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Evaluation of Viable Cells in *Pseudomonas aeruginosa* Biofilms by Colony Count and Live/Dead Staining

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[Abstract] *Pseudomonas aeruginosa* is a human pathogen capable to form robust biofilms. *P. aeruginosa* biofilms represent a serious problem because of the adverse effects on human health and industry, from sanitary and economic points of view. Typical strategies to break down biofilms have been long used, such as the use of disinfectants or antibiotics, but also, according to their high resistance to standard antimicrobial approaches, alternative strategies employing photocatalysis or control of biofilm formation by modifying surfaces, have been proposed. Colony forming units (cfu) counting and live/dead staining, two classic techniques used for biofilm quantification, are detailed in this work. Both methods assess cell viability, a key factor to analyze the microbial susceptibility to given treatment, then, they represent a good approach for evaluation of an antibiofilm strategy.

Keywords: *Pseudomonas aeruginosa*, Biofilms, Viability, Colony count, Live/dead staining, Biofilm quantification, Antibiofilm strategy, Photocatalytic killing

[Background] Bacterial biofilms, complex structures attached to surfaces, are matrices composed by proteins, DNA, polysaccharides and water networks, in which cells are embedded (Costerton *et al.*, 1995). *Pseudomonas aeruginosa* is a versatile bacterium that can be found in terrestrial and aquatic environments, or as human pathogen, either as free cells or as cells in robust biofilms. *P. aeruginosa* biofilms represent a serious problem because of the adverse effects on human health and industry (Nickel *et al.*, 1985; Gibson *et al.*, 1999; Willcox *et al.*, 2001; Ramsey and Wozniak, 2005; Rajasekar *et al.*, 2010; Mulcahy *et al.*, 2014) and their high resistance to antibacterial agents (Mah and O'Toole, 2001; Mah *et al.*, 2003).Because of their resistance and robustness, *P. aeruginosa* biofilms represent a model for biofilm studies (Ciofu and Tolker-Nielsen, 2019). The development of *P. aeruginosa* biofilms is regulated by a complex genetic program; in addition, biofilm formation is modified by environmental factors (O'Toole *et al.*, 2000; Di Bonaventura *et al.*, 2007; Ben Said *et al.*, 2011; Gambino and Cappitelli, 2016; Pezzoni *et al.*, 2018).

Factors related to the high resistance of biofilms include impaired diffusion of antibacterial compounds, reduced sensitivity due to slow growth rate of cells in biofilms, emergence of resistant bacterial phenotypes, presence of antioxidant products in the biofilm matrix, among others (Pezzoni *et al.*, 2014; Hall and Mah, 2017). Strategies employed to combat biofilms include chemical and physical treatments such as application of disinfectants, antibiotics and ultrasound (Bridier *et al.*, 2011; Wu *et al.*, 2014; Gnanadhas *et al.*, 2015). The high resistance of biofilm cells to commonly used disinfectants and the risk to human health and the environment by the use of increasing bactericide doses prompted to

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redirect research to safer strategies, such as the use of photocatalytic techniques, proposed as inexpensive, safe and effective (Dalrymple *et al.*, 2010; Gamage McEvoy and Zhang, 2010; Pezzoni *et al.*, 2020). On the other hand, since adhesion of microorganisms to surfaces depends on the surface topography and roughness, preventing biofilm formation by tuning surfaces at nanoscale level is a major opportunity in the development of antibiofilm strategies (Katsikogianni and Missirlis, 2004; Pezzoni *et al.*, 2017).

In order to deepen in the understanding of biofilm properties and/or evaluate the effectiveness of antibacterial treatments, several techniques have been employed to evaluate the amount of live bacteria in biofilms (Welch *et al.*, 2012). Among them, two known methods are the counting of the number of colony forming units (cfu) and the live/dead staining (Merritt *et al.*, 2005; Smith and Hunter, 2008; Tsvetanova, 2020). Both methods provide valuable but different information. In the cfu method, a viable cell is one capable to form a colony. On the other hand, the live/dead staining evaluates membrane integrity. The live/dead staining method employs the fluorescent stains SYTO 9 and Propidium iodide (PI), and viability is evaluated by fluorescence microscopy. PI is a red dye that only enters cells with permeabilized cytoplasmic membrane, while SYTO 9 is a green dye that stains all types of cells. According to this criterion, green cells (intact membrane) are live and red cells (disrupted membrane) are dead. While the colony count method requires biofilm disruption and a subsequent step of cell culture to visualize individual colonies, the live/dead method can be applied on entire biofilms and allows us to evaluate the morphological aspects of the biofilm. Since the two methods are based on different criteria, the data obtained from them will not be identical but they will follow the same tendency upon a given antibacterial treatment (Berney *et al.*, 2006; Bosshard *et al.*, 2010; Pezzoni *et al.*, 2014 and 2020). In this work, we detailed how to apply both methods to *P. aeruginosa* biofilms.

Materials and Reagents

1. 14 ml sterile Borosilicatetubes (Pyrex®, catalog number:SLW1622/09M))
2. 1.5 ml sterile Eppendorf centrifuge tubes (Eppendorf, catalog number: 022364111)
3. Sterile pipette tips 1-200, 100-1,000 (Corning®, catalog numbers: S4860, S9032)
4. Sterile Petri dish glass plates (40 mm and 80 mm diameter) (Brand®, catalog numbers: BR455701, BR455732)
5. Sterile cotton plugs (Nunn Finer, catalog number: 562)
6. Glass coupons (approximately 15 mm x 15mm x 1mm)
   Obtained from glass slides cut with an emery stone. To prepare the coupons, the slides are sliced gently with successive passes of the emery stone. You can wet the area to facilitate the cut. Finally, the glass slide is wrapped with a towel and the final cut is made by pressing hard on the marked area.
7. Glass slides (YEGREN®, catalog number: 7101)
8. Emery stone (Value-Tec, catalog number 52-003094)
9. Tupper container (20 cm x 30 cm x 6 cm)

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10. Rake shaped glass spreaders (Chemglass Life Science, catalog number: CLS-1350-01)
11. Aluminum foil (Reynolds Wrap Heavy Duty Aluminum Foil, catalog number: 625)
12. Paper towel (WypAll* X 60 Jumbo Roll, KCWW, Kimberly-Clark, catalog number: 30218593)
13. Inoculating loop (Decon™, catalog number: MP 19025)
14. *P. aeruginosa* strain PAO1 (B.H. Holloway)

    Pseudomonas aeruginosa PAO1 is maintained in glycerol stocks in liquid nitrogen. Stocks are prepared by mixing by inversion 130 µl of sterile glycerol and 770 µl of an overnight bacterial culture grown in LB medium. Bacteria are streaked with a sterile inoculating loop from glycerol stocks onto solid LB plates and grow overnight at 37 °C in an incubation stove. Single colonies are used as inoculums.
15. Glycerol (Sigma-Aldrich, catalog number: G5516-500ML)
16. Distilled water (G-BIOSCIENCES, catalog number: 786-1713)
17. Etanol 96% (EMSURE® Reag, catalog number: 159010)
18. Inmersion oil (Leica, standar and type “F”)
19. Tryptone (OXOID, catalog number: LP0042)
20. Yeast extract (Merck, catalog number: 103753)
21. Granulated agar (Difco, catalog number: 214530)
22. Sodium Chloride (NaCl) (Biopack, catalog number: 1646.08)
23. SYTO™ 9 Green Fluorescent Nucleic Acid Stain (Invitrogen, catalog number: S34854)
24. Propidium Iodide (Invitrogen, catalog number: P1304MP)
25. LB medium (see Recipes)
26. LB agar solid medium (see Recipes)
27. 4 M NaCl solution(see Recipes)
28. Saline solution (see Recipes)
29. 3.5 µM SYTO 9 and 20 µM PI solution (see Recipes)

Equipment

1. 125 ml sterile Erlenmeyers flasks (Duran®, catalog number: 2121628)
2. 2-20 µl, 20-100 µl,100-1,000 µl Kartell pluripet micropipettes (Kartell LABWARE, catalog numbers: 13000, 13210, 13220) and 1-10 ml Acura® manual micropipette (Socorex Swiss, catalog number: 825/835)
3. Sterile 50, 100 and 1,000 ml borosilicate measuring cylinders (VILABO, catalog numbers: 3501114, 3501115, 3501118)
4. Sterile 100 ml glass beakers (Brand®, catalog number: BR91224)
5. Spatula (Arnaldochapini®, catalog number: 1929)
6. Stainless steel dissecting tweezer (Fisherbrand, catalog number: 12-000-132)
7. Conventional incubator shaker (New Brunswick Scientific Co., INC, model: G25)
8. UV-Vis Spectrophotometer (Biotraza, model: 752)

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9. Autoclave (Hirayama HICLAVE™, model: HVE-50)
10. Hot air oven sterilizer (DalvoIntrumentos, model: OHR/T)
11. Vortex (Velp, model: ZV3, 201251076)
12. Epifluorescence microscope (Olympus, model: BX51)
13. Incubation stove (Precision, Scientific Group, model 4, catalog number: 31483)
14. Bunsen burner (Eisco™, catalog number: CH0091B)

Software

1. ImageJ software (Rasband, 1997)

Procedure

The steps of biofilm formation and biofilm evaluation are summarized in **Figure 1**.

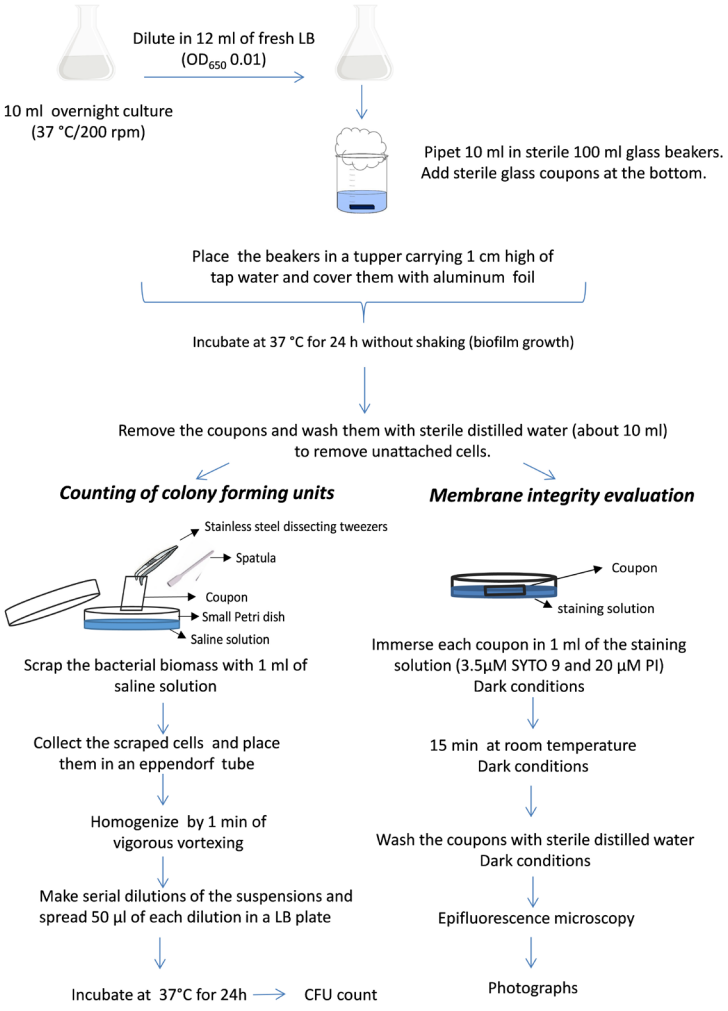

Figure 1. Schematic diagram of the experimental procedure

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A. Biofilm formation

1. Pipet 10 ml of LB medium in sterile 125 ml Erlenmeyer flasks. Then pick, using an inoculation loop, PAO1 colonies from an LB media plate, and shake the loop until the fragment is visibly floating in the liquid LB medium. Grow overnight at 37 °C with shaking (200 rpm) in an incubator shaker. Keep the Bunsen burner on during the entire procedure. Work slowly, carefully, and at all times within this sterile area created by the Bunsen burner.
2. Dilute this overnight culture (desired OD_{650} 2.5-3) in 12 ml of fresh LB medium in a new sterile 125 ml Erlenmeyer flask to achieve a final OD_{650} of about 0.01. Keep the Bunsen burner on during the entire procedure. Work slowly, carefully, and at all times within this sterile area created by the Bunsen burner.
3. Pipet 10 ml of these suspensions in sterile100 ml glass beakers. By using a sterile stainless steel dissecting tweezer, place a sterile glass coupon horizontally at the bottom of each beaker to allow biofilm formation on it. Cap the beaker with a cotton plug (Figure 2).

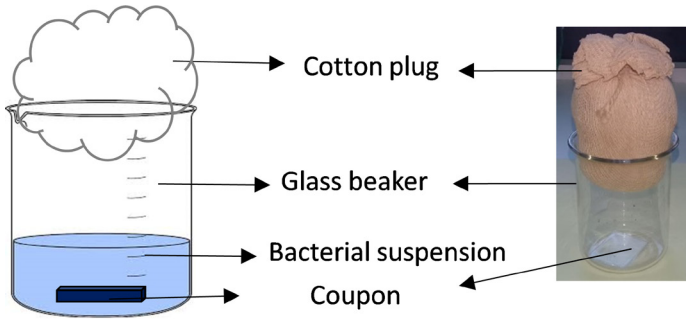

Figure 2. Biofilm formation device

4. Place the glass beakers in a tupper carrying 1 cm high of tap water and cover them with aluminum foil to create a humid chamber.
5. Incubate at 37 °C in an incubation stove for 24 h without shaking.

Note: Immediately before placing the coupons into the beakers, they must be sterilized by dipping into a flask with 96% ethanol and flaming on fire. Repeat this procedure between coupons.

B. Biofilm evaluation

Counting of colony forming units (cfu)

1. Prepare fresh LB agar plates by placing 25 ml of solid LB medium in 80 mm Petri dishes. After solidification, allow them to dry open and upside down in a stove for at least 30 min at 55 °C.
2. Remove the coupons from the beakers with a sterile stainless steel dissecting tweezer.
3. Wash the coupons carefully by letting sterile distilled water (about 10 ml) drop down gently on them to remove unattached cells.
4. Hold the coupons vertically with the tweezer and scrap for 1 min the bacterial biomass from them with a sterile spatula in a 40 mm Petri plates containing 1 ml of saline solution (Figure 3).

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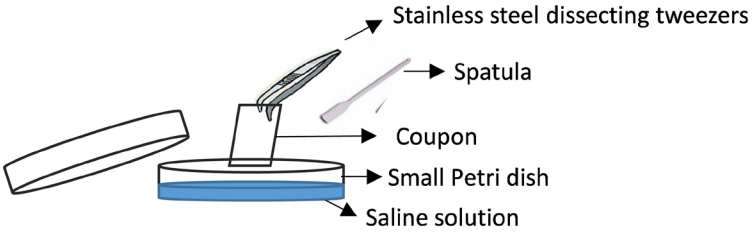

Figure 3. Coupon scraping

5. Wash the coupons by pipetting the saline solution in the small Petri dish to remove all attached bacteria.
6. Place the bacterial suspensions in 1.5 ml sterile Eppendorf tubes.
7. Homogenize by 1 min of vigorous vortexing (2,400 rpm).
8. Make serial dilutions of the suspensions. To do this, add 0.5 ml of the initial sample to a sterile borosilicate glass tube carrying 4.5 ml of saline solution and mix by vortexing (10^{-1} dilution). Then, take 0.5 ml of 10^{-1} dilution and pass it to a new tube carrying 4.5 ml of saline solution to make the next dilution (10^{-2} dilution) and so on until 10^{-5} dilution.
9. Spread 50 µl of each dilution in a sterile dried LB plate by using a sterile rake shaped spreader.
10. Incubate the plates at 37 °C for 24 h in an incubation stove.
11. Count the colonies formed onto the LB agar in the Petri plates. Only plates containing about 20-200 colonies must be taken into account.
12. Determine the number of the viable cells in cfu cm^{-2}. First, the total cfu in a coupon is calculated according the following formula: no. of colonies count in the plate x dilution factor x total volume of biofilm biomass (1 ml)/volume plated (0.05 ml). This value must be divided by the total surface of the coupon to obtain the number of cfu per cm^2. At least three coupons are needed for statistical analysis.

Note: The stainless steel dissecting tweezer and the rake shaped glass spreader must be sterilized by dipping into a flask with 96% ethanol and flaming on fire between each use. Spatulas are immersed in 96% ethanol and dried in an oven before used.

Membrane integrity evaluation

1. Remove the coupons by taking them with a pair of stainless steel dissecting tweezer as for cfu counting procedure.
2. Wash the coupons carefully by letting sterile distilled water (about 10 ml) drop down gently on them to remove unattached cells.
3. Immerse each coupon in 1 ml of a solution containing 3.5 µM SYTO 9 and 20 µM PI in distilled water placed in a small Petri plate. This procedure must be performed in dark conditions.
4. Incubate for 15 min at room temperature in the dark.
5. Wash the coupons with sterile distilled water to remove the staining solution. To do this, dip the coupon in a 100 ml glass beaker carrying 20 ml of distilled water for a few seconds and then let

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dry at room temperature on a paper towel. This procedure must be performed in dark conditions.

6. Observe the coupon under an epifluorescence microscope with 100x objective lens and immersion oil.
7. Take at least 6 representative photographs per slide.
8. Use the ImageJ Program (ImageJ software [Rasband, 1997]) to calculate the percentage of red areas in the images:
   a. Open the image with the ImageJ Program.
   b. Select the red areas using any of the drawing/selection tools (*i.e.*, rectangle, circle, polygon or freeform).
   c. Then, from the Analyze menu, select Set measurements and look of the area value.
   d. Repeat the procedure with all the red areas.
   e. Calculate the percentage of red areas based on the total area of the image.

Notes:

1. *After washing the slides, the stained biofilms could be stored under dark conditions at room temperature for at least 24 h to observe them by epifluorescence microscopy.*
2. *Avoid all contact with SYTO9 and PI; they need to be handled carefully and with gloves. The generation of waste should be avoided or minimized whenever possible. All waste must be discarded as "special waste" under the safety standards of hazardous waste.*
3. *The staining and microscope procedures do not need to maintain sterility.*

Data analysis

As example for the described methodologies, results of the effect of different photocatalytic titania surfaces on *P. aeruginosa* biofilms are shown (Pezzoni *et al.*, 2020). Surfaces were obtained through sol-gel and evaporation-induced self-assembly, by combining titania and surfactants under controlled conditions. They were: non-mesoporous (NM) and mesoporous titania surfaces with different pore sizes, which were achieved based on the use of surfactants Brij-58 (MB) and Pluronics-F127 (MF). In addition, two structural forms of titania were assayed: amorphous and anatase. Biofilms were grown on these surfaces and submitted to ultraviolet-A (UVA) radiation to promote the photocatalytic killing. The biofilms were exposed for 180 min at a fluence rate of 20 W m^{-2} (total dose 216 Kj m^{-2}).

Note: Data presented in Figures 4 and 5 were reported in: Pezzoni, M., Catalano P. N., Delgado, D. C., Pizarro, R. A., Bellino, M. G. and Costa, C. S. (2020). Antibiofilm effect of mesoporous titania coatings on Pseudomonas aeruginosa biofilms. J Photochem Photobiol B 203:111762.

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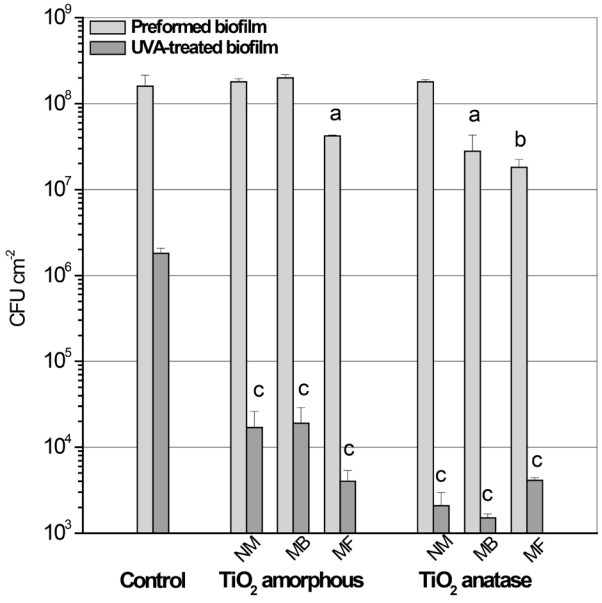

Figure 4. Counting of cfu showing: i) the effect of different titania surfaces on biofilm formation (pre-formed biofilm), and ii) the effect of photocatalytic treatment (UVA-treated biofilm) on these biofilms. 24 h biofilms (Preformed biofilm) were obtained on Control (non-TiO_2) or titania coated surfaces (amorphous NM, MB or MF and anatase NM, MB or MF) and exposed to UVA at a fluence rate of 20 W m^{-2} (UVA-treated biofilm). Appropriate dilutions of the bacterial biomass were plated for cfu count before and after UVA exposure to determine efficiency of biofilm formation and cell survival. Error bars represent the standard deviations of a least three independent experiments. a ($P < 0.05$) and b ($P < 0.005$) represent significant difference between preformed biofilms grown on titania and the control surface before UVA exposure. c ($P < 0.0005$) represents significant difference between biofilms grown on each titania surface and the control surface after UVA exposure.

Surface	Control	TiO_2 amorphous			TiO_2 anatase		
		NM	MB	MF	NM	MB	MF
Dark							
% red cells	1.7 ± 0.5	2.7 ± 0.5	3.3 ± 0.5	2.7 ± 0.5	2.3 ± 0.5	3.3 ± 0.5	3.7 ± 0.5
UVA							
% red cells	8.3 ± 1.7	50.3 ± 1.2	58.7 ± 2.9	62.7 ± 2.1	78.3 ± 1.2	85.7 ± 1.7	76 ± 3.3

Figure 5. Evaluation of membrane integrity as indicator of the effect of photocatalytic treatment on biofilms obtained on different surfaces. Biofilms grown on Control (no TiO_2) or titania coated surfaces (amorphous NM, MB or MF and anatase NM, MB or MF) were maintained in the dark or exposed to UVA at fluence rate of 20 W m^{-2} for 180 min.

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Representative epifluorescence images of biofilms stained with the stains SYTO 9 and PI are shown. The experiments were repeated at least three times. Each value is the mean of three independent tests. The scale bars represent 150 nm.

Recipes

1. LB medium
   a. Dissolve 10 g tryptone, 5 g yeast extract, 5 g NaCl
   b. Bring the volume up to 1,000 ml in distilled water
   c. Autoclave at 1atm for 20 min
2. LB solid medium
   a. Dissolve 10 g tryptone, 5 g yeast extract, 5 g NaCl, 15 g agar
   b. Bring the volume up to 1,000 ml in distilled water
   c. Autoclave at 1atm for 20 min
3. 4 M NaCl
   a. Dissolve 46.7 g NaCl in 200 ml of distilled water
   b. Autoclave at 1atm for 20 min
4. Saline solution
   Mix 7.5 ml sterile 4 M NaCl in 300 ml of sterile distilled water
5. 3.5 µM SYTO 9 and 20 µM PI solution
   0.7 µl of 5 mM SYTO 9 (stock solution; stored at -20 °C protected from the light)
   26 µl of 0.77 mM PI (stock solution; stored at 4 °C protected from the light)
   1,000 µl of distilled water
   Prepare fresh for every experiment
   The solution can be kept in the dark at room temperature during the experiment

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The counting of colony forming units procedure is based on a previously protocol of Merritt, Kadouri and OToole (2005). The live/dead staining method for membrane integrity evaluation is a modification of a protocol published by Smith and Hunter (2008).

Competing interests

The authors declare no conflict of interest.

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