Journal of Visualized Experiments

Rapid screening of bacteria-specific compounds for interrogating distal lung infection in the intensive care unit by confocal laser endomicroscopy
--Manuscript Draft--

Manuscript Number:	JoVE56284R2
Full Title:	Rapid screening of bacteria-specific compounds for interrogating distal lung infection in the intensive care unit by confocal laser endomicroscopy
Article Type:	Invited Methods Article - JoVE Produced Video
Keywords:	Optical probe; bacterial infection; confocal laser endomicroscopy; distal lung; translation; intensive care unit; optical imaging
Manuscript Classifications:	3.1: Bacterial Infections and Mycoses; 3.8: Respiratory Tract Diseases; 5.1: Diagnosis; 5.1.370.350: Diagnostic Imaging; 5.1.370.350.557: Molecular Imaging; 5.5.601: Molecular Probe Techniques; 5.7.632: Optical Devices; 5.7.632.750: Optical Fibers
Corresponding Author:	Bethany Mills University of Edinburgh Edinburgh, Edinburgh UNITED KINGDOM
Corresponding Author Secondary Information:	
Corresponding Author E-Mail:	beth.mills@ed.ac.uk
Corresponding Author's Institution:	University of Edinburgh
Corresponding Author's Secondary Institution:	
First Author:	Bethany Mills
First Author Secondary Information:	
Other Authors:	Ahsan R Akram
	Emma Scholefield
	Mark Bradley
	Kevin Dhaliwal
Order of Authors Secondary Information:	
Abstract:	Improving the speed and accuracy of bacterial detection is important for patient stratification and to ensure the appropriate use of antimicrobials. To achieve this goal, the development of diagnostic techniques to recognize bacterial presence in real-time at the point-of-care are required. Optical imaging for direct identification of bacteria within the host is an attractive approach. Several attempts at chemical probe design and validation have been investigated, however none have yet been successfully translated into the clinic. Here we describe a method for ex vivo validation of bacteria-specific probes for identification of bacteria within the distal lung, imaged by fibered confocal fluorescence microscopy (FCFM). Our model used ex vivo human lung tissue and a clinically approved confocal laser endomicroscopy (CLE) platform to screen novel bacteria-specific imaging compounds, closely mimicking imaging conditions expected to be encountered with patients. Therefore, screening compounds by this technique provides confidence of potential clinical tractability.
Author Comments:	
Additional Information:	
Question	Response

If this article needs to be "in-press" by a certain date, please indicate the date below and explain in your cover letter.	

Dear Constantinos Katevatis,

Thank you for your editorial comments for our 'Rapid screening of bacteria-specific probes for interrogating distal lung infection in the intensive care unit by confocal laser endomicroscopy' manuscript.

We have taken every effort to address each of your comments, and have included all track changes in the revised manuscript. Our responses to each of your points are detailed below.

We look forward to hearing from you.

Best wishes

Dr Bethany Mills

Research Fellow in Optical Imaging

PROTEUS Hub, Room E2.32
University of Edinburgh
Queens Medical Research Institute
47 Little France Crescent
Edinburgh
EH16 4TJ
Tel: +44 (0)131 242 9180
Website: http://www.proteus.ac.uk
Twitter: https://twitter.com/edPROTEUS
Facebook: EPSRC Proteus | Facebook

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• Please review and address the revisions and comments made on the most current manuscript file.

These have been addressed individually within the manuscript file.

• The language in the manuscript is not publication grade. Please take the time to proofread or send it for copyediting.

The manuscript has now undergone further proofreading to correct for grammatical errors.

• Please use American-English.

American English has now been implemented.

• Please add the following to the Table of Materials: FCFM imaging fiber software, data analysis software, etc.

This software was listed in the original table. It is called 'IC-viewer'.

• After providing the necessary details, please highlight up to 2.75 pages of the protocol that you would like to film. Please note that complete sentences must be highlighted.

The highlighted text is less than 2.75 pages.

• Figure 1 – Please remove commercial language 'Cellvizio'.

The commercial language has been removed.

• Figure 1/2/3 – Please provide higher resolution images.

We have provided figures at the highest resolution possible.

1   **TITLE:**
2   Rapid Screening of Bacteria-specific Compounds for Interrogating Distal Lung Infection in
3   the Intensive Care Unit by Confocal Laser Endomicroscopy
4
5   **AUTHORS & AFFILIATIONS:**
6   Bethany Mills[1], Ahsan R. Akram[1], Emma Scholefield[1], Mark Bradley[2], Kevin Dhaliwal[1]
7
8   [1]EPSRC Proteus Hub, MRC Centre of Inflammation Research, Queen's Medical Research
9   Institute, University of Edinburgh, 47 Little France Crescent, EH16 4TJ, Edinburgh, UK.
10 [2]School of Chemistry, EaStChem, University of Edinburgh, Joseph Black Building, David
11 Brewster Road, Edinburgh, EH9 3FJ, UK.
12
13 **AUTHOR EMAILS:**
14 Bethany Mills (beth.mills@ed.ac.uk)
15 Ahsan R. Akram (ahsan.akram@ed.ac.uk)
16 Emma Scholefield (emma.scholefield@ed.ac.uk)
17 Mark Bradley (mark.bradley@ed.ac.uk)
18 Kevin Dhaliwal (kev.dhaliwal@ed.ac.uk)
19
20 **CORRESPONDING AUTHOR:**
21 Kev Dhaliwal (Kev.Dhaliwal@ed.ac.uk)
22 Tel: +44 (0)131 242 9180
23
24 **KEYWORDS:**
25 Optical probe, bacterial infection, confocal laser endomicroscopy, distal lung, translation,
26 intensive care unit, optical imaging.
27
28 **SHORT ABSTRACT:**
29 This technique describes an efficient screening process for evaluating bacteria-specific
30 optical imaging agents within *ex vivo* human lung tissue, by fibered confocal fluorescence
31 microscopy for the rapid identification of small molecule chemical probe-candidates with
32 translatable potential.
33
34 **LONG ABSTRACT:**
35 Improving the speed and accuracy of bacterial detection is important for patient
36 stratification and to ensure the appropriate use of antimicrobials. To achieve this goal, the
37 development of diagnostic techniques to recognize bacterial presence in real-time at the
38 point-of-care is required. Optical imaging for direct identification of bacteria within the host
39 is an attractive approach. Several attempts at chemical probe design and validation have
40 been investigated, however none have yet been successfully translated into the clinic. Here
41 we describe a method for *ex vivo* validation of bacteria-specific probes for identification of
42 bacteria within the distal lung, imaged by fibered confocal fluorescence microscopy (FCFM).
43 Our model used *ex vivo* human lung tissue and a clinically approved confocal laser
44 endomicroscopy (CLE) platform to screen novel bacteria-specific imaging compounds,
45 closely mimicking imaging conditions expected to be encountered with patients. Therefore,
46 screening compounds by this technique provides confidence of potential clinical tractability.
47

INTRODUCTION:

This technique describes a rapid screening process for evaluating bacteria-specific optical imaging agents within *ex vivo* human lung tissue by CLE using FCFM for the rapid identification of compounds with potential clinical utility for visualizing bacteria in the distal lung *in situ*.

There is an urgent global requirement to ration antimicrobial prescribing in the era of rising antimicrobial resistance[1]. To this end, the development of diagnostic methods which act to identify bacterial infection with high specificity, sensitivity, and in real-time are highly sought[2]. Current techniques to confirm a diagnosis of pneumonia in critically unwell patients, such as those within intensive care units (ICUs), often rely on interpreting non-specific clinical or radiological features alongside bacterial culture techniques from aspirated fluids/tissues, which can take up to 3 days to produce results. Furthermore, bacterial culture from fluid instilled into the distal lung and retrieved is prone to contamination from more proximal airways[3] and is often culture negative due to concomitant antimicrobial therapy or poor sampling techniques. Additionally, molecular techniques such as polymerase chain reaction are overly sensitive when used on aspirated fluids, risking overtreatment of patients. An emerging diagnostic approach is molecular optical imaging, making *in situ* molecular pathology of tissues a possibility; however, the development and validation of optical imaging compounds is required. Nonetheless, direct visualization of bacteria, via activatable optical probes is potentially a very powerful method to allow the study of the presence and evolution of pneumonia in the patient, and importantly, could be used to study host-pathogen interactions in response to therapies in real-time *in situ.*

CLE is an established investigative procedure in multiple diseases[4], including within the fields of gastroenterology[5], oncology[6,7], and for interrogating airways and alveolar sacs[8,9]. It enables point-of-care structural imaging of diseased tissue using a fiber imaging bundle, which passes through the working channel of a clinical endoscope and forms direct contact with the tissue surface to be imaged by confocal microscopy. However, one limitation that remains is the need for generic contrast agents. Therefore, the use of disease specific probes, such as specific bacterial agents, could vastly expand the utility of this modality by directly visualizing bacteria at the site of suspected infection. Optical agents offer many advantages over other techniques by enabling real-time, high resolution imaging with diagnostic potential. Moreover, optical probes offer the prospect of multiplexing for interrogating multiple targets, all achieved at a relatively low cost. A number of optical agents are under development for such a purpose, however none have yet been successfully implemented within the clinic[10]. We have synthesized a library of small molecule chemical probes with specificity towards bacteria and developed a rapid, effective pipeline for evaluation of probe function for detecting bacterial pneumonia *in situ*[11].

To identify suitable probe candidates, the following prerequisites had to be fulfilled prior to interrogation of the probe on *ex vivo* human lung tissue by FCFM: i) aqueous solubility, ii) specificity and selectivity for rapidly labeling clinically relevant bacteria, iii) a high signal-to-noise ratio, and iv) resistance to degradation within the lung environment. The latter was assessed by bronchoalveolar lavage fluid (BALF) from patients with acute respiratory distress syndrome (ARDS), which is a condition that is characterized by proteolytic and inflammatory environments in the lung in the ICU. Moreover, the probes had to have a

95 suitable fluorophore for detection by a clinically approved optical CLE imaging device within
96 human lung alveolar tissue.

98 The pipeline to interrogate each of these prerequisites was as follows (at each stage, only
99 probes that passed were carried forward to the next): (1) a library of probes to be
100 investigated was synthesized; (2) each probe was added to a panel of live bacteria for
101 confocal laser scanning microscopy (CLSM) to ensure bacterial labeling; (3) selectivity of
102 bacterial labeling over mammalian cells in co-cultures with primary human neutrophils was
103 established by CLSM; (4) stability and successful labeling of bacteria in the presence of the
104 ARDS patient BALF was determined by CLSM and Matrix Assisted Laser
105 Desorption/Ionization-Time of Flight (MALDI-TOF) Mass Spectrometry; (5) optimal
106 concentration of candidates was determined by CLSM, ensuring selectivity for bacteria over
107 mammalian cells was maintained; (6) candidates were imaged by FCFM in suspension and
108 on *ex vivo* human lung alveolar tissue to ensure stability and that the signal-to-noise was
109 adequate for detection. Step 6 is described in detail within this protocol. Methodology for
110 steps 1–5 has been previously reported[11].

112 **PROTOCOL:**
113 All human lung tissue was obtained following informed consent and the study was approved
114 by the Regional Ethics Committee.

116 1. Preparation of Biological Samples

118 1.1. Preparation of probes

120 1.1.1. Make up a 1 mM stock solution of each probe (*e.g.*, Calcein AM, UBI-3, UBI-10, *etc.*)
121 in sterile dH_2O, using a fine balance to weigh the freeze-dried probe compound[11]. Calculate
122 the volume of dH_2O to add based on the weighed mass and molecular weight of the probe.

124 1.2. Preparation of bacterial cultures

126 Note: For this method, *Staphylococcus aureus* was used as the exemplar strain. Any
127 appropriate bacterial strain can be selected.

129 1.2.1. Select a single colony of desired bacterial strain from a fresh Lysogeny Broth (LB)
130 agar plate using a sterile loop. Inoculate the colony into 10 mL of LB in a 50 mL centrifuge
131 tube by dipping the end of the loop into the culture media. Incubate at 37 °C, 250 rpm for
132 16 h (or overnight).

134 1.2.2. Determine the OD_{595} of the overnight culture by adding 100 μL of the overnight
135 culture to 900 μL LB in a 1 mL cuvette. Measure the OD at 595 nm (using a cuvette with 1
136 mL LB as a blank) in a spectrophotometer. Multiply the obtained OD by 10 to obtain the OD
137 for the overnight culture.

139 1.2.3. Subculture the overnight culture. Do this by adjusting the optical density at 595 nm
140 (OD_{595}) to 0.1 in 10 mL of fresh LB. Calculate the required volume of overnight culture to be

141 added to 10 mL fresh LB to adjust the OD_{595} to 0.1. Incubate the culture at 37 °C, 250 rpm
142 until the culture reaches mid-log phase (OD_{595} 0.6–0.8), approximately 4 h.
143
144 1.2.4.   Measure the culture optical density (step 1.2.2) and harvest 1×10^8 colony forming
145 units (CFU) (OD_{595} 1 ~ 1×10^8 CFU/mL) of the bacterial culture into a 1.5 mL microtube (*e.g.*,
146 if the bacterial culture is OD_{595} 0.6, collect 1.67 mL). Centrifuge the culture at 10,000 x g at
147 room temperature for 1 min to pellet the bacteria. Wash the pellet twice in phosphate
148 buffered saline (PBS) by resuspending (pipetting up and down carefully) the pellet in 1 mL
149 PBS, centrifuging as above, discarding the supernatant, and repeating. Take care not to
150 dislodge the bacterial pellet when removing the supernatant. Resuspend the final pellet in 1
151 mL PBS.
152
153 Note: Prepare as many samples as required for each labeling procedure. The protocol may
154 be paused here for up to 1 h, followed by step 1.2.5.1, 1.2.5.2, or 1.2.5.3.
155
156 1.2.5.   Bacterial staining
157
158 1.2.5.1.   To label the bacteria with Calcein AM, add the dye to a final concentration of
159 1 µM into the washed bacterial culture. Incubate the culture for 30 min at 37 °C with
160 shaking at 300 rpm. Wash the counterstained bacterial suspension in PBS 3 times by
161 centrifugation as in step 1.2.4 to remove excess dye. Resuspend in 1 mL PBS, dilute 100 µL
162 1:1 in PBS to obtain 200 µL of OD_{595} 0.5 Calcein AM stained bacteria. The protocol may be
163 paused here for up to 1 h.
164
165 1.2.5.2.   To label the bacteria with test probes (*e.g.*, UBI-3 or UBI-10), dilute 100 µL of
166 the bacterial culture 1:1 in PBS to obtain OD_{595} 0.5 in 200 µL PBS. Add either of the probes
167 to a final concentration of 10 µM. Invert the microtube several times to ensure thorough
168 mixing of the bacteria and the probe.
169
170 Note: Imaging should be performed immediately after the addition of the probe to mimic
171 the clinical scenario.
172
173 1.2.5.3.   To prepare the control unstained bacterial samples, dilute 100 µL of the
174 culture 1:1 with PBS to obtain 200 µL of unstained OD_{595} 0.5 sample.
175
176 1.3.   Preparation of *ex vivo* human lung tissue
177
178 Note: Human lung tissue samples were obtained from patients undergoing surgical
179 resection for lung carcinoma. All tissue used for imaging was obtained from samples of
180 normal lung tissue away from the cancerous growth. Samples were taken fresh from the
181 operating theatre and stored in microtubes or centrifuge tubes at -80 °C until use.
182
183 1.3.1.   Immediately before imaging, remove the human lung tissue sample from the freezer
184 on dry ice. At room temperature, allow the tissue to thaw slightly; just enough to be sliced
185 with a scalpel into 1 x 4 mm sections.
186

187 Note: The level of thawing is important; too frozen and the tissue will not slice without
188 chipping off, too thawed and the tissue is too soft to slice.
189
190 1.3.2. Using forceps, place the sliced human lung tissue into wells of a 96-well clear flat-
191 bottom tissue culture plate. Return any unused human lung tissue immediately to the
192 storage container and place on dry ice for transport back to the -80 °C freezer.
193
194 1.3.3. Add 100 µL PBS to each lung tissue sample with a pipette. Use the pipette tip to
195 ensure all the tissue is covered in the PBS (and not stuck to the walls of the well). The tissue
196 will swell slightly and may float. Leave the PBS on the sample for a few minutes to allow any
197 blood to leach from the tissue into the solution. Remove as much of the PBS as possible. The
198 tissue may block the end of the pipette tip; try to angle the plate/tip placement in order to
199 prevent this.
200
201 1.3.4. Pipette 100 µL of unstained, Calcein AM labeled, or test-probe labeled bacteria to
202 each well containing lung tissue. Also set up controls with lung tissue and 100 µL PBS. To
203 these control wells, probes without bacteria can be added to measure any increase in
204 background fluorescence and/or non-specific activation of the probe by lung tissue alone. A
205 well with lung tissue and PBS should also be included.
206
207 2.   Imaging with the CLE Device with FCFM
208
209 2.1.   Set up of CLE device
210
211 Note: Prepare the CLE system 20 min before calibration to allow the laser to warm up.
212
213 2.1.1. Press the on/off switch on the back of the transformer of the system and turn on the
214 designated computer. Press the on/off button on the front of the laser scanning unit (LSU).
215 Confirm the appearance of the green light, indicating that the unit is switched on.
216
217 2.1.2. Double click on the CLE software icon. Enter the login details and wait for the LSU to
218 initialize (10–30 s).
219
220 2.1.3. For use of new FCFM imaging fibers, installation with the supplied CD is necessary.
221 Insert the installation CD into the computer CD drive and follow the onscreen instructions.
222
223 2.1.4. Clean the FCFM imaging fiber connector unit with a fiber cleaner. Rub the connector
224 on the cleaning ribbon in a figure eight motion to remove any dust/dirt. Remove the
225 protective yellow cap from the front of the LSU.
226
227 2.1.5. Prepare the LSU hub by gently rotating the silver hub anti-clockwise until it stops.
228 Insert the FCFM imaging fiber connector into the hub with the flat side of the connector
229 facing upwards. Hold the fiber in place and rotate the silver hub clockwise until it clicks
230 twice. Complete the connection by rotating the silver hub clockwise by a further 45 °.
231
232 Note: If the fiber is not recognized, check that the FCFM imaging fiber has been installed and
233 connected in the correct orientation.

234

2.1.6.   Follow the instructions that will pop-up for completing the FCFM imaging fiber calibration. There are 3 steps: (1) FCFM imaging fiber test (steps 2.1.7-2.1.8), (2) background acquisition (step 2.1.9), (3) fiber detection (step 2.1.10).

2.1.7.   Press the 'start laser' button on the screen. The laser will center.

2.1.8.   Select fresh vials (yellow: calibrate; red: clean; blue: rinse) from the calibration kit and follow the onscreen instructions: place the distal end of the FCFM imaging fiber into the yellow vial and watch for the increase in fluorescence on the monitor, then place the fiber tip into the red vial (without stirring). Wait for the fluorescence (as shown on the computer monitor) to disappear. Finally rinse the fiber tip in the blue vial.

Note: If the fluorescence does not disappear, clean the FCFM imaging fiber with 8% H_2O_2 and lens cleaning tissues and begin again. Repeat the process until satisfactory results are obtained (the image quality is clear, and no marks from dirt are apparent).

2.1.9.   Place the FCFM imaging fiber into the blue vial. Press 'start laser' followed by 'calculate' when this becomes an option.

2.1.10. Place the FCFM imaging fiber into the yellow vial. Press 'start laser' followed by 'calculate' when this becomes an option.

2.1.11. During the automated calibration, clean the distal end of the FCFM imaging fiber by placing in the red vial for > 10 s, followed by the blue vial for > 4 s, as indicated by the software.

2.2.   Data collection with CLE

2.2.1.   Following setup, a window to select storage location and file prefix will open. Select the desired folder for data storage, and name the prefix accordingly.

2.2.2.   Place the foot pedals so that they can be easily accessed by the operator. Left pedal: laser on/off; center pedal: pause; right pedal: record/stop.

Note: The laser controls can also be accessed through onscreen controls.

2.2.3.   Click 'start' onscreen or press the left foot-pedal to turn on the laser. This will start acquisition and obtain images using 100% laser power and a frame rate of 12 frames/s (default settings).

Note: For other applications, these settings can be altered on screen if necessary, depending on the sample type.

2.2.4.   Image each of the bacterial suspension samples.

280   2.2.4.1.   Insert the distal end of the FCFM imaging fiber and move the fiber slowly
281   through the suspension to interrogate the sample.
282
283   2.2.4.2.   Record videos of any length (up to 10 min) by pressing the right foot pedal or
284   selecting the onscreen record controls, as the fiber moves slowly around the sample.
285
286   2.2.4.3.   Clean the distal end of the FCFM imaging fiber with 8% H_2O_2 and lens cleaning
287   tissues between samples.
288
289   Note: Typical video lengths of 10–30s are sufficient for *in vitro* imaging.
290
291   2.2.5.   Image each of the lung tissue samples.
292
293   2.2.5.1.   Insert the distal end of the FCFM imaging fiber into the sample, ensuring that
294   direct contact between the end of the fiber and the tissue is made. Gently move the end of
295   the imaging fiber around to interrogate the sample.
296
297   Note: Lifting the end of the fiber away from the tissue will remove the tissue from the focal
298   plane; however, this may be used to image labeled bacteria that are not adhered to the
299   tissue.
300
301   2.2.5.2.   Record videos of any length (up to 10 min) by pressing the right foot pedal or
302   selecting the onscreen record controls, as the fiber moves slowly around the sample.
303
304   Note: Typically, video lengths of 30s are sufficient for *ex vivo* imaging on tissue.
305
306   2.2.5.3.   Clean the distal end of the FCFM imaging fiber with lens cleaning tissues and
307   8% H_2O_2 between samples.
308
309   2.3.   Turning off the system
310
311   2.3.1.   Switch the laser off by pressing the left foot pedal or by clicking the on-screen
312   button.
313
314   2.3.2.   Disconnect the FCFM imaging fiber from the CLE device by turning the silver LSU hub
315   anticlockwise until it stops. Remove the FCFM imaging fiber from the LSU hub by gently
316   pulling the fiber connector from the LSU.
317
318   2.3.3.   Clean and disinfect the FCFM imaging fiber with 8% H_2O_2 and lens cleaning tissues.
319   Return the protective caps to the proximal end of the FCFM imaging fiber and the front of
320   the LSU unit. Place the fiber gently in the storage box.
321
322   2.3.4.   Close the data capture software and copy any saved files to an external USB device.
323   Shut down the computer and turn off the LSU device by pressing the front panel I/O for 3 s
324   until the green light disappears.
325
326   2.3.5.   Dispose of human lung tissue and bacteria according to local regulations.

327

3.   Data Analysis

3.1.   Open the software and import the files for analysis by selecting the appropriate directory on the computer through the 'Go to' icon on the software dashboard. Alternatively, files can be dragged and dropped into the software dashboard.

3.2.   Double click on each video file to open them. The videos will automatically play with optimized color lookup table (LUT) and color table adjust. Disable the automatic intensity scaling, by clicking on the wand button above the intensity scale bar. The feature is disabled when there is no black shadowing around the button.

Note: Automatic intensity scaling must be disabled to prevent continuous contrast enhancement throughout each video, making it impossible to compare and analyze videos from the same data set.

3.3.   Select the desired intensity scaling by moving the minimum and maximum bars to give the best contrast. Use the histogram tool when selecting the intensity scaling to ensure the broadest dynamic range is captured.

Note: Ensure that the dynamic range is such that the images are not saturated (*i.e.* limit the white regions of the image, which indicate saturation), so that low intensity features are not missed.

3.4.   Once the desired scaling has been achieved, right click over the dropdown menu button listed as 'Default (Green)'. Select the option to save the LUT. Save the LUT to a desired location.

3.5.   For each other video within the data set, apply the same LUT by right clicking over the 'Default (Green)' drop down menu and select 'Load LUT'. Select the appropriate file to apply consistent intensity scaling to all videos within a dataset.

3.6.   Export processed videos by clicking on the 'movie reel' button. Select the desired video format, *e.g.* 'for presentation purposes', which will produce a .mpg file. Press 'Export' and chose file location to save the video file. Snapshots of single frames can be exported by clicking the 'camera' button. It is possible to save a .png, .bmp, or .jpg file. Choose the file destination and press save.

Note: Videos can then be imported into any software for preparation of presentations or further quantification. Labeled bacteria are visualized as green 'blinking' dots in the video. The lung tissue structure will be apparent as ordered fluorescent strands, with alveolar space appearing black.

REPRESENTATIVE RESULTS:
In this study, we have demonstrated a method for the rapid-screening of novel bacteria-specific probes in an *ex vivo* human alveolar lung tissue model of infection using a clinically approved CLE device.

374

375 CLE by FCFM is well suited for obtaining structural information within the distal lung, as this
376 region (due to a high abundance of elastin and collagen) is naturally highly fluorescent when
377 excited with a 488 nm laser[8]. Conversely, the alveolar space does not fluoresce, and as such
378 enables high contrast between tissue structure and air space to be visualized (**Figure 1**).

379

380 The addition of disease related probes or contrast agents, such as bacteria-specific probes
381 should enable functional information about disease processes to be obtained in real-time.
382 We have previously described the synthesis and initial *in vitro* screening of a library of
383 bacteria-specific probes[11]; where bacterial-specificity, proteolytic stability, and retention
384 within the bacterial membrane over time was determined. A promising bacteria-specific
385 probe (UBI-10) was identified within the study, as well as one that showed poor retention
386 within the bacterial cell membrane (UBI-3). These were compared to a control of
387 commercial counterstaining (Calcein AM) that was used to labeled *S. aureus*.

388

389 Unstained, Calcein AM, UBI-3, and UBI-10 labeled *S. aureus* were imaged in suspension by
390 FCFM with 100% 488 nm laser power and a frame rate of 12 frames/s (**Figure 2**). Where
391 unlabeled bacteria in PBS were imaged, no fluorescent signal was detectable. This is in
392 contrast to when labeled bacteria were imaged. Where bacterial suspensions with UBI-3 or
393 UBI-10 were imaged by FCFM, it was apparent that the general background fluorescence of
394 the solution was elevated compared to PBS only controls, this is because NBD (the probe
395 fluorophore) does emit a small amount of fluorescence signal in aqueous solution, however,
396 bright punctate dots are clearly visible throughout the solution, without the need for a wash
397 step. This is due to an increase in fluorescence signal emitted from NDB in a polar
398 environment *i.e.*, the bacterial membrane. Calcein AM is not an activatable probe, so a wash
399 step after bacterial staining was required to remove the high fluorescent background of
400 unbound probe in the solution. Like UBI-3 and UBI-10 labeled bacteria, bacteria labeled with
401 Calcein AM were detected in solution by FCFM as bright green punctate dots. As the data
402 are collected in video format, these dots appear to 'twinkle' as they move between cores
403 and in-and-out of focus, a characteristic trait of imaging labeled bacteria by this method.

404

405 The labeled bacteria were subsequently added to small slices of *ex vivo* human lung tissue
406 and imaged again by FCFM (**Figure 3**). Where only PBS or unlabeled *S. aureus* was added to
407 the lung tissue, only the lung tissue autofluorescent structure was detected (seen as bright
408 green strands of collagen and elastin and dark areas of alveolar space). No punctate dots
409 were detected for these control conditions. Similarly, only lung tissue structure (and no
410 punctate dots) was visualized for the lung tissue condition with *S. aureus* plus UBI-3;
411 indicating that this probe was not retained stably within the bacterial cell membrane *i.e.*, it
412 was washed out and/or is degraded in the presence of native proteolytic enzymes within
413 the lung tissue (as previously demonstrated[11]).

414

415 However, bright punctate dots were visible in both the Calcein AM labeled positive control
416 *S. aureus* sample, and with the most promising bacteria-specific probe (UBI-10) *S. aureus*
417 sample. The 'twinkling' dots were visible despite the strong tissue autofluorescence (**Figure
418 3**). Thus, the results obtained by FCFM were in concurrence with the *in vitro* pre-screening
419 of the panel of bacteria-specific probes by CLSM, and demonstrated a clinically relevant
420 detection method for imaging infections in real-time.

421

The results presented here demonstrate that the lung is an appropriate organ system for imaging by FCFM due to its distinctive autofluorescence. The bright distinctive structures allow the CLE operator to determine that they are in the alveolar space. These regions, coupled with the dark alveolar air sacs provide the perfect backdrop for imaging fluorescently labeled bacteria with high contrast.

Although the detection of bacteria presented within this study is determined qualitatively by visualizing bright punctate dots, it could be possible to quantify the number of punctate dots frame by frame using a secondary software in order to further characterize probe libraries.

FIGURE LEGENDS:

Figure 1. Static image of human lung tissue autofluorescence. Confocal laser endomicroscopy (CLE) image of *ex vivo* human lung tissue using fibered confocal fluorescence microscopy (FCFM), at 488 nm excitation, 100% laser power, and 12 frames/s. Elastin and collagen are highly fluorescent (false colored green), whereas alveolar space is not, and appears as black regions. This figure has been modified from Akram *et al*.[11]

Figure 2. Confocal laser endomicroscopy (CLE) image of labeled *S. aureus* in suspension. Fibered confocal fluorescence microscopy (FCFM) was used to image pre-labeled bacteria, at 488 nm excitation, 100% laser power, and 12 frames/s. Labeled bacteria show as highly fluorescent punctate dots (false colored green). This figure has been modified from Akram *et al*.[11]

Figure 3. Confocal laser endomicroscopy (CLE) image of *ex vivo* human lung tissue with labeled *S. aureus*. Fibered confocal fluorescence microscopy (FCFM) was used to image *ex vivo* human lung tissue and labeled *S. aureus*, at 488 nm excitation, 100% laser power and 12 frames/s. Labeled bacteria show as highly fluorescent punctate dots (false colored green) within the lung tissue sample when labeled with Calcein AM or UBI-10. The highest contrast is observed where bacteria are imaged within the alveolar space. This figure has been modified from Akram *et al*.[11]

DISCUSSION:

Lower respiratory tract infections account for the second highest burden of disease globally[12,13], and a substantial rise in the number of infections attributed to antimicrobial resistant bacteria has been reported[14]. Pneumonia remains a common cause for hospitalization. In the ICU, the development of a pneumonia is compounded by diagnostic uncertainty and is associated with an extremely high mortality rate[15]. During the onset of pneumonia, bacteria proliferate within the alveolar space of the distal lung, an area that is relatively sterile, with minimal microbiota in health.

This method describes relatively late stage *ex vivo* validation of bacteria-specific optical imaging probes[11], but the design, synthesis, and probe evaluation prior to beginning this validation step is imperative, as previously shown[11].

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CLE is an emerging clinical technique for interrogating disease states *in situ* in real-time. It offers many advantages over traditional techniques for investigating suspected pulmonary pathology, which may involve a biopsy and collection of the lavage fluid. Biopsies are invasive and can cause morbidity and mortality in ventilated patients, and collected lavage fluid is often contaminated with bacteria from the upper airways. The use of CLE in the detection of pneumonia is however somewhat limited due to the poor availability of compatible imaging probes which may provide functional information of disease, despite many concerted efforts[10]. Combining CLE with optical agents offers the prospect of diagnosing pneumonia faster and less invasively compared to current standard practice.

The critical steps to this protocol are in the sample preparation and setup of the CLE platform. Obtaining human tissue relevant to the final clinical application is also important, such as human lung tissue as demonstrated within this study. It is necessary to use human tissue because the extent of tissue autofluorescence shows large inter-species variation, and may therefore mislead the sensitivity of the bacteria-probe being imaged. Additionally, obtaining ethics for retrieval and use of human lung tissue is essential. From a technical level, correct cleaning, attachment of the imaging fiber to the imaging LSU platform, and calibration is essential for good resolution and consistent imaging, as is ensuring equivalent numbers of bacteria are added to each lung tissue sample. To further expand the utility of this method for screening panels of probes, repeating the procedure with a range of pathogens, such as those likely to be causative agents of pneumonia is necessary.

The largest limitation of this technique is that the clinically approved CLE device has only one laser (488 nm). Therefore, currently, the selection of fluorophore for probe design is limited for use with this system, though clinically approved single color devices do exist with excitation wavelengths of 660 nm and near-infrared. It is highly desirable to have a second laser line implemented within the same device to enable a probe to be developed with a spectrally distinct fluorophore to improve bacteria-probe sensitivity over the level of tissue autofluorescence. Whilst dual-color CLE devices are under development, they are either not clinically approved and/or their cost is significant[16].

CLE *in vitro* using pathogenic bacteria and *ex vivo* human lung tissue to screen potential probes bridges the gap between conventional *in vitro* techniques such as flow cytometry and CLSM, and clinical utility. This step offers confidence when selecting promising compounds to carry forward to be coupled with clinical CLE imaging; and will provide indication as to whether the tested probe maintains target specificity, or demonstrates any off-target labeling, such as binding directly to tissue, or shows instability with host proteolytic enzymes. It would also be pertinent to add each of the activatable probes directly to samples of human lung tissue plus bacteria, to fully characterize the speed of probe binding and activation in real-time.

We believe that our pipeline for rapidly screening novel bacteria-specific probes to assess their potential for imaging within the distal lung of patients will result in much faster translation to the clinic. This is largely because the bacteria-specific probe could be delivered locally within the lung through a catheter inserted down the working channel of a bronchoscope, meaning that microdose (< 100 µg) amounts could be delivered. Therefore, systemic delivery and biodistribution of the compound is not a concern, as is the case for

many other infection targets within the body, or with nuclear imaging. Moreover, delivering the imaging probe in such a small dose reduces the risk of toxicity related complications (although toxicity screening would be required for translation). Following instillation of the probe, the catheter could then be replaced by the FCFM fiber and the same region of the lung interrogated by CLE, much the same way we have performed within this method. Imaging should be performed rapidly following installation of the probe before the probe washes away to undetectable concentrations.

It is also important to note that screening of disease-identifying probes by this technique should not be limited to bacterial-imaging agents, but could also extend to the validation of probes with alternative targets, such as inflammation. This approach should also be adaptable to other disease locations within the body where imaging via FCFM is permissible.

DISCLOSURES:
KD: Founder Director of Edinburgh Molecular Imaging. Received consultancy from Mauna Kea Technologies as advisor.
MB: Founder Director of Edinburgh Molecular Imaging.

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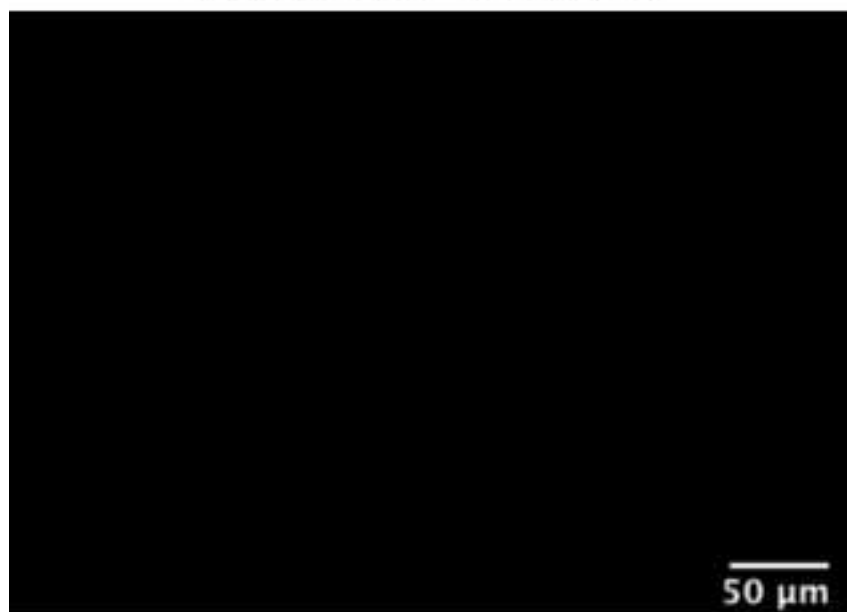575   **165** (7), 867-903, doi:10.1164/ajrccm.165.7.2105078, (2002).
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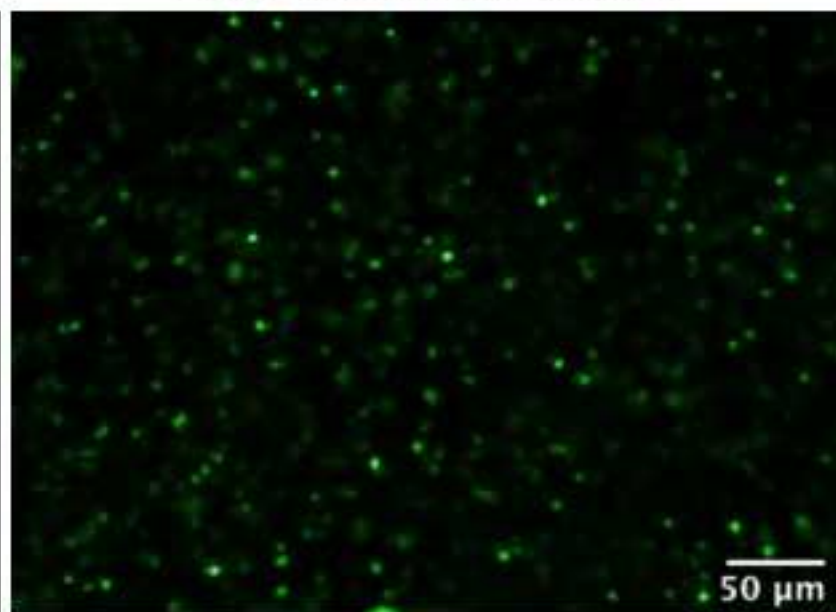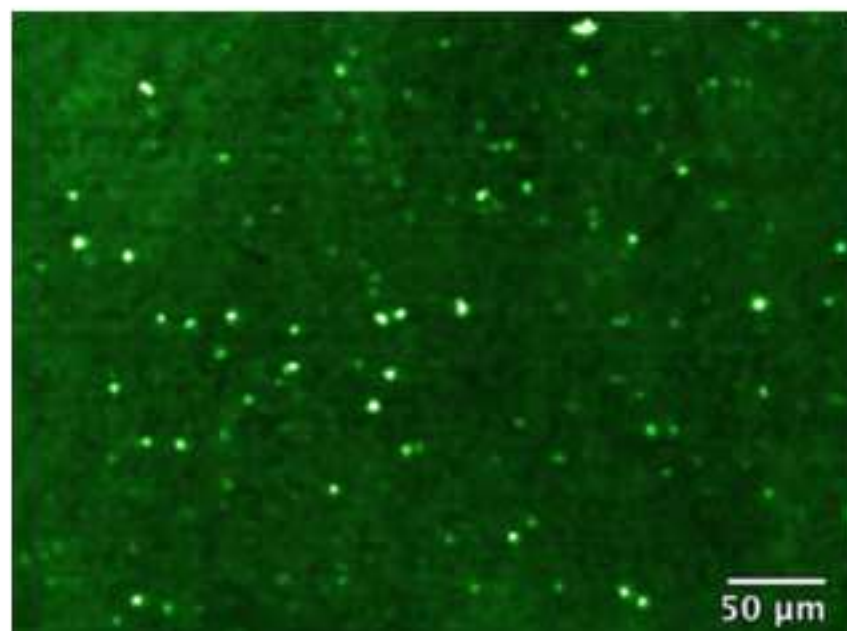579

Figure

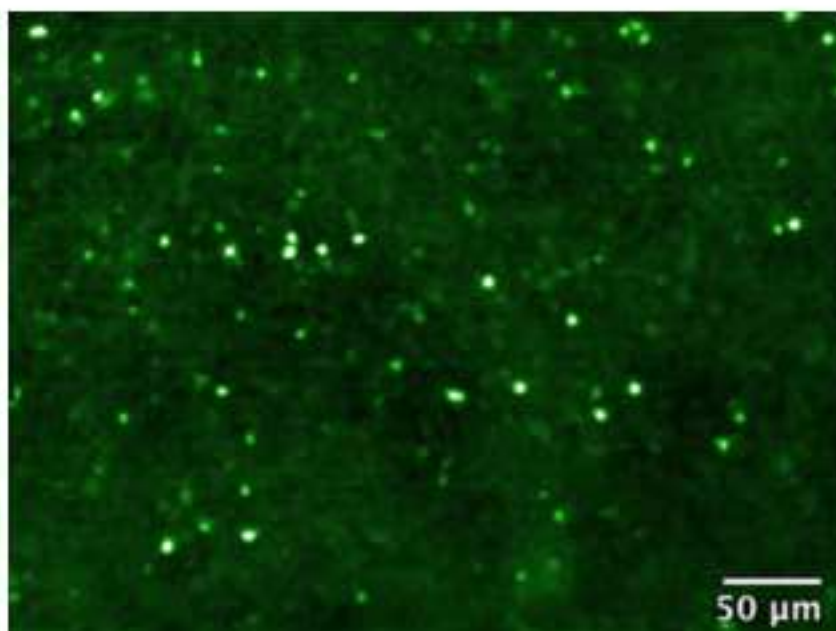## *Ex vivo* human lung autofluorescence
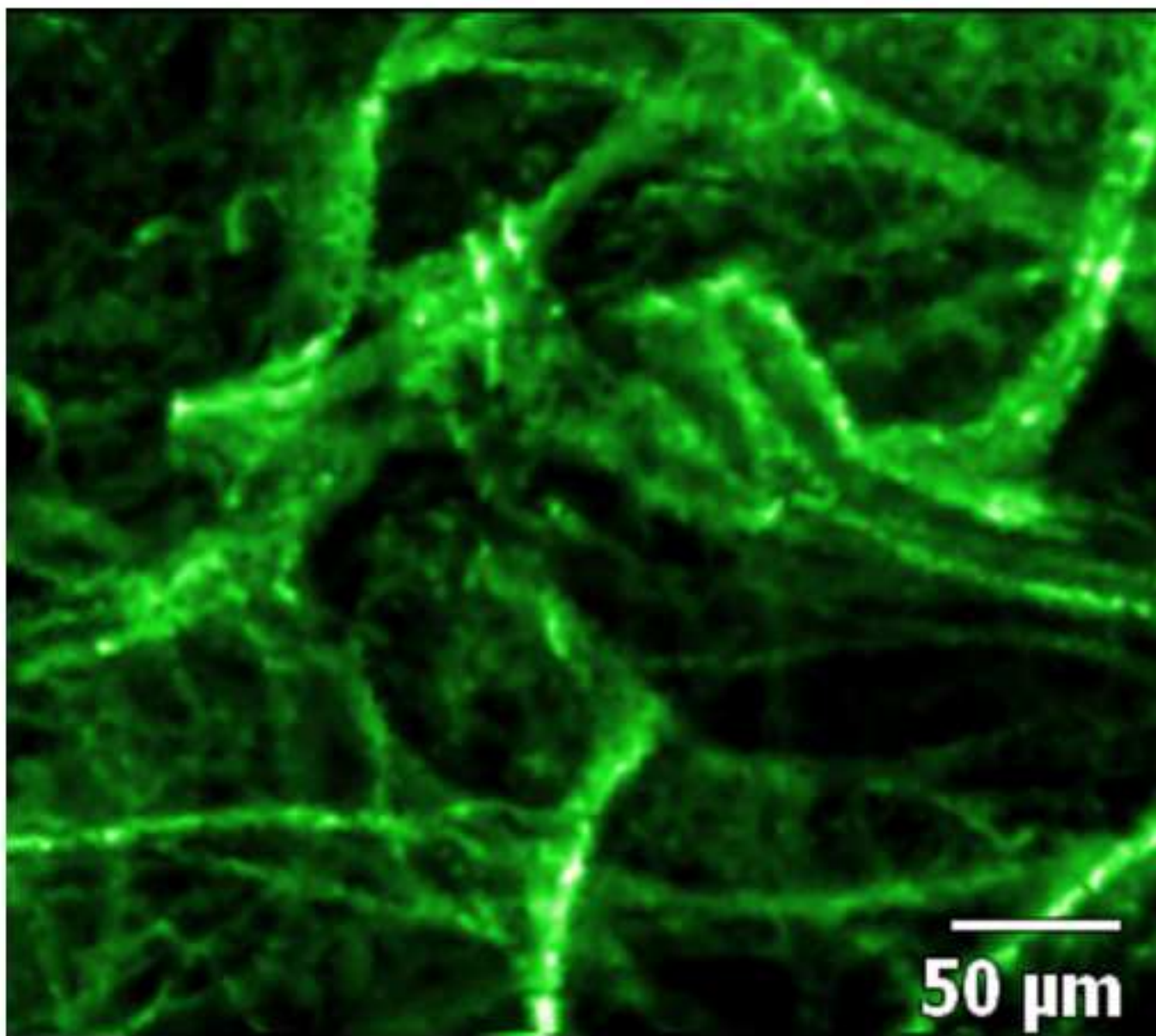
S. aureus (unstained)

S. aureus Calcein AM

S. aureus UBI-3
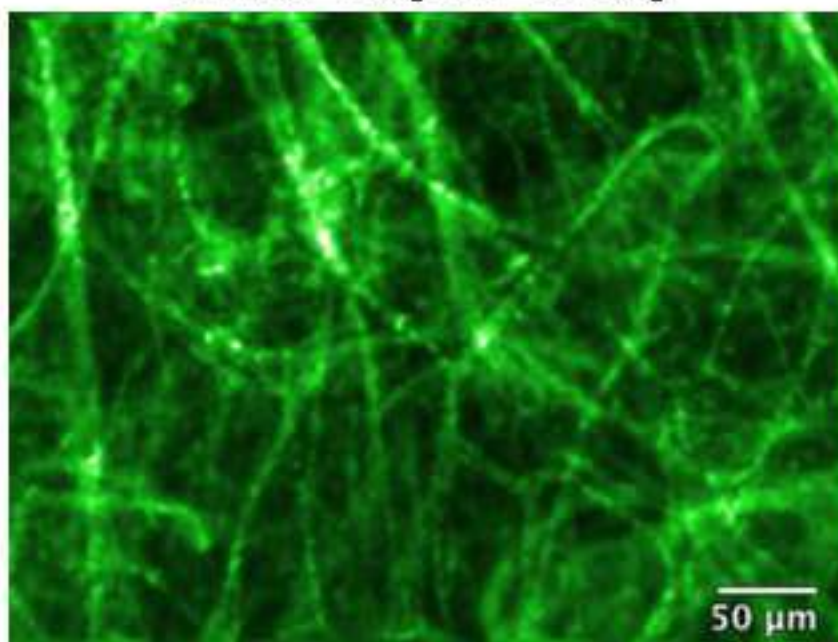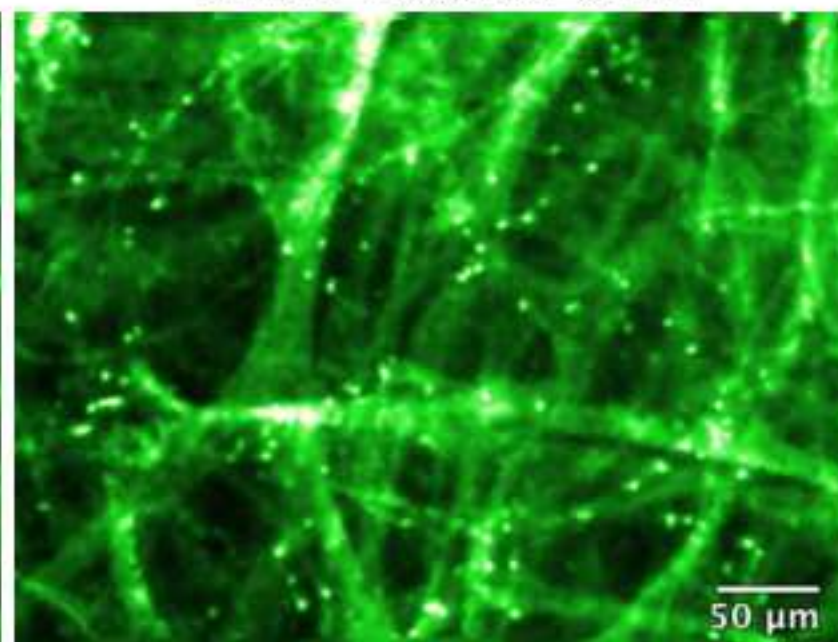
S. aureus UBI-10
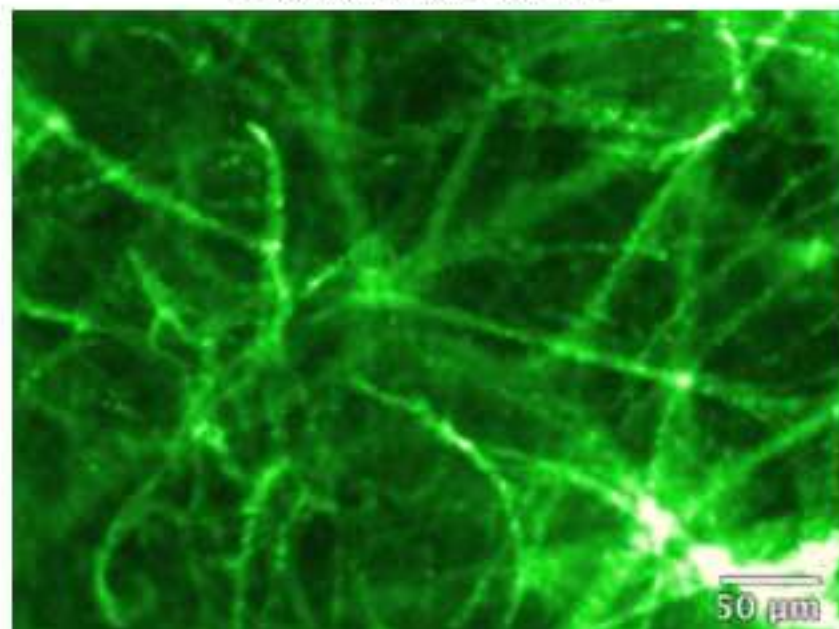
Table of Materials/Equipment
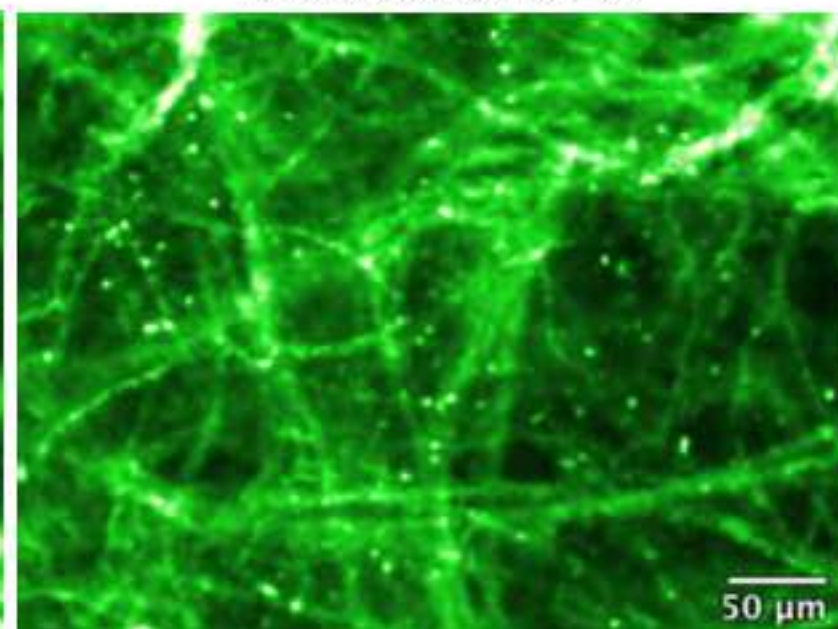
Name of Material/ Equipment	Company	Catalog Number
1-14 Microfuge	SciQuip	90616
96-well plate	Corning	3370
Calcein AM	Sigma Aldrich	17783
Cellvizio 488 nm Research CLE	Mauna Kea Technologies	LC-0001-488
Cletop-S	Cletop	14110601
Eppendorf (1.5 mL)	Eppendorf	30120086
Eppendorf Research Plus Pipettes	Fisher Scientific	11568663
Falcon tube (50 mL)	Scientific Lab Supplies	352070
Gibco Phosphate Buffered Saline	Thermo Fisher Scientific	10010023
IC-Viewer	Mauna Kea Technologies	LW-0001
Incu-Shake midi	Sciquip	SQ-4020
Lysogeny Broth	Sigma Aldrich (Miller)	L3522
non-standard research AlveoFlex 488 nm	Mauna Kea Technologies	MP-0002-AF3
Quanti Kit 488 nm	Mauna Kea Technologies	LQ-0005
S. aureus ATCC 25923	ATCC	25923
Semi-micro spectrophotometry cuvette	Sigma Aldrich	C5416-100EA
Thermomixer comfort	Eppendorf	41102422
UV 1101 Biotech photometer	Biochrom WPA	

Comments/Description

Small benchtop microcentrifuge

Assay plate

Commercial fluorescent dye

Confocal laser endomicroscopy device

Fibre cleaner

1.5 mL microfuge tube

Micro pipettes

50 ml centrifugation tube

PBS - wash media

Data collection and processing software for the research CellVizio 488 nm system

Floor standing shaking incubator

LB Growth media for *S. aureus*

Fibred confocal fluorescence microscopy fibre

Calibration kit for the CellVizio 488 system

Bacterial strain used in this study

For spectrophotometry

Benchtop heater with shaking

Spectrophotometer

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Thank you for your editorial comments for our 'Rapid screening of bacteria-specific probes for interrogating distal lung infection in the intensive care unit by confocal laser endomicroscopy' manuscript.

We have taken every effort to address each of your comments, and have included all track changes in the revised manuscript. Our responses to each of your points are detailed below.

We look forward to hearing from you.

Best wishes

Dr Bethany Mills

Research Fellow in Optical Imaging

PROTEUS Hub, Room E2.32
University of Edinburgh
Queens Medical Research Institute
47 Little France Crescent
Edinburgh
EH16 4TJ
Tel: +44 (0)131 242 9180
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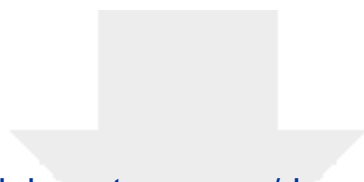
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