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Quantification of Bacterial Attachment to Tissue Sections

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[Abstract] Here we describe a method to test bacterial adhesion to paraffin embedded tissue sections. This method allows examining binding of different bacterial strains, transfected with a fluorescent protein reporter plasmid to various tissues, to better understand different mechanisms such as colonization. This assay provides a more physiological context to bacterial binding, than would have been achieved using adhesion assays to cell lines. The sections can be imaged using fluorescent microscopy and adhesion of various bacterial strains can be quantified and tested, simultaneously.
Keywords: Host-pathogen interactions, Bacterial attachment, Bacterial colonization

[Background] Many types of bacteria, both commensal and pathogenic, express various adhesion molecules, allowing them binding to different surfaces of the host (Gur *et al*., 2015; Abed *et al*., 2016; Isaacson *et al*., 2017). This adhesion is crucial, as it is the first step of colonization and plays a role in both competition and survival, in different environments (Schilling *et al*., 2001). Many of these adhesins are lectins, binding sugar moieties on glycoproteins on various kinds of cells, such as epithelial cells and others (Abed *et al*., 2016; Isaacson *et al*., 2016). Over the years, many groups studying host-pathogen interactions used cell lines and tissue culture in order to try to understand bacterial adhesion to cells. Tissue sections give a more physiological context to the colonization study, as they provide organization and structures that are almost impossible to obtain using *in vitro* tissue culture. Furthermore, in immortalized or cancerous cells, the expression pattern of surface molecules, to which bacteria can bind, might be altered. In order to better understand physiological context of bacterial adherence, in both normal and pathological conditions, we chose to employ bacterial attachment to tissue sections.

Materials and Reagents

1. Plastic 50 ml tubes for centrifugation (Greiner Bio One International, catalog number: 227270)
2. 1.5 ml tubes for transformation
3. Petri dishes for bacteria (FL MEDICAL, catalog number: 29052)
4. Inoculation loop, 10 µl (Greiner Bio One International, catalog number: 731171)
5. Ice box with ice
6. Slide jars for washing

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7. Superfrost Plus glass slides (Thermo Fisher Scientific, Thermo Scientific™, catalog number: J1800AMNT)
8. Coverslip (Bar Naor, catalog number: BNBB024050A1)
9. Pipette tips (20-200 µl, 100-1,000 µl)
10. *Escherichia coli* strain of interest (for example CFT073)
11. Plasmids for fluorescent protein reporter expression (see references for examples)
12. Calcium chloride (Sigma-Aldrich, catalog number: C5670)
13. Glycerol anhydrous (Avantor Performance Materials, J.T. Baker, catalog number: 2136)
14. Phospho-buffered saline (PBS 10x) (HyLabs, catalog number: BP-507/1Ld)
15. Paraformaldehyde (PFA) (Bar Naor, catalog number: BN15711)
16. Xylene (Sigma-Aldrich, catalog number: 534056)
17. Ethanol (Sigma-Aldrich, catalog number: E7023)
18. ProLong™ Glass Antifade Mountant (Thermo Fisher Scientific, Invitrogen™, catalog number: P36980)
19. Hoechst 33258 (Sigma-Aldrich, catalog number: 94403)
20. Dehydrated culture media, LB Broth (BD, Difco™, catalog number: 244620)
21. Agar purified for microbiology (Sigma-Aldrich, catalog number: 05038)
22. Erythromycin (Sigma-Aldrich, catalog number: E6376)
23. Ampicillin (Bio Basic, catalog number: AB0028)
24. Tris (Avantor Performance Materials, J.T. Baker, catalog number: 4109-1)
25. Sodium chloride (Avantor Performance Materials, J.T. Baker, catalog number: 3624-19)
26. Polyoxyethylene 20 sorbitan monolaurate (Tween 20) (Sigma-Aldrich, catalog number: 93774)
27. Bovine serum albumin (BSA) (VWR, Ameresco, catalog number: 97061-420)
28. Fetal bovine serum (FBS) (Biological Industries, catalog number: 04-0071A)
29. Triton X-100 (Avantor Performance Materials, J.T. Baker, catalog number: X198-07)
30. LB medium (see Recipes)
31. LB agar plates with antibiotics (see Recipes)
32. TBSS solution (10x) (see Recipes)
33. Blocking solution (see Recipes)

Equipment

1. Pipettes
2. Autoclave
3. Spectrophotometer (600 nm wavelength)
4. Shaker
5. Micro centrifuge
6. Incubator
7. Thermoblock

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8. Chemical hood
9. Fluorescence microscope (TL-Nikon)

Software

1. ImagePro Analyzer 7.0 software
2. Software for statistical analysis (GraphPad Prism software version 6.0 or later, for example)

Procedure

The procedure outline is described in Figure 1.

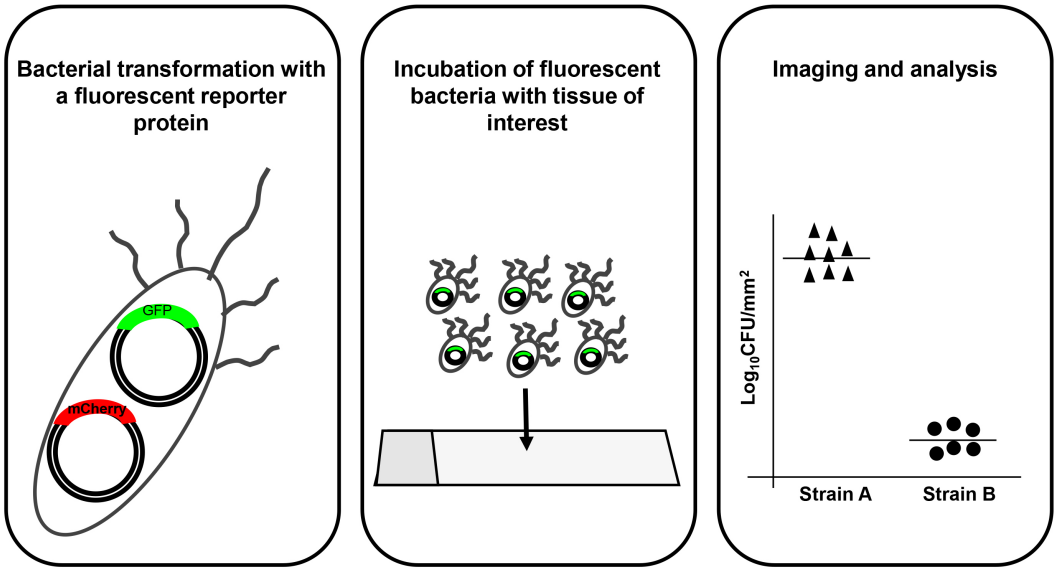

Figure 1. Protocol outline. General outline of the protocol describing the three main stages of the assay. The left panel shows preparation of fluorescent protein expressing *E. coli*. The middle panel outlines the tissue adhesion test and the right panel shows an output of analysis of data acquired during imaging.

A. Preparation of competent bacteria
   1. Grow *E. coli* strain of interest in 5 ml of sterile LB medium (see Recipes) in a 50 ml tube, overnight (12-18 h) at 220 rpm shaking at 37 °C.
   2. Inoculate 500 µl of the overnight starter culture into 50 ml of preheated LB (37 °C) and grow for two hours, at 220 rpm shaking at 37 °C until OD_{600} of 0.3 to 0.4.
   3. Centrifuge at 4 °C, 3,220 *x g* for 10 min.
   4. Discard supernatant, keep pellet on ice for 10 min.
   5. Suspend pellet in 20 ml of 0.1 M cold $CaCl_2$.
   6. Leave for 25 min on ice.

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7. Centrifuge again as indicated in Step A3, discard supernatant and suspend pellet in 2 ml of 0.1 M $CaCl_2$ + 15% glycerol.
   Note: Glycerol should be autoclaved and the $CaCl_2$ solution should be filtered prior to use.
8. Incubate on ice for 90 min.

B. Bacterial transformation
1. Take 100 µl of competent bacteria into a 1.5 ml tube and add 30 ng of the plasmid of choice encoding for–either GFP (Hansen *et al*., 2001) or mCherry (Sason *et al*., 2009).
2. Incubate for 20 min on ice.
3. Transfer tubes to a thermoblock heated to 42 °C for 90 sec.
4. Move tubes to ice for 5 min.
5. Add 1 ml sterile LB and shake for 1 h at 37 °C, 220 rpm.
6. Centrifuge at 4,830 *x g* for 5 min.
7. Resuspend pellet in 150 µl of fresh LB and seed on an LB agar plate supplemented with appropriate antibiotic for selection, according to resistance encoded on the plasmid of choice (here ampicillin and erythromycin, see Recipes).
8. Incubate plate overnight at 37 °C.
9. The next day–pick a single colony, grow in 5 ml LB (supplemented with appropriate antibiotics, see Recipes section) overnight (12-18 h) at 220 rpm shaking at 37 °C.
   Note: In order to avoid loss of fluorescent signal, it is strongly recommended that bacteria expressing fluorescent proteins should be protected from light at all times.
10. Bring bacterial culture to OD_{600} of 1 (dilute in sterile 1x PBS).

C. Tissue binding assay

This assay uses 4 µm thick paraffin embedded section of tissues fixated in PFA, mounted on glass slides (see Materials and Reagents).
1. Fill three staining jars with these three solutions and perform deparaffinization as described (Figure 2A):
   a. Xylene–5 min, 5 min, 2 min.
   b. Ethanol 100%–5 min, 5 min, 2 min.
   c. Ethanol 96%–3 min, 2 min, 2 min.
   Note: Deparaffinization is done in a chemical hood.
2. Cover sections with blocking solution (see Recipes) and incubate at room temperature for 6 h.
3. Suspend 50 µl of bacteria at OD_{600} = 1 in 950 µl blocking solution, after discarding blocking solution, lay the bacterial suspension gently on slide.
4. Incubate overnight at 4 °C in a wet chamber (line chamber with wet tissues), protected from light.

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Next day washing:

5. Prepare 2 staining jars filled with 1x PBS and another staining jar containing PBS with 0.05% Tween 20 (PBST, Figure 2B).
6. Wash twice with PBS for 5 min per wash. Transfer the slides to PBST and wash for 10 min (Figure 2B).

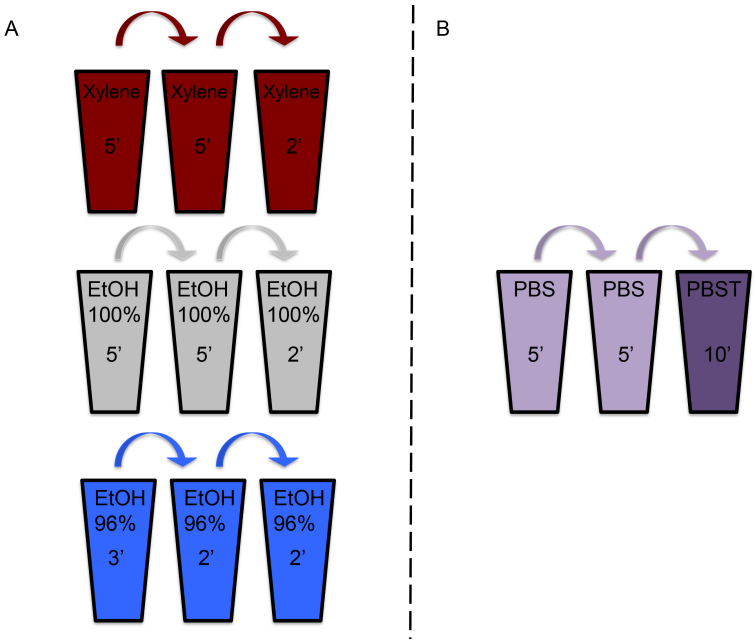

Figure 2. Washing/deparaffinization illustration. Prepare three washing jars filled with the solution indicated on the jar in the illustration and keep the slides in the jar for the indicated amount of time. Figure 2A illustrates the first round of slide deparaffinization in preparation for the binding assay. Figure 2B illustrates washing after overnight incubation to wash off unbound bacteria, dark purple jar contains PBS supplemented with tween (PBST).

7. Dilute Hoechst 33258 1:5,000 in 1x PBS at approximately 200 µl per slide, apply and incubate for 20 min at room temperature. Protect from light.
8. Apply mounting medium to slide and cover with a coverslip.
9. Imagining can be done under a fluorescence microscope using a 60x magnification.
   Note: Scan at least four fields per slide.

Data analysis

1. Images obtained from the fluorescence microscope are converted to 8 bit images by fluorescence microscopy image analysis software (see Figure 3, for example).
2. Fluorescent bacteria should be quantified (for each field) by two different experimenters for a total tissue area of 1,600 µm^2.

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3. Convert field to mm^2. Each fluorescent bacterium counted represents a colony forming unit (CFU), data are represented as $Log_{10}CFU/mm^2$.
   Example: For convenience purposes, this example will refer to a field of 100 μm^2.

   $$(CFU \text{ in } 100\ \mu m^2) = (CFU \text{ in } 0.1\ mm^2)$$
   $$10x\ (CFU \text{ in } 100\ \mu m^2) = CFU \text{ in } 1\ mm^2$$

   If the counted CFU in 100 μm^2 is 1,000, the CFU in mm^2 will be 10,000 (or, 10^4) and therefore the $log_{10}CFU/mm^2$ is 4.
4. Each spot of a single bacterium is referred to as a CFU.

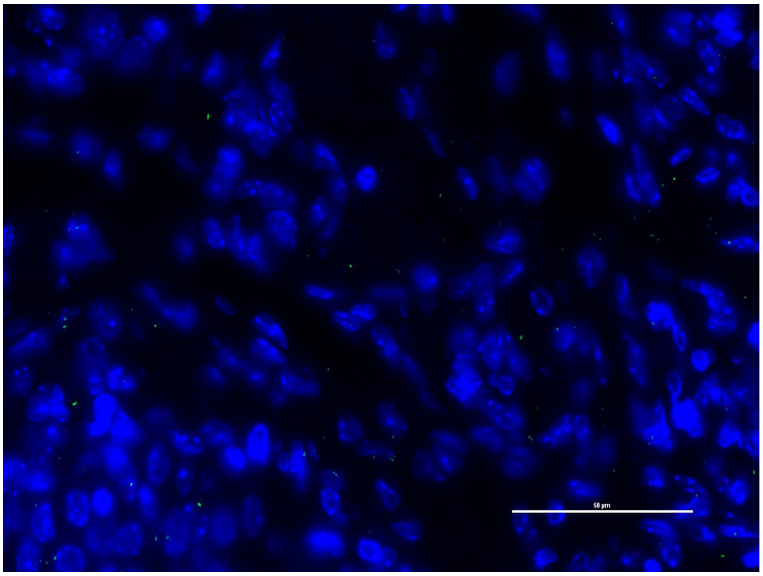

Figure 3. Fluorescent microscope image of GFP expressing uropathogenic *E. coli* (UPEC) adhesion paraffin embedded bladder tissue section. Scale bar = 50 µm.

Recipes

1. LB medium
   1 L of double distilled water (DDW)
   20 g LB dehydrated culture media
   Mix until dissolved
   Autoclave at 121 °C for 30 min and aliquot
2. LB agar plates with antibiotics
   1 L of DDW
   20 g LB dehydrated culture media
   15 g of purified agar
   Mix and autoclave. Agar will dissolve during autoclave heating

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Let cool until LB agar can be handled, before it gets solidified

Add antibiotics (erythromycin at 6 mg/ml and ampicillin at 1 mg/ml) and pour plates

3. TBSS solution (10x)

   500 ml 0.5 M Tris (pH 7.4)

   800 ml 2 M NaCl

   2 ml Tween 20

   Add DDW up to 2 L

   Mix well

4. Blocking solution

   100 ml 1x TBSS

   15 g BSA

   15 ml FBS

   5.75 ml 5% Triton X-100

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