Journal of Visualized Experiments

# Detection of tissue-resident bacteria in bladder biopsies by 16S rRNA fluorescence in situ hybridization

--Manuscript Draft--

| | |
|---|---|
| **Article Type:** | Invited Methods Article - JoVE Produced Video |
| **Manuscript Number:** | JoVE60458R1 |
| **Full Title:** | Detection of tissue-resident bacteria in bladder biopsies by 16S rRNA fluorescence in situ hybridization |
| **Section/Category:** | JoVE Biology |
| **Keywords:** | Urinary Tract Infection; Fluorescence in situ hybridization; 16S rRNA; Bacteria; confocal microscopy; pathogenesis |
| **Corresponding Author:** | Nicole De Nisco, Ph.D.<br><br>UNITED STATES |
| **Corresponding Author's Institution:** | |
| **Corresponding Author E-Mail:** | Nicole.DeNisco@utdallas.edu |
| **Order of Authors:** | Nicole J. De Nisco, Ph.D. |
| | Michael L. Neugent |
| | Jashkaran Gadhvi |
| | Kelli L. Palmer |
| | Philippe E. Zimmern |
| **Additional Information:** | |
| **Question** | **Response** |
| Please indicate whether this article will be Standard Access or Open Access. | Open Access (US$4,200) |
| Please indicate the **city, state/province, and country** where this article will be **filmed**. Please do not use abbreviations. | Richardson, Texas, United States of America |

**TITLE:**

# Detection of Tissue-Resident Bacteria in Bladder Biopsies by 16S rRNA Fluorescence In Situ Hybridization

**AUTHORS AND AFFILIATIONS:**

Michael L. Neugent[1]*, Jashkaran Gadhvi[1]*, Kelli L. Palmer[1], Philippe E. Zimmern[2], Nicole J. De Nisco[1]

[1]Department of Biological Sciences, University of Texas at Dallas, Richardson, TX, USA

[2]Department of Urology, University of Texas Southwestern Medical Center, Dallas, TX, USA

*These authors contributed equally.

**Email addresses of co-authors:**

Michael L. Neugent (Michael.Neugent@utdallas.edu)

Jashkaran Gadhvi (Jashkaran.Gadhvi@utdallas.edu)

Kelli L. Palmer (Kelli.Palmer@utdallas.edu)

Philippe E. Zimmern (Philippe.Zimmern@utsouthwestern.edu)

**Corresponding author:**

Nicole J. De Nisco (Nicole.DeNisco@utdallas.edu)

**KEYWORDS:**

urinary tract infection, bladder, fluorescence in situ hybridization, 16S rRNA, bacteria, confocal microscopy, pathogenesis

**SUMMARY:**

This protocol is for the unbiased detection of tissue-associated bacteria in patient biopsies by 16S rRNA in situ hybridization and confocal microscopy.

**ABSTRACT:**

Visualization of the interaction of bacteria with host mucosal surfaces and tissues can provide valuable insight into mechanisms of pathogenesis. While visualization of bacterial pathogens in animal models of infection can rely on bacterial strains engineered to express fluorescent proteins such as GFP, visualization of bacteria within the mucosa of biopsies or tissue obtained from human patients requires an unbiased method. Here, we describe an efficient method for the detection of tissue-associated bacteria in human biopsy sections. This method utilizes fluorescent in situ hybridization (FISH) with a fluorescently labeled universal oligonucleotide probe for 16S rRNA to label tissue-associated bacteria within bladder biopsy sections acquired from patients suffering from recurrent urinary tract infection. Through use of a universal 16S rRNA probe, bacteria can be detected without prior knowledge of species, genera, or biochemical characteristics, such as lipopolysaccharide (LPS), that would be required for detection by immunofluorescence experiments. We describe a complete protocol for 16S rRNA FISH from biopsy fixation to imaging by confocal microscopy. This protocol can be adapted for use in almost any type of tissue and represents a powerful tool for the unbiased visualization of clinically-

relevant bacterial-host interactions in patient tissue. Furthermore, using species or genera-specific probes, this protocol can be adapted for the detection of specific bacterial pathogens within patient tissue.

**INTRODUCTION:**

The urinary tract, consisting of the urethra, bladder, ureters and kidneys is constantly exposed to bacteria that comprise the urinary microbiome as well as invading uropathogens, like uropathogenic *E. coli* (UPEC), from the gastrointestinal tract[1,2]. A layer of hydrated mucus consisting of glycosaminoglycans and an impermeable plaque of glycosylated uroplakin proteins expressed on the surface of the superficial cells form a barrier that routinely protects the bladder epithelium from invasion by adherent bacteria[3,4]. During urinary tract infection (UTI), these barriers are disturbed or destroyed, facilitating attachment to and invasion of the bladder epithelium by uropathogenic bacteria[5,6]. Work in murine models has revealed that many uropathogenic bacteria including UPEC, *Klebsiella pneumoniae,* and *Enterococcus faecalis* can form replicative intracellular communities (IBCs) within the cytoplasm of superficial cells and quiescent intracellular reservoirs (QIRs) within transitional epithelial cells[7-9]. Although UPEC has been identified within shed epithelial cells from human UTI patients, the interaction of uropathogens with the bladder mucosa in humans had not been previously visualized[10].

We adapted a common technique, fluorescence in situ hybridization (FISH), to detect bacteria within the mucosa of bladder biopsies obtained from postmenopausal patients undergoing cystoscopy with electrofulguration of trigonitis (CEFT) for the advanced management of antibiotic-refractory recurrent UTI[11]. Using a universal probe for 16S rRNA, we were able to objectively detect bacterial species associated with the bladder mucosa of recurrent UTI patients and determine their position within the bladder wall[12]. The universal 16S rRNA nucleotide probe was previously designed to target a conserved region of the bacterial 16S rRNA[13], which corresponds to positions 388-355 of the E. coli 16s rRNA. The 16S rRNA and scramble probe sequences have been previously validated and published for use in the mouse gastrointestinal tract[14,15]. The sequences and properties of the probes are described in **Table 1**. It is essential to use two sequential sections in this protocol, one for the 16S rRNA probe and one for the scramble probe, to be able to distinguish between true and background signal as the bladder epithelium, collagen and elastin exhibit autofluorescence[16]. In this protocol, the 16S rRNA and scramble probes were designed with fluorescent Alexa Fluor 488 labels on both the 3' and 5' termini via N-hydroxysuccinimide (NHS) ester linkages to increase fluorescent signal.

Although this protocol was developed for use on human bladder biopsy sections, it can readily be adapted for use on paraffin-embedded sections from any tissue where bacteria are believed to reside. Unlike immunohistochemistry experiments that target specific antigens (e.g., lipopolysaccharide) on the bacterial surface, this method requires no prior knowledge of antigens expressed by the tissue-associated bacteria[10,17]. Use of the universal 16S rRNA probe allows the unbiased detection of all bacterial species within the sample but does not allow determination of their identity. To determine the identify of detected bacteria, species or genus-specific 16S or 23S rRNA probes must be used. This protocol will also not detect fungal pathogens, such as *Candida albicans,* associated with host tissue. For detection of fungal pathogens, 28S or 18S rRNA

probes must be used[18].

**PROTOCOL:**

The study protocol was approved by and followed the guidelines of the Institutional Biosafety and Chemical Safety Committees of UT Dallas and UT Southwestern Medical Center. The use of biopsies from human subjects in this protocol was approved by and followed the guidelines of the Institutional Review Boards of the UT Dallas and the UT Southwestern Medical Center. All individuals involved with biopsy collection and processing have current human subject protection (HSP) and HIPPA training.

**1. Tissue preparation for fixation and paraffin embedding**

NOTE: Biopsies were taken from consenting women undergoing cystoscopy with electro-fulguration of trigonitis for the advanced management of recurrent urinary tract infection (rUTI). rUTI is defined as ≥3 UTIs in a 12-month period. Biopsy collection was performed in the operating room while the patient was under anesthesia after obtaining informed patient consent per UTSW IRB protocol STU 082010-016. All samples were coded and de-identified before experimentation.

1.1. Obtain a cold-cup (~1 $mm^3$) biopsy from the bladder trigone with urologic forceps via flexible cystoscope and place immediately into a sterile 2 mL cryovial containing 1.5 mL of 4% v/v paraformaldehyde (PFA) prepared in 1x sterile phosphate buffered saline (PBS).

NOTE: 4% PFA in 1x PBS can be made in advance and stored at -20 °C until needed.

1.2. Fix biopsy for 6 h at room temperature or for 16-24 h at 4 °C.

NOTE: Fixation duration should be calculated based on the size of the tissue sample and over-fixation should be avoided. It is not advised to use glutaraldehyde as a fixative as it introduces autofluorescence.

1.3. In a sterilized hood or biosafety cabinet, remove fixative by pipetting and replace with 1.5 mL of sterile 1x PBS. Keep the samples at 4 °C overnight or immediately perform tissue processing and paraffin embedding[19].

1.4. Use a sterilized microtome to section tissue blocks at 5 μm thickness and adhere paraffin tissue sections to charged glass microscope slides. A minimum of two slides per biopsy will be required for the 16S rRNA FISH protocol.

NOTE: The biopsy tissue should be cross sectionally arranged relative to the cutting plane in order to ensure visualization of urothelial layers in all sections. It should also be noted that section thickness should be optimized for bacterial community detection. Thinner sections or sampling of multiple serial sections may be required in the case of infections where tissue-resident bacteria are extremely scarce (e.g., <1 tissue-resident bacterium per 1000 mammalian cells).

## 2. Fluorescence in situ hybridization with universal 16S rRNA probes

NOTE: Two slides per biopsy are required. One slide is needed for the universal 16S rRNA probe and one slide for a control probe with a scrambled sequence. This is important for distinguishing true signal from background signal during microscopy since the bladder epithelium is auto-fluorescent in multiple channels. In addition to the scramble probe, blocking with 0.1% Sudan Black B prior to mounting may reduce background autofluorescence inherent in the tissue[20].

### 2.1. Preparation of reagents and fume hood

2.1.1. Clean an empty fume hood (or appropriately fitted biosafety cabinet) with 70% ethanol.

2.1.2. Prepare the hybridization buffer comprised of 0.9 M sodium chloride (NaCl), 20 mM Tris-HCl (pH 7.2), 0.1% sodium dodecyl sulfate (SDS) in 10 mL of sterile-filtered water.

NOTE: Sterile-filtered water may be prepared by passing autoclaved distilled water through a 0.22 μM filter. The hybridization buffer can be stored at room temperature, but the SDS may precipitate from solution. If SDS precipitates, warm the solution in a 50 °C water bath prior to use.

2.1.3. Prepare at least 100 mL each of 95% and 90% ethanol in sterile-filtered water in washed, autoclaved bottles.

2.1.4. Dissolve fluorescently labeled lyophilized probes in 10 mM Tris-HCl (pH 8.0) and 1 mM ethylenediaminetetraacetic acid (EDTA) buffer (TE) prepared in filter-sterilized nuclease free water to a final concentration of 100 μM. Prepare a dilution of 1 μM in TE buffer for use in this protocol. Store both 100 μM concentrated stock and 1 μM stock reconstituted probes at -20 °C protected from light.

NOTE: Do not dissolve fluorescently labeled probes in water. Buffering is required to prevent hydrolysis of the NHS ester bond conjugating the fluorophore to the nucleotide probe.

2.1.5. Clean five Coplin jars with 70% ethanol, allow to dry, label as follows: xylenes I, xylenes II, 95% EtOH, 90% EtOH, dd$H_2O$, and fill with 100 mL of the appropriate solution. This will help avoid confusion in later steps.

### 2.2. Tissue de-paraffinization and rehydration

2.2.1. In the hood, place two slides per biopsy into a vertical slide rack.

2.2.2. Place a vertical slide rack into the xylenes I Coplin jar (containing 100 mL of xylenes) for 10 min.

2.2.3. Remove the slide rack from xylenes I and blot the bottom on a paper towel to remove excess xylenes. Place into the xylenes II Coplin jar for 10 min.

NOTE: Never work with xylenes outside of a certified fume hood.

2.2.4. Rehydrate deparaffinized tissue sections in successive ethanol washes (95% and 90%) for 10 min each in the respectively labeled Coplin jars.

NOTE: During this stage, warm Hybridization Buffer to 50-56 °C in a water bath

2.2.5. Remove the slide rack from the 90% ethanol wash, blot the bottom on paper towels to remove excess ethanol, and place in the dd$H_2O$ Coplin jar containing 100 mL of filter-sterilized dd$H_2O$ for 10 min.

2.2.6. While waiting for the dd$H_2O$ wash, dilute the probes to 10 nM in hybridization buffer to create the staining solution. Prepare 150 μL of staining solution per slide.

NOTE: Protect staining solution from light by wrapping the tubes in aluminum foil and storing in a drawer. When working with fluorescently labeled probes on the benchtop, consider turning off overhead lights when able. Probes diluted in hybridization buffer should not be re-used.

**2.3. Hybridization and counter-staining**

2.3.1. Prepare a humidifying chamber for each probe by placing soaked, crumpled Kimwipe and sterile water to the reservoir of a P1000 tip box. Place the tip holder cartridge on-top – this is where the slides will sit.

NOTE: It is important to use a humidifying chamber to prevent the biopsy sections from drying out during hybridization. It should be noted that commercially available humidifier chambers are designed to maintain a stable, humid atmosphere. However, the technique detailed here sufficiently controls humidity at substantially less cost.

2.3.2. Remove the slides from the slide rack and place onto a fresh paper towel (tissue-side up). Use a Kimwipe to dry the slide. Be careful to only gently dab near (not on) the biopsy section to wick away water. Using a hydrophobic pen, draw a border around the biopsy section and place the slide tissue-side up in the humidifying chamber.

NOTE: Work quickly so that the tissues do not dry out before hybridization.

2.3.3. Place the humidifying chamber into an incubator set to 50 °C. Pipette 50-150 μL of the staining solution directly on top of the tissue so that the rectangle made by the hydrophobic border around the tissue is filled. Be careful to not add too much solution as to overflow the hydrophobic border. Close the box gently.

2.3.4. Incubate overnight (~16 h) at 50 °C in the dark. If the incubator has a window, cover it with aluminum foil to create a dark environment.

NOTE: A hybridization temperature below the melting temperature of the FISH probe is required for reliable signal. 50 °C is the optimal temperature for the universal 16S rRNA probe but may not be optimal for other probes.

2.3.5. The following morning, prepare at least 500 mL of Wash buffer comprised of 0.9 M NaCl, 20 mM Tris-HCl (pH 7.2) in $ddH_2O$ and filter-sterilize into a sterile bottle with a vacuum bottle-top filter. Warm to 50-56 °C in a water bath.

2.3.6. Remove the slides from the humidifying chambers and carefully wick away any remaining hybridization solution with a Kimwipe. Place the slides in a vertical staining rack.

2.3.7. Place the staining rack into an aluminum foil-wrapped Coplin jar containing 100 mL of pre-warmed Wash buffer for 10 min. If the Coplin jars are not opaque (e.g., glass), place them in the dark during incubation steps – perhaps under a box or in a drawer.

2.3.8. Repeat the wash step twice with fresh Wash buffer in new Coplin jars.

2.3.9. During the wash steps, prepare the counter-stain by diluting a 100 μg/mL stock solution of Hoechst 33342 1:1,000 in wash buffer. To the same tube, add Alexa-555 wheat germ agglutinin (WGA) to a final concentration of 5μg/mL and Alexa-555 Phalloidin to a final concentration of 33 nM. Store in the dark until ready for use.

NOTE: Hoechst, Alexa-555 WGA, and Alexa-555 Phalloidin label DNA, mucin/uroplakins, and actin, respectively and may be stored long-term in the dark per the manufacturer's instructions. Fluorescent labels used for probes and counterstains can be customized for the filter sets available for the microscope to be used.

2.3.10. Remove the slides from the last wash and gently wick away excess wash buffer with a Kimwipe. Place the slides tissue-side up on a paper towel and add 50-150 μL of counter-stain directly on top of the tissue so that the hydrophobic border is filled, but not overflowing. Cover up to four slides with a cryobox-top and incubate for 10 min at room temperature.

2.3.11. Place the sides back into the staining rack and wash twice more in Coplin jars with fresh Wash buffer, for 10 min each.

2.3.12. Thoroughly dry the slides after the last wash and place tissue-side up on a paper towel under a cryobox-top. Squeeze one drop of mounting media directly on top of the tissue. Gently place an appropriately sized coverslip (will depend on biopsy size) on top. Gently press out any bubbles as they will interfere with imaging and allow the cover-slipped slides to cure overnight in the dark.

2.3.13. The next day, seal the edges of the coverslip to the slide with a light coat of clear nail polish. Allow to dry for 10 min in the dark and then store in the dark at 4 °C for confocal microscopy.

## 3. Visualization of 16S rRNA FISH by confocal microscopy

NOTE: For this protocol, best results are achieved with a laser-scanning confocal microscope with 63X and 100X objectives. Proper filter sets for visualization of Hoechst, Alexa-488, and Alexa-555 fluorescence are required. However, standard fluorescent microscopy can be used if a confocal microscope is unavailable. This protocol is for a laser scanning confocal microscope.

3.1. Switch on the confocal microscope and the computer software associated with the microscope per manufacturer instructions.

3.2. Load the slide and visualize with the 10X objective in the blue (DAPI/Hoechst) channel. Focus carefully until nuclei are visible.

3.3. Once focused, change the objective to high magnification (63X or 100X). Add oil on top of the cover slip. Refocus with the new objective, making sure that the objective lens comes in contact with oil while focusing.

NOTE: Use only fine focusing at high magnification (63X or 100X). Oil should only be used for an oil objective.

3.4. Quickly assess each slide through the eye-piece in the green (eGFP/Alexa-488) channel to determine which slides are FISH positive and which are FISH negative.

NOTE: It is best if this initial assessment/scoring is done blinded by a separate individual to reduce experimental bias.

3.5. To image the stained biopsies, start with a FISH positive slide and switch to the computer visualization mode. Select the 405 (Hoechst), 488 (Alexa-488), 555 (Alexa-555) channels. Set the pinhole using the longest wavelength channel, in this case 555. Find the correct focal plane for visualization of labeled bacteria in the 488 channel. Without changing the focal plane, set the gain, laser power, and offset for each channel such that the signal is not saturated, and the background is not over-corrected. Acquire the image in all three channels.

NOTE: Use the same settings for the 488 channel to image every slide in an experiment. The laser power may require adjustment in the 405 and 555 channels if the optimal focal plane for bacterial visualization changes between fields.

3.6. Repeat on additional fields until acquiring images of the entire epithelial surface.

NOTE: You may have to change the focal plane slightly to visualize labeled bacteria in different fields, but never change the gain, laser power, or offset for the 488 channel between fields. If working on a confocal microscope, it may be informative to capture a Z-stack so that the three-dimensional localization of the bacteria within the tissue may be analyzed.

3.7. Process images and quantify labeled bacteria within or associated with the tissue using ImageJ or similar software. Minimal processing of the images is recommended (e.g., splitting/merging channels and converting into image files), although background correction may be performed if necessary. All corrections or other alterations should remain consistent between images.

**REPRESENTATIVE RESULTS:**

The protocol has been optimized for the unbiased detection of bacteria associated with the bladder mucosa in paraffin-embedded bladder biopsy sections. **Figure 1** depicts representative confocal micrographs from an experiment using this protocol on sections of bladder biopsies obtained from women with recurrent urinary tract infection. Two serial sections were hybridized with either the universal 16S rRNA (upper panels) or scramble (lower panels) probes. Images from the same region of the tissue were taken and bacteria (green) are clearly visible in the tissue hybridized with the 16S rRNA probes and not with the scramble probe. **Figure 2** represents a false positive result. Signal corresponding to autofluorescent collagen or elastin is detected in the 405 and 488 channels in both the 16S rRNA and scramble probe-hybridized biopsy sections highlighting the importance of always using a scramble probe control.

**FIGURE AND TABLE LEGENDS:**

**Table 1. FISH probe sequences and characteristics.** Tm indicates melting temperature and NHS is an abbreviation for N-hydroxysuccinimide.

**Figure 1. Representative confocal micrographs of FISH of universal 16S rRNA and scramble probe in a human bladder biopsy.** Actin and Mucin are labeled in red, cellular nuclei are labeled in blue, and bacteria in green. Tissue-associated bacteria are only detected with the 16SrRNA probe and not the scramble. Images taken at 63X magnification. Scale bar = 20 μm.

**Figure 2. Representative confocal micrographs of false-positive green autofluorescence in a human bladder biopsy.** Actin and mucin are labeled in red, cellular nuclei are labeled in blue, and bacteria and autofluorescent components of the extracellular matrix (e.g., collagen and elastin) are green. Green fluorescence is observed with both the 16S rRNA and scramble probes indicating a false-positive result. Images are taken at 63X magnification. Scale bar = 20 μm.

**DISCUSSION:**

Here, we describe a protocol for the detection of tissue-associated bacteria in human bladder biopsies by 16S rRNA FlSH. This protocol can be easily adapted for biopsies taken from other tissues, such as the gastrointestinal tract or skin, and can be extended to tissues harvested from a variety of mammalian model organisms. The protocol described here can also be adapted to

for the use of multiple fixation (e.g., formalin, ethanol, methacarn) and tissue preparation techniques (e.g., paraffin or resin embedded, and cryopreserved tissues). The double-labeled universal 16S rRNA probe allows for the unbiased detection of all bacterial species present within tissue and can provide valuable insight into how pathogens and the microbiota spatially interact with mucosal surfaces in disease and healthy states. Using resources such as probeBase, PhylOPDb or the PROBE_DESIGN tool of the ARB software package for the selection or design of species or genera-specific 16S or 23S rRNA probes, this protocol can be adapted for the detection of specific bacterial species or genera within tissue[15,21,22]. An important future direction for this method is multiplexing using species- or genera-specific probes labeled with different, discrete fluorophores for evaluation of microbial diversity within the bladder mucosa.

The primary limitation of this method for use on human specimens is the availability of biopsied tissue. Institutional Review Board approval and informed patient consent are required to obtain biopsies and direct collaboration with the clinician performing the procedure is necessary for optimal sample collection and access to patient metadata. The CEFT procedure itself destroys the bladder epithelium so we were able to justify biopsy of these areas before the procedure. A fume hood or appropriately fitted biosafety cabinet is required for this protocol due to the use of toxic xylenes in the deparaffinization step and the need to maintain a sterile environment throughout the procedure. A fluorescent microscope, preferably confocal, with a 63X or a 100X objective and appropriate filter sets for visualization of Hoechst, Alexa-555, and Alexa-488 is required for this protocol. The representative results depicted in **Figure 1** were imaged using a laser scanning confocal microscope. Similar laser scanning microscopes should produce comparable images. This protocol is limited by its ability to only detect tissue-associated bacteria and not, for example, fungi. Probes specific the fungal 18S or 28S rRNA must be used to identify fungal pathogens within tissue[18].

Critical steps to this protocol include maintaining a sterile environment throughout the procedure and ensuring that the tissue does not dry out between hybridization and staining steps. If the tissue dries out during the procedure, the signal may be dampened or the tissue may fall off of the slide during a wash step. It is also critical to always use two serial sections for this protocol – one for the 16S rRNA probe and one for the scramble probe. Without this control, it may be very difficult to distinguish false positives and the data obtained may not be useful or informative. If this protocol is being adapted for use with a probe other than the universal 16S rRNA probe, care must be taken to select an appropriate hybridization temperature, approximately 5 °C lower than the predicted melting temperature of the probe. To maintain signal intensity, the tissue must not be exposed to light for long periods of time after the probe has been added and must not be overexposed during microscopy. Lastly, during microscopy the same settings for the channel corresponding to the fluorophore conjugated to the FISH probe must be kept consistent between the experimental (16S rRNA probe) and control (scramble probe) slides. Visualizing the spatial relationship of bacteria within mucosal surfaces of patient-derived tissues is critical to understanding and building clinically relevant hypotheses about the host-pathogen interactions underlying infectious disease.

**ACKNOWLEDGMENTS:**

We would like to thank Kim Orth and Marcela de Souza Santos for protocol advice and Amanda Arute for technical support. This work was partially supported by the Cecil H. and Ida Green Chair in Systems Biology Science held by K.P.

**DISCLOSURES:**

The authors have nothing to disclose.

**REFERENCES:**

1 Abraham, S. N. & Miao, Y. The nature of immune responses to urinary tract infections. *Nature Reviews Immunology.* **15** (10), 655-663, doi:10.1038/nri3887, (2015).

2 Wolfe, A. J. *et al.* Evidence of uncultivated bacteria in the adult female bladder. *Journal of Clinical Microbiology.* **50** (4), 1376-1383, doi:10.1128/JCM.05852-11, (2012).

3 Grist, M. & Chakraborty, J. Identification of a mucin layer in the urinary bladder. *Urology.* **44** (1), 26-33 (1994).

4 Wu, X. R., Kong, X. P., Pellicer, A., Kreibich, G. & Sun, T. T. Uroplakins in urothelial biology, function, and disease. *Kidney International.* **75** (11), 1153-1165, doi:10.1038/ki.2009.73, (2009).

5 Ingersoll, M. A. & Albert, M. L. From infection to immunotherapy: host immune responses to bacteria at the bladder mucosa. *Mucosal Immunology.* **6** (6), 1041-1053, doi:10.1038/mi.2013.72, (2013).

6 Flores-Mireles, A. L., Walker, J. N., Caparon, M. & Hultgren, S. J. Urinary tract infections: epidemiology, mechanisms of infection and treatment options. *Nature Reviews Microbiology.* **13** (5), 269-284, doi:10.1038/nrmicro3432, (2015).

7 Lewis, A. J., Richards, A. C. & Mulvey, M. A. Invasion of Host Cells and Tissues by Uropathogenic Bacteria. *Microbiology Spectrum.* **4** (6), doi:10.1128/microbiolspec.UTI-0026-2016, (2016).

8 Horsley, H. *et al. Enterococcus faecalis* subverts and invades the host urothelium in patients with chronic urinary tract infection. *PLoS One.* **8** (12), e83637, doi:10.1371/journal.pone.0083637, (2013).

9 Rosen, D. A. *et al.* Utilization of an intracellular bacterial community pathway in Klebsiella pneumoniae urinary tract infection and the effects of FimK on type 1 pilus expression. *Infection and Immunity.* **76** (7), 3337-3345, doi:10.1128/IAI.00090-08, (2008).

10 Rosen, D. A., Hooton, T. M., Stamm, W. E., Humphrey, P. A. & Hultgren, S. J. Detection of intracellular bacterial communities in human urinary tract infection. *PLoS Med.* **4** (12), e329, doi:10.1371/journal.pmed.0040329, (2007).

11 Hussain, S. A., Alhalabi, F. & Zimmern, P. E. Long-term efficacy of fulguration of trigonitis for recurrent urinary tract infections in women. *Urological Science.* **26** (3), 197-201, doi:10.1016/j.urols.2015.08.005, (2015).

12 De Nisco, N. J. *et al.* Direct Detection of Tissue-Resident Bacteria and Chronic Inflammation in the Bladder Wall of Postmenopausal Women with Recurrent Urinary Tract Infection. *Journal of Molecular Biology.* doi:10.1016/j.jmb.2019.04.008, (2019).

13 Amann, R. I. *et al.* Combination of 16S rRNA-targeted oligonucleotide probes with flow cytometry for analyzing mixed microbial populations. *Applied and Environmental Microbiology.* **56** (6), 1919-1925 (1990).

14 Vaishnava, S. *et al.* The antibacterial lectin RegIIIgamma promotes the spatial segregation of microbiota and host in the intestine. *Science.* **334** (6053), 255-258, doi:10.1126/science.1209791, (2011).
15 Greuter, D., Loy, A., Horn, M. & Rattei, T. probeBase--an online resource for rRNA-targeted oligonucleotide probes and primers: new features 2016. *Nucleic Acids Research.* **44** (D1), D586-589, doi:10.1093/nar/gkv1232, (2016).
16 Monici, M. Cell and tissue autofluorescence research and diagnostic applications. *Biotechnology Annual Review.* **11** 227-256, doi:10.1016/S1387-2656(05)11007-2, (2005).
17 Mysorekar, I. U. & Hultgren, S. J. Mechanisms of uropathogenic Escherichia coli persistence and eradication from the urinary tract. *Proceedings of the National Academy of Sciences of the United States of America.* **103** (38), 14170-14175, doi:10.1073/pnas.0602136103, (2006).
18 Frickmann, H., Lakner, A., Essig, A. & Poppert, S. Rapid identification of yeast by fluorescence in situ hybridisation from broth and blood cultures. *Mycoses.* **55** (6), 521-531, doi:10.1111/j.1439-0507.2012.02214.x, (2012).
19 Fischer, A. H., Jacobson, K. A., Rose, J. & Zeller, R. Paraffin embedding tissue samples for sectioning. *Cold Spring Harbor Protocols.* **2008** pdb prot4989, doi:10.1101/pdb.prot4989, (2008).
20 Sun, Y. *et al.* Sudan black B reduces autofluorescence in murine renal tissue. *Archives of Pathology & Laboratory Medicine.* **135** (10), 1335-1342, doi:10.5858/arpa.2010-0549-OA, (2011).
21 Ludwig, W. *et al.* ARB: a software environment for sequence data. *Nucleic Acids Research.* **32** (4), 1363-1371, doi:10.1093/nar/gkh293, (2004).
22 Jaziri, F. *et al.* PhylOPDb: a 16S rRNA oligonucleotide probe database for prokaryotic identification. *Database (Oxford).* **2014** (0), bau036, doi:10.1093/database/bau036, (2014).

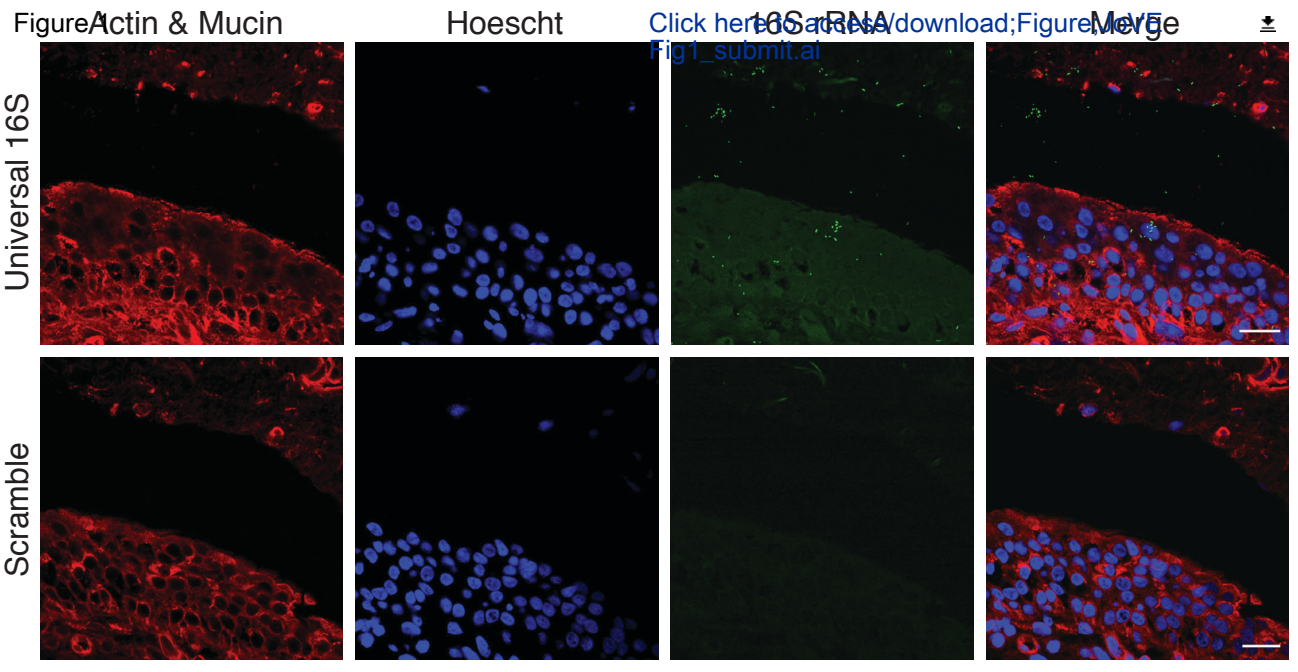

Figure 2



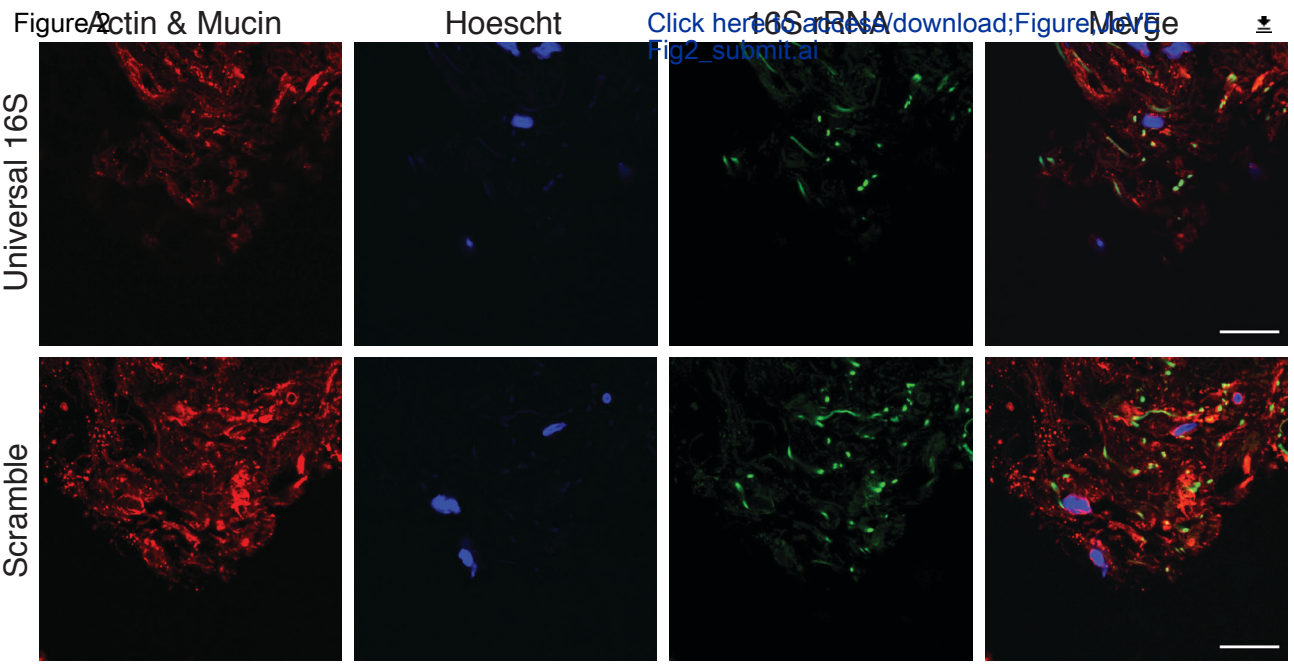

Table 1



| Probe | Sequence | Tm | Fluorophore | Linkage |
|---|---|---|---|---|
| Universal 16S rRNA | 5'-GCTGCCTCCCGTAGGAGT-3' | 54.9 | Alexa-488 (5' and 3') | NHS Ester |
| Scramble | 5'-ACTCCTACGGGAGGCAGC-3' | NA | Alexa-488 (5' and 3') | NHS Ester |

| Name of Material/ Equipment | Company | Catalog Number | Comments/Description |
|---|---|---|---|
| Alexa-555 Phalloidin | Invitrogen | A34055 | Staining Actin |
| Alexa-555 Wheat Germ Agglutinin (WGA) | Invitrogen | W32464 | Staining Mucin |
| Bottle top filters | Fisher Scientific | 09-741-07 | Sterilization |
| Coplin Jar | Simport | M900-12W | Deparaffinization/washing |
| Coverslips | Fisher Scientific | 12-548-5M | Microscopy |
| Ethanol | Fisher Scientific | 04-355-224 | Rehydration |
| Ethylenediaminetetraacetic Acid, Disodium Salt Dihydrate | Fisher Scientific | S311-500 | TE |
| Frosted Slides | Thermo Fisher Scientific | 12-550-343 | |
| Hoechst 33342, Trihydrochloride, trihydrate | Invitrogen | H21492 | Staining Nucleus |
| Hydrophobic marker | Vector Laboratories | H-4000 | Hydrophobic barrier |
| Kimwipes | Fisher Scientific | 06-666-11 | |
| Oil Immersol 518 F | Fisher Scientific | 12-624-66A | Microscopy |
| Paraformaldehyde (16%) | Thermo Scientific | TJ274997 | Fixation |
| ProLong Gold antifade reagent | Invitrogen | P36934 | Mounting medium |
| Sodium Chloride | Fisher Scientific | BP358-10 | Hybridization buffer/PBS |
| Sodium Dodecyl Sulphate | Fisher Scientific | BP166-500 | Hybridization buffer |
| Sodium Phosphate Dibasic Hepahydrate | Fisher Scientific | S373-500 | PBS |
| Sodium Phosphate Monobasic Monohydrate | Fisher Scientific | S369-500 | PBS |
| Syringe | VWR | 75486-756 | Sterilization |
| Tris-HCl | Fisher Scientific | BP152-5 | TE/Hybridization buffer |
| Xylene | Fisher Chemical | X3P-1GAL | Deparaffinization |
| 0.22 micron syringe filter | Fisher Scientific | 09-754-29 | Sterilization |

1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

# ARTICLE AND VIDEO LICENSE AGREEMENT

| | |
|---|---|
| Title of Article: | Detection of tissue-resident bacteria in bladder biopsies by 16S rRNA fluorescence in situ hybridization |
| Author(s): | Michael L. Neugent, Jashkaran Gadhvi, Kelli L. Palmer, Philippe Zimmern, Nicole J. De Nisco |

Item 1: The Author elects to have the Materials be made available (as described at http://www.jove.com/publish) via:

☐ Standard Access ☑ Open Access

Item 2: Please select one of the following items:

☑ The Author is **NOT** a United States government employee.

☐ The Author is a United States government employee and the Materials were prepared in the course of his or her duties as a United States government employee.

☐ The Author is a United States government employee but the Materials were NOT prepared in the course of his or her duties as a United States government employee.

## ARTICLE AND VIDEO LICENSE AGREEMENT

1. **Defined Terms.** As used in this Article and Video License Agreement, the following terms shall have the following meanings: "**Agreement**" means this Article and Video License Agreement; "**Article**" means the article specified on the last page of this Agreement, including any associated materials such as texts, figures, tables, artwork, abstracts, or summaries contained therein; "**Author**" means the author who is a signatory to this Agreement; "**Collective Work**" means a work, such as a periodical issue, anthology or encyclopedia, in which the Materials in their entirety in unmodified form, along with a number of other contributions, constituting separate and independent works in themselves, are assembled into a collective whole; "**CRC License**" means the Creative Commons Attribution-Non Commercial-No Derivs 3.0 Unported Agreement, the terms and conditions of which can be found at: http://creativecommons.org/licenses/by-nc-nd/3.0/legalcode; "**Derivative Work**" means a work based upon the Materials or upon the Materials and other pre-existing works, such as a translation, musical arrangement, dramatization, fictionalization, motion picture version, sound recording, art reproduction, abridgment, condensation, or any other form in which the Materials may be recast, transformed, or adapted; "**Institution**" means the institution, listed on the last page of this Agreement, by which the Author was employed at the time of the creation of the Materials; "**JoVE**" means MyJove Corporation, a Massachusetts corporation and the publisher of The Journal of Visualized Experiments; "**Materials**" means the Article and / or the Video; "**Parties**" means the Author and JoVE; "**Video**" means any video(s) made by the Author, alone or in conjunction with any other parties, or by JoVE or its affiliates or agents, individually or in collaboration with the Author or any other parties, incorporating all or any portion of the Article, and in which the Author may or may not appear.

2. **Background.** The Author, who is the author of the Article, in order to ensure the dissemination and protection of the Article, desires to have the JoVE publish the Article and create and transmit videos based on the Article. In furtherance of such goals, the Parties desire to memorialize in this Agreement the respective rights of each Party in and to the Article and the Video.

3. **Grant of Rights in Article.** In consideration of JoVE agreeing to publish the Article, the Author hereby grants to JoVE, subject to **Sections 4** and **7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Article in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Article into other languages, create adaptations, summaries or extracts of the Article or other Derivative Works (including, without limitation, the Video) or Collective Works based on all or any portion of the Article and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and(c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. If the "Open Access" box has been checked in **Item 1** above, JoVE and the Author hereby grant to the public all such rights in the Article as provided in, but subject to all limitations and requirements set forth in, the CRC License.

4. **Retention of Rights in Article.** Notwithstanding the exclusive license granted to JoVE in **Section 3** above, the Author shall, with respect to the Article, retain the non-exclusive right to use all or part of the Article for the non-commercial purpose of giving lectures, presentations or teaching classes, and to post a copy of the Article on the Institution's website or the Author's personal website, in each case provided that a link to the Article on the JoVE website is provided and notice of JoVE's copyright in the Article is included. All non-copyright intellectual property rights in and to the Article, such as patent rights, shall remain with the Author.

5. **Grant of Rights in Video – Standard Access.** This **Section 5** applies if the "Standard Access" box has been checked in **Item 1** above or if no box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby acknowledges and agrees that, Subject to **Section 7** below, JoVE is and shall be the sole and exclusive owner of all rights of any nature, including, without limitation, all copyrights, in and to the Video. To the extent that, by law, the Author is deemed, now or at any time in the future, to have any rights of any nature in or to the Video, the Author hereby disclaims all such rights and transfers all such rights to JoVE.

6. **Grant of Rights in Video – Open Access.** This **Section 6** applies only if the "Open Access" box has been checked in **Item 1** above. In consideration of JoVE agreeing to produce, display or otherwise assist with the Video, the Author hereby grants to JoVE, subject to **Section 7** below, the exclusive, royalty-free, perpetual (for the full term of copyright in the Article, including any extensions thereto) license (a) to publish, reproduce, distribute, display and store the Video in all forms, formats and media whether now known or hereafter developed (including without limitation in print, digital and electronic form) throughout the world, (b) to translate the Video into other languages, create adaptations, summaries or extracts of the Video or other Derivative Works or Collective Works based on all or any portion of the Video and exercise all of the rights set forth in (a) above in such translations, adaptations, summaries, extracts, Derivative Works or Collective Works and (c) to license others to do any or all of the above. The foregoing rights may be exercised in all media and formats, whether now known or hereafter devised, and include the right to make such modifications as are technically necessary to exercise the rights in other media and formats. For any Video to which this **Section 6** is applicable, JoVE and the Author hereby grant to the public all such rights in the Video as provided in, but subject to all limitations and requirements set forth in, the CRC License.

7. **Government Employees.** If the Author is a United States government employee and the Article was prepared in the course of his or her duties as a United States government employee, as indicated in **Item 2** above, and any of the licenses or grants granted by the Author hereunder exceed the scope of the 17 U.S.C. 403, then the rights granted hereunder shall be limited to the maximum rights permitted under such statute. In such case, all provisions contained herein that are not in conflict with such statute shall remain in full force and effect, and all provisions contained herein that do so conflict shall be deemed to be amended so as to provide to JoVE the maximum rights permissible within such statute.

8. **Protection of the Work.** The Author(s) authorize JoVE to take steps in the Author(s) name and on their behalf if JoVE believes some third party could be infringing or might infringe the copyright of either the Author's Article and/or Video.

9. **Likeness, Privacy, Personality.** The Author hereby grants JoVE the right to use the Author's name, voice, likeness, picture, photograph, image, biography and performance in any way, commercial or otherwise, in connection with the Materials and the sale, promotion and distribution thereof. The Author hereby waives any and all rights he or she may have, relating to his or her appearance in the Video or otherwise relating to the Materials, under all applicable privacy, likeness, personality or similar laws.

10. **Author Warranties.** The Author represents and warrants that the Article is original, that it has not been published, that the copyright interest is owned by the Author (or, if more than one author is listed at the beginning of this Agreement, by such authors collectively) and has not been assigned, licensed, or otherwise transferred to any other party. The Author represents and warrants that the author(s) listed at the top of this Agreement are the only authors of the Materials. If more than one author is listed at the top of this Agreement and if any such author has not entered into a separate Article and Video License Agreement with JoVE relating to the Materials, the Author represents and warrants that the Author has been authorized by each of the other such authors to execute this Agreement on his or her behalf and to bind him or her with respect to the terms of this Agreement as if each of them had been a party hereto as an Author. The Author warrants that the use, reproduction, distribution, public or private performance or display, and/or modification of all or any portion of the Materials does not and will not violate, infringe and/or misappropriate the patent, trademark, intellectual property or other rights of any third party. The Author represents and warrants that it has and will continue to comply with all government, institutional and other regulations, including, without limitation all institutional, laboratory, hospital, ethical, human and animal treatment, privacy, and all other rules, regulations, laws, procedures or guidelines, applicable to the Materials, and that all research involving human and animal subjects has been approved by the Author's relevant institutional review board.

11. **JoVE Discretion.** If the Author requests the assistance of JoVE in producing the Video in the Author's facility, the Author shall ensure that the presence of JoVE employees, agents or independent contractors is in accordance with the relevant regulations of the Author's institution. If more than one author is listed at the beginning of this Agreement, JoVE may, in its sole

jove JOURNAL OF VISUALIZED EXPERIMENTS
1 Alewife Center #200
Cambridge, MA 02140
tel. 617.945.9051
www.jove.com

# ARTICLE AND VIDEO LICENSE AGREEMENT

discretion, elect not take any action with respect to the Article until such time as it has received complete, executed Article and Video License Agreements from each such author. JoVE reserves the right, in its absolute and sole discretion and without giving any reason therefore, to accept or decline any work submitted to JoVE. JoVE and its employees, agents and independent contractors shall have full, unfettered access to the facilities of the Author or of the Author's institution as necessary to make the Video, whether actually published or not. JoVE has sole discretion as to the method of making and publishing the Materials, including, without limitation, to all decisions regarding editing, lighting, filming, timing of publication, if any, length, quality, content and the like.

12. **Indemnification.** The Author agrees to indemnify JoVE and/or its successors and assigns from and against any and all claims, costs, and expenses, including attorney's fees, arising out of any breach of any warranty or other representations contained herein. The Author further agrees to indemnify and hold harmless JoVE from and against any and all claims, costs, and expenses, including attorney's fees, resulting from the breach by the Author of any representation or warranty contained herein or from allegations or instances of violation of intellectual property rights, damage to the Author's or the Author's institution's facilities, fraud, libel, defamation, research, equipment, experiments, property damage, personal injury, violations of institutional, laboratory, hospital, ethical, human and animal treatment, privacy or other rules, regulations, laws, procedures or guidelines, liabilities and other losses or damages related in any way to the submission of work to JoVE, making of videos by JoVE, or publication in JoVE or elsewhere by JoVE. The Author shall be responsible for, and shall hold JoVE harmless from, damages caused by lack of sterilization, lack of cleanliness or by contamination due to the making of a video by JoVE its employees, agents or independent contractors. All sterilization, cleanliness or decontamination procedures shall be solely the responsibility of the Author and shall be undertaken at the Author's expense. All indemnifications provided herein shall include JoVE's attorney's fees and costs related to said losses or damages. Such indemnification and holding harmless shall include such losses or damages incurred by, or in connection with, acts or omissions of JoVE, its employees, agents or independent contractors.

13. **Fees.** To cover the cost incurred for publication, JoVE must receive payment before production and publication of the Materials. Payment is due in 21 days of invoice. Should the Materials not be published due to an editorial or production decision, these funds will be returned to the Author. Withdrawal by the Author of any submitted Materials after final peer review approval will result in a US$1,200 fee to cover pre-production expenses incurred by JoVE. If payment is not received by the completion of filming, production and publication of the Materials will be suspended until payment is received.

14. **Transfer, Governing Law.** This Agreement may be assigned by JoVE and shall inure to the benefits of any of JoVE's successors and assignees. This Agreement shall be governed and construed by the internal laws of the Commonwealth of Massachusetts without giving effect to any conflict of law provision thereunder. This Agreement may be executed in counterparts, each of which shall be deemed an original, but all of which together shall be deemed to me one and the same agreement. A signed copy of this Agreement delivered by facsimile, e-mail or other means of electronic transmission shall be deemed to have the same legal effect as delivery of an original signed copy of this Agreement.

A signed copy of this document must be sent with all new submissions. Only one Agreement is required per submission.

**CORRESPONDING AUTHOR**

| | |
|---|---|
| Name: | Nicole J. De Nisco |
| Department: | Department of Biological Sciences |
| Institution: | University of Texas at Dallas |
| Title: | Assistant Professor |
| Signature: | Nicole J De Nisco |
| Date: | 06/20/2019 |

Please submit a **signed** and **dated** copy of this license by one of the following three methods:

1. Upload an electronic version on the JoVE submission site
2. Fax the document to +1.866.381.2236
3. Mail the document to JoVE / Attn: JoVE Editorial / 1 Alewife Center #200 / Cambridge, MA 02140

**Response to Reviewers**

We would like to thank the editors and reviewers for their insightful comments regarding our submission, *Detection of tissue-resident bacteria in bladder biopsies by 16S rRNA fluorescence in situ hybridization.* We have taken all the comments into account and have amended the manuscript to address the reviewer's concerns.

**Reviewer #1:**

Major Concerns:
I have no major concerns.

Minor Concerns:
line 70 - positions noted as 388-355, is this correct?

Author Response:

Based on the original report and uses of these probes (ref 13), these are the correct positions of the probe target in the E coli 16s rRNA. However, the original submission of this manuscript does not properly refer to the enumerated positions belonging to the E coli 16s rRNA. We have amended the text to include this distinction as follows.

Introduction (Lines 73-74): The universal 16S rRNA nucleotide probe was previously designed *to target a conserved region* of the bacterial 16S rRNA[13], *which corresponds to positions 388-355 of the E. coli 16s rRNA*

lines 119-121 - sections are 5um thick - won't this mean that single bacteria in general can't be seen in adjacent sections? Particularly if the intracellular population consists of only a few bacteria. This should be noted as a limitation, again something that may not be as big of a deal for UTI (where in animal models large collections of bacteria can be seen) but may limit the generality of the method or the value of the controls for other infections.

Author Response:

Thank you for this suggestion. We have added text to the manuscript to address this valid concern.

Note, Section 1.4 (lines 138-142)

NOTE: The biopsy tissue should be cross sectionally arranged relative to the cutting plane in order to ensure visualization of urothelial layers in all sections. *It should also be noted that section thickness should be optimized for bacterial community detection. Thinner sections or sampling of multiple serial sections*

*may be required in the case of infections where tissue-resident bacteria are extremely scarce (e.g. <1 tissue-resident bacterium per 1000 mammalian cells).*

lines 123-124 - "longitudinally arranged" - maybe use cross section vs. en face

Author Response:

We appreciate the nomenclature advice on this point. To address the reviewer's comment, we have amended the text as follows.

Note, Section 1.4 (lines 138-139)

NOTE: The biopsy tissue should be *cross sectionally* arranged relative to the cutting plane in order to ensure visualization of urothelial layers in all sections

lines 233-234 - what volume and concentrations are "small, concentrated aliquots"?

Author Response:

Thank you for raising this question. We realize that different manufacturers will have different storage recommendations. To address the reviewer's comment, we have changed the text as follows.

Note, Section 2.3.9

NOTE: Hoechst, Alexa-555 WGA, and Alexa-555 Phalloidin may be stored long-term in the dark *per the manufacturer's instructions.*

**Reviewer #2:**

Major Concerns:

1. The authors provide description of the fixation conditions for newly procured tissues. However, in some cases, investigators may seek to use established tissue banks that utilize formalin or other fixative agents. Can the authors please provide comment on the use of other fixatives with this protocol? The authors should also caution against overfixation and the autofluorescence introduced with the use of glutaraldehyde.

Author Response:

We appreciate the reviewer posing this question and suggestion. To address this comment, we have amended the text as follows.

Line 126:

*NOTE: Fixation duration should be calculated based on the size of the tissue sample and over-fixation should be avoided. It is not advised to use glutaraldehyde as a fixative as it introduces autofluorescence.*

2. Can frozen samples be used?

Author Response:

While this protocol is intended to be implemented on formalin fixed paraffin imbedded samples, the use of frozen tissues is feasible. We have included discussion to address this as follows.

Discussion (Lines 362-365): *"The protocol described here can also be adapted to for the use of multiple fixation (e.g. formalin, ethanol, methacarn) and tissue preparation techniques (paraffin or resin embedded, and cryopreserved tissues)."*

3. The use of the scrambled probe is a nice addition to ensure appropriate interpretation. However, the scrambled probe is not on the same section, thus the investigator must infer the signal on the other slide. Please consider the use of dual fluorescent reporters on the same section to ensure appropriate interpretation. Also, there are systems available that will reduce the background inherent fluorescence that can be applied to the sample prior to the probes. These are commercially available through Molecular Probes and other sources.

Author Response:

*Thank you for this observation. For bladder tissue, we do not believe that using the scramble and 16S rRNA probe with different fluorescent labels is superior to using the probes with the same label on serial sections. Different channels require different settings for gain, laser power, and background correction. It would therefore be difficult to interpret true signal from autofluorescence if the probes were labeled with difference fluorophores. Collagen and elastin are the main producers of autofluorescence in bladder tissue and their presence often transcends several serial sections. As for the autofluorescence quenchers we assume the reviewer is referring to solutions like Sudan Black B. This is a good idea. To our knowledge this method has not been specifically used to quench bladder-specific autofluorescence, but since it has worked well in other tissues, we have added it as an optional step in the note on line 150 (see below). However, we do not believe this should be used in lieu of the scramble probe, but perhaps in addition to it (the scramble probe also helps to control against signal from non-specific binding of the probe). To address this concern, we have added the following to the text as well as a new reference.*

*NOTE: Two slides per biopsy are required. One slide is needed for the universal 16S rRNA probe and one slide for a control probe with a scrambled sequence. This is important for distinguishing true signal from background signal during microscopy since the bladder epithelium is auto-fluorescent in multiple channels. In addition to the scramble probe, blocking with 0.1% Sudan Black B prior to mounting may reduce background autofluorescence inherent in the tissue[20]*

*20. Sun, Y. et al. Sudan black B reduces autofluorescence in murine renal tissue. Arch Pathol Lab Med. **135** (10), 1335-1342, doi:10.5858/arpa.2010-0549-OA, (2011).*

4. With at 5 micron section, the majority of the bacteria will be intact within the section. Are there steps to ensure that these intact bacteria are sufficiently permeable for access by the probe? What measures have been taken to ensure that the probe is effective within the center of the section?

Author Response:

*We agree with this observation, however the purpose of this protocol is to detect bacteria within tissue and not necessarily to detect every single bacterium so no additional steps have been taken to permeabilize the bacteria besides the dehydration and rehydration with ethanol (a method commonly used to permeabilize both Gram positive and Gram negative bacteria) as part of the embedding and deparaffinizing procedure. We have performed Z-stacks and have observed bacteria in multiple layers in many 5 micron sections. However, we realize that with infection models in which bacterial load is exceptionally low, thinner sections may be needed to detect extremely rare tissue-resident bacteria. We have amended the note in section 1.4 (line 138) accordingly (please see below).*

NOTE: The biopsy tissue should be cross sectionally arranged relative to the cutting plane in order to ensure visualization of urothelial layers in all sections. *It should also be noted that section thickness should be optimized for bacterial community detection. Thinner sections or sampling of multiple serial sections may be required in the case of infections where tissue-resident bacteria are extremely scarce (e.g. <1 tissue-resident bacterium per 1000 mammalian cells).*

5. There is significant subjectivity to microscopy and the use of software to analyze the samples. There should be more direction for the reader to know how to discriminate background from true signal, appropriate threshold cutoffs, number of bacteria to constitute a community, how to discriminate intracellular from extracellular. Which ImageJ plugins are recommended?

Author Response:

*We agree with the reviewer that there is significant subjectivity to microscopy, however we believe further discussion of analysis is too field (and microscope)-specific and thus outside the scope of this method article. We do not process our images further in ImageJ beyond splitting the channels and converting the files into image files. We have added the following sentence to 3.6 (starting line 324):*

> *3.6 Process images and quantify labeled bacteria within or associated with the tissue using ImageJ or similar software. Minimal processing of the images is recommended (e.g. splitting/merging channels and converting into image files), although background correction may be performed if necessary. All corrections or other alterations should remain consistent between images.*

*Likewise, how many bacteria constitute a certain type of community will be field-specific. Also, this method is not sufficient to conclude if detected bacteria are intracellular or extracellular, which is why we refer to the bacteria as tissue-resident. To make this differentiation, the bacteria must be co-localized with compartmental markers, which would require a hybrid FISH-immunofluorescence protocol.*

6. In Figure 1, please define recurrent urinary tract infection, is the causative agent known? If the same settings were used to capture the images for the green channel, why is the background so different between the experimental and the scramble probes? Please indicate which bacteria would be excluded from analysis (there is signal in the lumen).

Author Response:

*We have added a definition of recurrent urinary tract infection to the Note on line 114 (see below). A variety of bacterial species can cause rUTI. We would like to direct you to the source manuscript for this method in JMB for more information about the etiological agents of rUTI.*

> *Note: Biopsies were taken from consenting women undergoing cystoscopy with electro-fulguration of trigonitis for the advanced management of recurrent urinary tract infection (rUTI). rUTI is defined as ≥3 UTIs in a 12-month period.*

*The same settings were used, and the images were not processed any further in ImageJ beyond splitting the channels and converting the files into image files. The difference is background is due to biological and perhaps experimental (e.g. maybe one tissue dried out more during hybridization) but does not affect the interpretation of the images as there is clearly only positive signal in the 16S rRNA probed sample.*

*Luminal bacteria would be scored but their localization would be marked as luminal instead of epithelial.*

7. In Figure 2, what portion of the bladder is represented? The distribution of nuclei does not seem to indicate that this is the epithelial side, and may be more representative of the connective tissue, muscle. It would be helpful if there were a diagram for orientation of the reader.

Author Response:

*Thank you for the comment. However, the purpose of this image is to illustrate autofluorescence caused by extracellular matrix and is not necessarily representative of the epithelium. The image is, however, representative of an artifact which an experimenter may readily encounter.*

Minor Concerns:

1. Avoid personification of bacteria and objects. Examples: Line 72, "their" Line 83 "bacterium's"

Author Response:

*Thank you. This has been corrected, however we note that "their" is a possessive for both human and nonhuman groups.*

2. Line 148. "Dissolved" should be "Dissolve"

Author Response:

*Thank you. This has been corrected*

3. Line 149, please correct the name of EDTA.

Author Response:

*Thank you. This has been corrected*

4. Line 150. Please indicate if the 1mM probe should be in hybridization buffer or the buffered water?

Author Response:

To address the reviewer's concern, we have added the following to the text.

(line 169) 2.1.4 Dissolve fluorescently-labeled lyophilized probes in 10 mM Tris-HCl (pH 8.0) and 1 mM ethylenediaminetetraacetic acid (EDTA) buffer *(TE)* prepared in filter-sterilized nuclease free water to a final concentration of 100 µM. *Prepare a dilution of 1 µM in TE buffer for use in this protocol*. *Store both 100 µM concentrated stock and 1 µM stock reconstituted probes at -20 ℃ protected from light.*

5. For light sensitive steps, please suggest turning off of the overhead lights, when feasible.

Author Response:

*To address the reviewer's concern, we have added the following to the text.*

*(line 209) NOTE: Protect staining solution from light by wrapping tubes in aluminum foil and storing in a drawer. When working with fluorescently labeled probes on the benchtop, consider turning off overhead lights when able. Probes diluted in hybridization buffer should not be re-used.*

6. For the humidifying chamber, the use of a pipet box is economical. However, there are also commercially available systems that are better sealed for humidity control, darkness and slide stability.

Author Response:

*To address the reviewer's concern, we have added the following to the text.*

*(line 219) NOTE: It is important to use a humidifying chamber to prevent the biopsy sections from drying out during hybridization. It should be noted that commercially available humidifier chambers are designed to maintain a stable, humid atmosphere. However, the technique detailed here sufficiently controls humidity at substantially less cost.*

7. Line 196, please clarify removal of the slides from the staining rack, this term has not yet been used, slide rack was used in prior steps.

Author Response:

*Thank you. This has been corrected*

8. Step 2.3.7. The jars could also be wrapped in foil.

Author Response:

*Thank you. This has been added*

9. For the counterstains, please indicate that other probes may be used according to the filter sets available for the microscope. Also, some objectives use water instead of oil for maintaining contact with the lens, please indicate appropriately as oil should not be placed on a water lens.

Author Response:

*To address the reviewer's concern, we have added the following to the text starting on line 264:*

> *NOTE: Hoechst, Alexa-555 WGA, and Alexa-555 Phalloidin may be stored long-term in the dark per the manufacturer's instructions. Fluorescent labels used for probes and counterstains can be customized for the filter sets available for the microscope to be used.*

*And the following sentence to the note starting on line 304:*

> *NOTE: Use only fine focusing at high magnification (63x or 100x). Oil should only be used for an oil objective.*

10. The protocol is written for capturing a single image, why not take a series of images through the Z plane for optimal analyses?

Author Response:

*To address the reviewer's concern, we have added the following to the text starting on line 321:*

> *NOTE: You may have to change the focal plane slightly to visualize labeled bacteria in different fields, but never change the gain, laser power, or offset for the 488 channel between fields. If working on a confocal microscope it may be informative to capture a Z-stack so that the three-dimensional localization of the bacteria within the tissue may be analyzed.*

11. Wheat Germ Agglutinin also binds to uroplakins, this should be included in the text.

Author Response:

*To address the reviewer's concern, we have added the following to the text starting on line 262*

*NOTE: Hoechst, Alexa-555 WGA, and Alexa-555 Phalloidin label DNA, mucin/uroplakins, and actin, respectively and may be stored long-term in the dark per the manufacturer's instructions.*

12. Please define CEFT

Author Response:

*CEFT is defined in the introduction on line 69*

**Reviewer #3:**

There is only one concern that the paper is very similar to multiple other protocols, including those published in JoVE (J Vis Exp. 2015; (99): 52836). That being said it does not take away the uniqueness of this study, as a more detailed protocol applied to the particular purpose (detection of bacteria in bladder mucosa) will certainly be useful for future researchers. But to differentiate this protocol from others, I would suggest to add more details and descriptions relevant to this particular tissue and problem. Authors already implicitly do this by adding (1) strict sterility and filtering notes, (2) drawing attention to using consecutive section to control for false positives, (3) discussing the orientation of the biopsy section to be able to relate to the urothelium layers. Perhaps some illustrative material during the video that will be produced and some additional discussion of the context would be helpful to make this protocol unique and provide the most benefit for the followers.
These, bladder-specific, issues all stem from the low-bacterial biomass in bladder microbiome in general and expectations (based on the available data) that there would not be many bacteria invading the urothelium cells. Therefore, these details of the protocol would be helpful to spell out explicitly so that the readers would be able to translate the method to other low-biomass microbiomes if needed.

Author Response:

*We would like to thank the reviewer for their insightful commentary. We agree that some illustrative material in the video can be added to highlight the uniqueness of this protocol and its broad applicability.*

Minor Concerns:

- abstract -'physical characteristics (LPS)' - LPS, while affecting cell surface physical properties, is

hardly a physical characteristic

Author Response:

We appreciate the Reviewer's comments here and have amended the abstract as follows.

> Through use of a universal 16S rRNA probe, bacteria can be detected without prior knowledge of species, genera, *or biochemical characteristics, such as lipopolysaccharide (LPS)*, that would be required for detection by immunofluorescence experiments.

- Page 2, line 55 - Infection is a very loaded term and perhaps this instance would be better as 'invasion'?

Author Response:

*Thank you. We have amended the text as per the Reviewer's suggestion.*

- Page 2, line 70 - defining positions of the probe within the 16S rRNA gene - why the number range is inverted? just because it is a complement? In addition, please add if not already the description that the probe is likely annealing to the RNA vs DNA and that might be important to selecting future more selective probes to perhaps other specific targets.

Author Response:

We appreciate the Reviewer's thorough observations here. The number range is inverted because it is the reverse compliment of positions corresponding to the E coli 16s rRNA. Based on the original report and uses of these probes (ref 13), these are the correct positions of the probe target in the E coli 16s rRNA. However, the original submission of this manuscript does not properly refer to the enumerated positions belonging to the E coli 16s rRNA. We have amended the text to include this distinction as follows.

> Introduction (Lines 73-74): The universal 16S rRNA nucleotide probe was previously designed *to target a conserved region* of the bacterial 16S rRNA[13], *which corresponds to positions 388-355 of the E. coli 16s rRNA*

- Page 3, line 117 - why not give the paraphinization protocol? is it because it is not modified at all?

Author Response:

*Yes, the paraffinization protocol is standard and not unique to this method. We have provided a reference for the reader to the standard protocol (line 132).*

- Page 4, lines 148-150 - since this is a detailed protocol it would be nice to describe how the stocks of the labeled oligos was prepared and stored before diluting it down to 1uM solution.

Author Response:

*Thank you. We have amended the text as per the Reviewer's suggestion.*

> 2.1.4 Dissolve fluorescently-labeled lyophilized probes in 10 mM Tris-HCl (pH 8.0) and 1 mM ethylenediaminetetraacetic acid (EDTA) buffer (TE) prepared in filter-sterilized nuclease free water to a final concentration of 100 μM. Prepare a dilution of 1 μM in TE buffer for use in this protocol. *Store both 100 μM concentrated stock and 1 μM stock reconstituted probes at -20 ℃ protected from light*.

- Page 5, line 184 - staining - word "solution" seems to be missing

Author Response:

*Thank you. This has been corrected*

- Page 6, lines 229, 233, and other instances in the manuscript - Please note which Hoechst stain is being used at least in the first instance of this dye.

Author Response:

*This information has been added to line 260 (Hoechst 33342).*

- Missing items from the table of reagents: mounting media (perhaps it is under brand name - but than it should be made clear in the table);

Author Response:

*This is in the table of reagents as ProLong Gold antifade reagent and is described as mounting medium in the description column.*

- Page 7, lines 269-270 - scanning through the tissue section at 63x magnification sounds like a very long procedure - is there a particular trick or strategy authors would recommend?

Author Response:

*These tissues are relatively small, so the initial scan does not take very long – about 10 minutes or so.*

- Page 7, lines 273-278 - What is the purpose of the pinhole adjusting? what would be the result and aim here? In general, based on the images, authors can use a larger pinhole as it might help to collect more signal to detect small/slightly stained objects.

Author Response:

*This imaging protocol was written for a confocal microscope. In confocal microscopy the pinhole acts as the spatial filter and the required pinhole diameter is variable for different wavelengths. When performing confocal microscopy with multiple fluorophores, it is best practice to set the pinhole for the longest wavelength to be monitored in a given experiment. This information can be found in the instructional manual of the confocal microscope*

- Page 10, Ref 19 - there is a 'pdb prot 4989' that seems to out of place here.

Author Response:

This seems to actually be part of the citation