figure 2A** – left two panels). Dead bacteria (red) were largely located at the outer layers whereas live GC (green) were located mainly in the core of ceftriaxone treated aggregates. This procedure was performed with MS11Opa+Pil-, MS11ΔOpa or MS11ΔLgtE, to examine aggregation size and survival rate (**Figure 2A**). MS11Opa+Pil+ was shown to form the largest and MS11Opa+Pil- the smallest aggregates (**Figure 2A**,**B**). MS11Opa+Pil+ aggregates were still alive in the core layer whereas GC in the small loose aggregates of MS11ΔOpaPil+, MS11Opa+Pil-, and MS11ΔLgtEPil+ were dead (**Figure 2A,C**). Based on the size and survival, a correlation graph can be plotted to examine the relationship of aggregation size and antibiotic survival (**Figure 2D**).

FIGURE AND TABLE LEGNEDS:

Table 1: Recipe for 1 L of GCK Agar Plate.

Table 2: Recipe for 1 L of 100x Kellogg's supplement.

Table 3: Recipe for 1 L of GCP Bacterial Growth Media.

Table 4: Minimum inhibitory concentration of GC strains treated with ceftriaxone. MS11Opa+Pil+, MS11ΔOpa, MS11ΔLgtE, and MS11Opa+Pil- were grown and suspended in GCP. Agar dilution test was then performed with serial concentration of ceftriaxone from 0.0016 – 0.25 μg/mL.

Figure 1: Representative data of survival rate of aggregated GC under ceftriaxone treatment by ATP-utilization assay. (**A**) Survival rate comparison of MS11Opa+Pil+ suspension without pre-aggregating, pre-aggregating for 6 h, or disrupting after pre-aggregating for 6 h. (**B**) Survival rate comparison of 6 h aggregated MS11Opa+Pil+ with MS11ΔOpaPil+, MS11Opa+Pil-, or MS11ΔLgtEPil+. Shown are the average values (±SD) obtained from three independent experiments. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. This figure was previously published [24] and is used with permission.

Figure 2: Representative data of live/dead bacteria distribution within aggregates under ceftriaxone treatment. (**A**) Pre- aggregated MS11Opa+Pil+, MS11ΔOpaPil+, MS11Opa+Pil-, or MS11ΔLgtEPil+ was either incubated in the presence or absence of 1 μg/mL ceftriaxone for 2 h. Aggregates were then stained to visualize viable (green) and dead (red) GC and visualized with confocal fluorescence microscope. Scale bar: 50 μm. Images were then analyzed for (**B**) aggregation size and (**C**) ratio of live-to-dead GC and (**D**) a correlation graph was then created.

Shown are the average values (SD) obtained from > 40 images of three independent experiments. ***$p < 0.001$; **$p < 0.01$; *$p < 0.05$. This figure was previously published [24]and is used with permission.

DISCUSSION:

Bacteria can form biofilms during infection of the human body. Traditional MIC testing may not reflect the concentration needed to eradicate bacteria in a biofilm. To test antimicrobials effects on a biofilm, methods based on biofilm biomass as well as plating CFUs can be erroneous due to the impact of biofilm structure. For example, the plating method only works if the biofilm can be disrupted. Hence, the CFU obtained may be lower than the actual number of viable bacteria. Visualizing dead and live bacteria within the biofilm have been established for measuring the survival of bacteria within biofilm, however, depending on the structure and density of biofilm, the staining may fail to penetrate into the biofilm and result in an inaccurate and underestimated survival rate.

The method here uses both a quantitative and a visualization assay to measure bacterial survival after treatment of aggregates. The advantage of this method is that it measures the bacterial survival in an environment that is closer to what is seen in a real infection. The survival rate differences between non-aggregated and aggregated bacteria will allow us to determine if the in vitro MIC correlates with the in vivo MIC.

The ATP utilization assay, which measures ATP production, can quantitatively measure the survival of aggregates. The assay is more sensitive and can differentiate survival between small differences in antibiotic concentration, compared to other similar assays. However, due to the high sensitivity of this assay, assurance of bacterial cell lysis is critical. Therefore, a sonication step was used. In addition, this method cannot be used to measure bacterial survival in vivo due to the large amount of ATP that host cells produce that can mask the bacterial ATP level. Moreover, the interaction of host cells with bacteria may affect bacterial ATP production. Using this assay on bacteria with pigments may be limiting as the pigments may interfere with the reading.

The live/dead stain has been widely used in biofilm studies. However, the penetration of the staining dyes may stain only the outermost bacteria, whereas the core may not be stained. Therefore, it can only be used for visualization but not quantification, due to uneven distribution of the dye. We used small aggregates for this staining and demonstrated this assay can be used to quantify the overall bacterial survival within the aggregates. Furthermore, negative and positive controls are needed for adjusting the concentration of the dye for different bacteria.

In combination with the MIC protocol, the ATP utilization assay and live/dead stain can serve as an effective method to quantitatively and visually analyze *N. gonorrhoeae* as well as other bacterial aggregations[25,26]. The application of the combined protocol could provide a better understanding of disease formation along with its versatile uses in drug screening based on bacteria biofilms. This method may reflect more accurately the *in vivo* MIC of an antibiotic.

ACKNOWLEDGEMENT:

This work was supported by a grant from National Institute of Health to D.C.S. and W.S. AI123340. L.-C.W., J.W., A.C., and E.N. were supported in part/participate in "The First-Year Innovation & Research Experience" program funded by the University of Maryland. The funders had no role in study design, data collection, and analysis, decision to publish, or preparation of the manuscript. We acknowledge the UMD CBMG Imaging Core for all microscopy experiments.

DISCLOSURE:

The authors have nothing to disclose.

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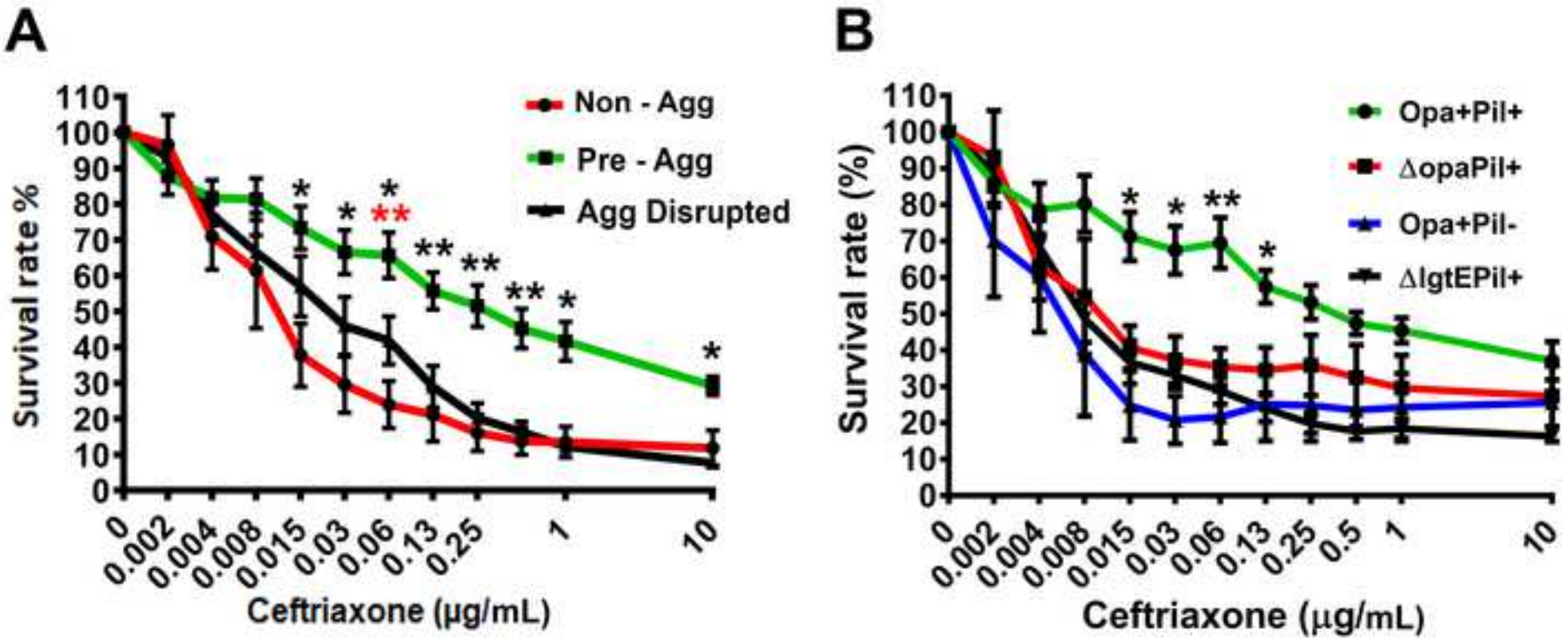

Figure 2

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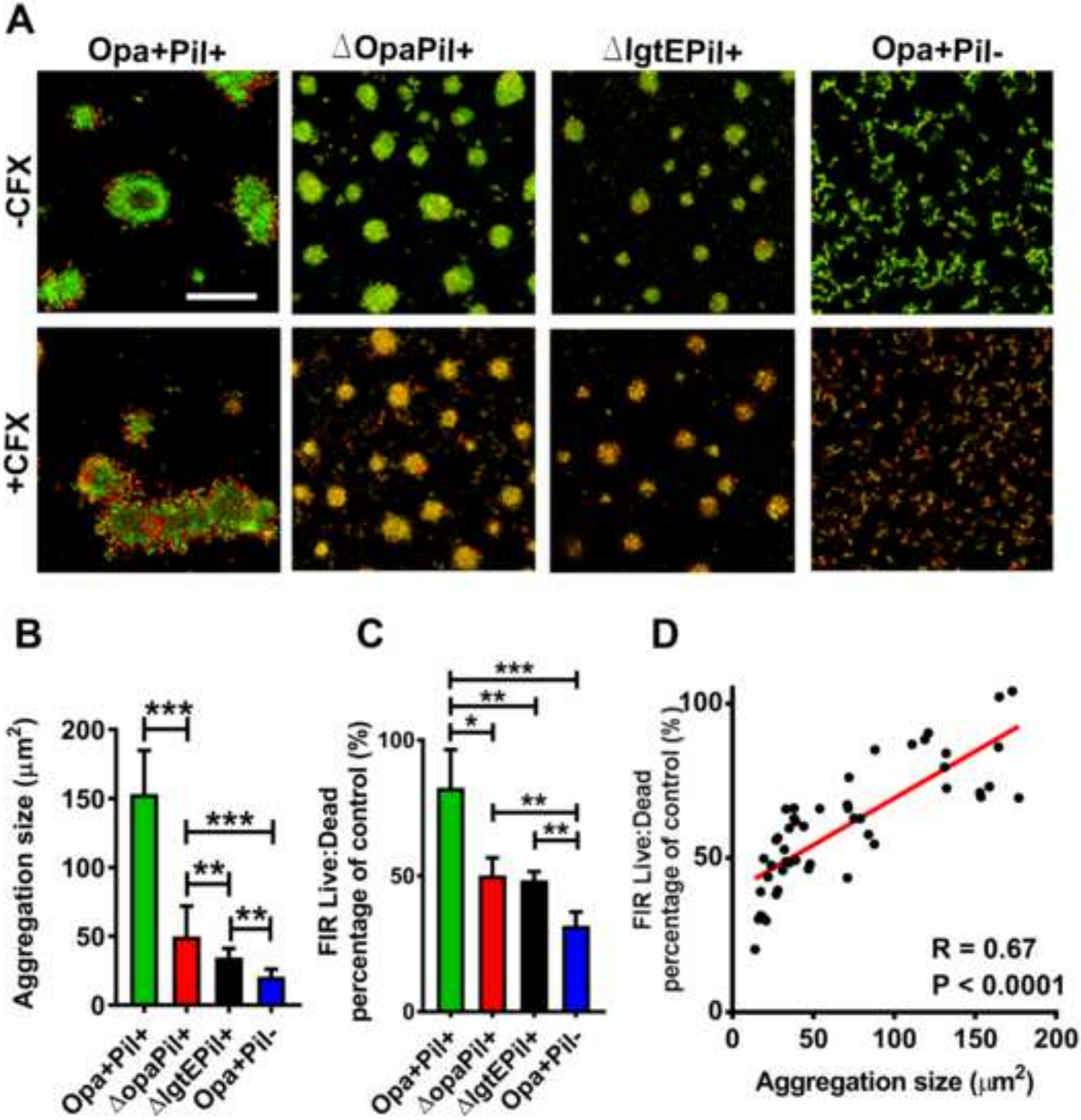

Weight/ Volume	Name of Material
36 g	Difco GC medium base
5 g	Agar
10 mL	100x Kellogg's supplement

Store at 4°C

Weight/ Volume	Name of Material
5 g	L-glutamine Crystalline Powder
0.5 g	Ferric nitrate, nonanhydrate
0.02 g	Thiamine pyrophosphate
400 g	Glucose

Store at 4°C

Weight/ Volume	Name of Material
15 g	Proteose peptone
1g	Soluble starch
4g	Potassium phosphate dibasic
1g	Potassium phosphate monobasic
5g	Sodium chloride

Store at room temperature

MIC	*Neisseria gonorrhoeae* strains			
	MS11Opa+Pil+	**MS11ΔOpa**	**MS11ΔLgtE**	**MS11Opa+Pil-**
Ceftriaxone (μg/mL)	0.016	0.016	0.016	0.016

Name of Material	Company	Catalog Number	Comments/Description
100x Kellogg's supplement			
Agar	United States Biological	A0930	
BacTiter Assay	Promega	G8232	
Ceftriaxone	TCI	C2226	
Difco GC medium base	BD	228950	
Ferric nitrate, nonahydrate	Sigma-Aldrich	254223-10G	
Glucose	Thermo Fisher Scientific	BP350-1	
L-glutamine Crystalline Powder	Fisher Scientific	BP379-100	
BacLight live/dead staining	Invitrogen	L7012	
MS11 Neisseria gonorrhoeae strain			kindly provided by Dr. Hern
Potassium phosphate dibasic (K_2HPO_4)	Fisher Scientific	P290-500	
Potassium phosphate monobasic (KH_2PO_4)	Fisher Scientific	BP329-1	
Proteose Peptone	BD Biosciences	211693	
Sodium chloride (NaCl)	Fisher Scientific	S671-10	
Soluble Starch	Sigma-Aldrich	S9765	
Thiamine pyrophosphate	Sigma-Aldrich	C8754-5G	

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Name of Equipment	**Company**
Petri Dishes	VWR
8-well coverslip-bottom chamber	Thermo Fisher Scientific
96-well tissue culture plates	Corning, Falcon
Biosafety Cabinet (NU-425-600 Class II, A2 Laminar Flow Biohazard Hood)	Nuaire
CO_2 Incubator	Fisher Scientific
Confocal microscope equipped with live imaging chamber	Leica
Corning 96 Well Black Polystyrene Micro	Corning
Glomax Illuminator	Promega
Pipette tips (0.1-10 µL)	Thermo Fisher Scientific
Pipette tips (1000 µL)	VWR
Pipette tips (200 µL)	VWR
Spectrophotometer Ultrospec 2000 UV	Pharmacia Biotech
Sterile 15 ml conical tubes	VWR
Sterile Microcentrifuge Tubes (1.7 mL)	Sorenson BioScience
Sterile polyester-tipped applicators	Fisher Scientific
Sonicator	Kontes

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Signature: L.C.Wang Date: Aug 21, 2018

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Point-to-point response to review comments on Protocol "Quantitative examination of antibiotic susceptibility of Neisseria gonorrhoeae aggregates using a Bactiter assay and Live/Dead Baclight stain"

We would like to thank the editor for carefully reviewing our protocol and providing suggestions. We describe how we address these comments in our revised manuscript below.

Editorial comments:

1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

We have thoroughly proofread the manuscript.

2. Table 2 is never mentioned in the manuscript. Please adjust the numbering of the all Tables.

We have corrected the numbering in the manuscript.

3. All tables should be uploaded separately to your Editorial Manager account in the form of an .xls or .xlsx file.

We have made tables into .xlsx files and uploaded to the submission site.

4. Please add the table titles of all tables in Figure and Table Legends.

We have added table titles in " Figure and Table Legends" section.

5. What are these superscripted numbers in AUTHORS? Do they mean different affiliations?

We have taken off the superscripted numbers.

6. Step 2.5: Please specify the concentrations used in the protocol.

We have added the concentrations we used in the protocol.

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Liang-Chun Wang <marknjoy@umd.edu>

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Monica He <monica.he@mdpi.com> Tue, Aug 14, 2018 at 8:58 PM
To: Liang-Chun Wang <marknjoy@umd.edu>
Cc: antibiotics@mdpi.com

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Kind regards,
Monica

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>
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>
> I am the corresponding author of " Neisseria gonorrhoeae Aggregation Reduces Its Ceftriaxone Susceptibility " in Antibiotics.
>
> Recently a method journal came to me and asked me to publish the methods I used in this antibiotics paper. I was wondering if I can reuse/adapt figures with proper citation of the paper.
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> The journal states that " *If a figure is adapted or republished from a previous publication, authors must cite the original article in the figure legend. Reprint permission for the previously published/adapted figure is required upon acceptance. "*
> *
> *
> Let me know if MDPI-Antibiotics is ok with that.
>
> Thank you,
> Mark
>
> On Thu, Jun 14, 2018 at 10:15 PM, Monica He <monica.he@mdpi.com <mailto:monica.he@mdpi.com>> wrote:
>
> Dear Mark,

You are welcome. It's my pleasure to process your paper. I hope we can cooperate again in the future. :)

Kind regards,
Monica

On 6/15/2018 10:06 AM, Liang-Chun Wang wrote:

Monica thank you a million for taking care of this manuscript.

Have a wonderful day,
Mark

On Thu, Jun 14, 2018, 9:53 PM Monica He <monica.he@mdpi.com
<mailto:monica.he@mdpi.com>
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