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TITLE:

Rapid Screening of Bacteria-specific Compounds for Interrogating Distal Lung Infection in the Intensive Care Unit by Confocal Laser Endomicroscopy

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KEYWORDS:

Optical probe, bacterial infection, confocal laser endomicroscopy, distal lung, translation, intensive care unit, optical imaging.

SHORT ABSTRACT:

This technique describes an efficient screening process for evaluating bacteria-specific optical imaging agents within *ex vivo* human lung tissue, by fibered confocal fluorescence microscopy for the rapid identification of small molecule chemical probe-candidates with translatable potential.

LONG 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 is 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.

48 **INTRODUCTION:**
49 This technique describes a rapid screening process for evaluating bacteria-specific optical
50 imaging agents within *ex vivo* human lung tissue by CLE using FCFM for the rapid
51 identification of compounds with potential clinical utility for visualizing bacteria in the distal
52 lung *in situ*.
53
54 There is an urgent global requirement to ration antimicrobial prescribing in the era of rising
55 antimicrobial resistance[1]. To this end, the development of diagnostic methods which act to
56 identify bacterial infection with high specificity, sensitivity, and in real-time are highly
57 sought[2]. Current techniques to confirm a diagnosis of pneumonia in critically unwell
58 patients, such as those within intensive care units (ICUs), often rely on interpreting non-
59 specific clinical or radiological features alongside bacterial culture techniques from aspirated
60 fluids/tissues, which can take up to 3 days to produce results. Furthermore, bacterial culture
61 from fluid instilled into the distal lung and retrieved is prone to contamination from more
62 proximal airways[3] and is often culture negative due to concomitant antimicrobial therapy or
63 poor sampling techniques. Additionally, molecular techniques such as polymerase chain
64 reaction are overly sensitive when used on aspirated fluids, risking overtreatment of
65 patients. An emerging diagnostic approach is molecular optical imaging, making *in situ*
66 molecular pathology of tissues a possibility; however, the development and validation of
67 optical imaging compounds is required. Nonetheless, direct visualization of bacteria, via
68 activatable optical probes is potentially a very powerful method to allow the study of the
69 presence and evolution of pneumonia in the patient, and importantly, could be used to
70 study host-pathogen interactions in response to therapies in real-time *in situ.*
71
72 CLE is an established investigative procedure in multiple diseases[4], including within the
73 fields of gastroenterology[5], oncology[6,7], and for interrogating airways and alveolar sacs[8,9]. It
74 enables point-of-care structural imaging of diseased tissue using a fiber imaging bundle,
75 which passes through the working channel of a clinical endoscope and forms direct contact
76 with the tissue surface to be imaged by confocal microscopy. However, one limitation that
77 remains is the need for generic contrast agents. Therefore, the use of disease specific
78 probes, such as specific bacterial agents, could vastly expand the utility of this modality by
79 directly visualizing bacteria at the site of suspected infection. Optical agents offer many
80 advantages over other techniques by enabling real-time, high resolution imaging with
81 diagnostic potential. Moreover, optical probes offer the prospect of multiplexing for
82 interrogating multiple targets, all achieved at a relatively low cost. A number of optical
83 agents are under development for such a purpose, however none have yet been
84 successfully implemented within the clinic[10]. We have synthesized a library of small
85 molecule chemical probes with specificity towards bacteria and developed a rapid, effective
86 pipeline for evaluation of probe function for detecting bacterial pneumonia *in situ*[11].
87
88 To identify suitable probe candidates, the following prerequisites had to be fulfilled prior to
89 interrogation of the probe on *ex vivo* human lung tissue by FCFM: i) aqueous solubility, ii)
90 specificity and selectivity for rapidly labeling clinically relevant bacteria, iii) a high signal-to-
91 noise ratio, and iv) resistance to degradation within the lung environment. The latter was
92 assessed by bronchoalveolar lavage fluid (BALF) from patients with acute respiratory
93 distress syndrome (ARDS), which is a condition that is characterized by proteolytic and
94 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.
97
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].
111
112 **PROTOCOL:**
113 All human lung tissue was obtained following informed consent and the study was approved
114 by the Regional Ethics Committee.
115
116 1. Preparation of Biological Samples
117
118 1.1. Preparation of probes
119
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.
123
124 1.2. Preparation of bacterial cultures
125
126 Note: For this method, *Staphylococcus aureus* was used as the exemplar strain. Any
127 appropriate bacterial strain can be selected.
128
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).
133
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.
138
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 x 10^8 colony forming
145 units (CFU) (OD_{595} 1 ~ 1 x 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
235 2.1.6. Follow the instructions that will pop-up for completing the FCFM imaging fiber
236 calibration. There are 3 steps: (1) FCFM imaging fiber test (steps 2.1.7-2.1.8), (2) background
237 acquisition (step 2.1.9), (3) fiber detection (step 2.1.10).
238
239 2.1.7. Press the 'start laser' button on the screen. The laser will center.
240
241 2.1.8. Select fresh vials (yellow: calibrate; red: clean; blue: rinse) from the calibration kit
242 and follow the onscreen instructions: place the distal end of the FCFM imaging fiber into the
243 yellow vial and watch for the increase in fluorescence on the monitor, then place the fiber
244 tip into the red vial (without stirring). Wait for the fluorescence (as shown on the computer
245 monitor) to disappear. Finally rinse the fiber tip in the blue vial.
246
247 Note: If the fluorescence does not disappear, clean the FCFM imaging fiber with 8% H_2O_2
248 and lens cleaning tissues and begin again. Repeat the process until satisfactory results are
249 obtained (the image quality is clear, and no marks from dirt are apparent).
250
251 2.1.9. Place the FCFM imaging fiber into the blue vial. Press 'start laser' followed by
252 'calculate' when this becomes an option.
253
254 2.1.10. Place the FCFM imaging fiber into the yellow vial. Press 'start laser' followed by
255 'calculate' when this becomes an option.
256
257 2.1.11. During the automated calibration, clean the distal end of the FCFM imaging fiber by
258 placing in the red vial for > 10 s, followed by the blue vial for > 4 s, as indicated by the
259 software.
260
261 2.2. Data collection with CLE
262
263 2.2.1. Following setup, a window to select storage location and file prefix will open. Select
264 the desired folder for data storage, and name the prefix accordingly.
265
266 2.2.2. Place the foot pedals so that they can be easily accessed by the operator. Left pedal:
267 laser on/off; center pedal: pause; right pedal: record/stop.
268
269 Note: The laser controls can also be accessed through onscreen controls.
270
271 2.2.3. Click 'start' onscreen or press the left foot-pedal to turn on the laser. This will start
272 acquisition and obtain images using 100% laser power and a frame rate of 12 frames/s
273 (default settings).
274
275 Note: For other applications, these settings can be altered on screen if necessary, depending
276 on the sample type.
277
278 2.2.4. Image each of the bacterial suspension samples.
279

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
328 3. Data Analysis
329
330 3.1. Open the software and import the files for analysis by selecting the appropriate
331 directory on the computer through the 'Go to' icon on the software dashboard.
332 Alternatively, files can be dragged and dropped into the software dashboard.
333
334 3.2. Double click on each video file to open them. The videos will automatically play with
335 optimized color lookup table (LUT) and color table adjust. Disable the automatic intensity
336 scaling, by clicking on the wand button above the intensity scale bar. The feature is disabled
337 when there is no black shadowing around the button.
338
339 Note: Automatic intensity scaling must be disabled to prevent continuous contrast
340 enhancement throughout each video, making it impossible to compare and analyze videos
341 from the same data set.
342
343 3.3. Select the desired intensity scaling by moving the minimum and maximum bars to
344 give the best contrast. Use the histogram tool when selecting the intensity scaling to ensure
345 the broadest dynamic range is captured.
346
347 Note: Ensure that the dynamic range is such that the images are not saturated (*i.e.* limit the
348 white regions of the image, which indicate saturation), so that low intensity features are not
349 missed.
350
351 3.4. Once the desired scaling has been achieved, right click over the dropdown menu
352 button listed as 'Default (Green)'. Select the option to save the LUT. Save the LUT to a
353 desired location.
354
355 3.5. For each other video within the data set, apply the same LUT by right clicking over
356 the 'Default (Green)' drop down menu and select 'Load LUT'. Select the appropriate file to
357 apply consistent intensity scaling to all videos within a dataset.
358
359 3.6. Export processed videos by clicking on the 'movie reel' button. Select the desired
360 video format, *e.g.* 'for presentation purposes', which will produce a .mpg file. Press 'Export'
361 and chose file location to save the video file. Snapshots of single frames can be exported by
362 clicking the 'camera' button. It is possible to save a .png, .bmp, or .jpg file. Choose the file
363 destination and press save.
364
365 Note: Videos can then be imported into any software for preparation of presentations or
366 further quantification. Labeled bacteria are visualized as green 'blinking' dots in the video.
367 The lung tissue structure will be apparent as ordered fluorescent strands, with alveolar
368 space appearing black.
369
370 **REPRESENTATIVE RESULTS:**
371 In this study, we have demonstrated a method for the rapid-screening of novel bacteria-
372 specific probes in an *ex vivo* human alveolar lung tissue model of infection using a clinically
373 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
422 The results presented here demonstrate that the lung is an appropriate organ system for
423 imaging by FCFM due to its distinctive autofluorescence. The bright distinctive structures
424 allow the CLE operator to determine that they are in the alveolar space. These regions,
425 coupled with the dark alveolar air sacs provide the perfect backdrop for imaging
426 fluorescently labeled bacteria with high contrast.
427
428 Although the detection of bacteria presented within this study is determined qualitatively
429 by visualizing bright punctate dots, it could be possible to quantify the number of punctate
430 dots frame by frame using a secondary software in order to further characterize probe
431 libraries.
432
433 **FIGURE LEGENDS:**
434 **Figure 1. Static image of human lung tissue autofluorescence**. Confocal laser
435 endomicroscopy (CLE) image of *ex vivo* human lung tissue using fibered confocal
436 fluorescence microscopy (FCFM), at 488 nm excitation, 100% laser power, and 12 frames/s.
437 Elastin and collagen are highly fluorescent (false colored green), whereas alveolar space is
438 not, and appears as black regions. This figure has been modified from Akram *et al*.[11]
439
440 **Figure 2. Confocal laser endomicroscopy (CLE) image of labeled *S. aureus* in suspension**.
441 Fibered confocal fluorescence microscopy (FCFM) was used to image pre-labeled bacteria,
442 at 488 nm excitation, 100% laser power, and 12 frames/s. Labeled bacteria show as highly
443 fluorescent punctate dots (false colored green). This figure has been modified from Akram
444 *et al*.[11]
445
446 **Figure 3. Confocal laser endomicroscopy (CLE) image of *ex vivo* human lung tissue with**
447 **labeled *S. aureus***. Fibered confocal fluorescence microscopy (FCFM) was used to image *ex*
448 *vivo* human lung tissue and labeled *S. aureus*, at 488 nm excitation, 100% laser power and
449 12 frames/s. Labeled bacteria show as highly fluorescent punctate dots (false colored green)
450 within the lung tissue sample when labeled with Calcein AM or UBI-10. The highest contrast
451 is observed where bacteria are imaged within the alveolar space. This figure has been
452 modified from Akram *et al*.[11]
453
454 **DISCUSSION:**
455 Lower respiratory tract infections account for the second highest burden of disease
456 globally[12,13], and a substantial rise in the number of infections attributed to antimicrobial
457 resistant bacteria has been reported[14]. Pneumonia remains a common cause for
458 hospitalization. In the ICU, the development of a pneumonia is compounded by diagnostic
459 uncertainty and is associated with an extremely high mortality rate[15]. During the onset of
460 pneumonia, bacteria proliferate within the alveolar space of the distal lung, an area that is
461 relatively sterile, with minimal microbiota in health.
462
463 This method describes relatively late stage *ex vivo* validation of bacteria-specific optical
464 imaging probes[11], but the design, synthesis, and probe evaluation prior to beginning this
465 validation step is imperative, as previously shown[11].
466

467 CLE is an emerging clinical technique for interrogating disease states *in situ* in real-time. It
468 offers many advantages over traditional techniques for investigating suspected pulmonary
469 pathology, which may involve a biopsy and collection of the lavage fluid. Biopsies are
470 invasive and can cause morbidity and mortality in ventilated patients, and collected lavage
471 fluid is often contaminated with bacteria from the upper airways. The use of CLE in the
472 detection of pneumonia is however somewhat limited due to the poor availability of
473 compatible imaging probes which may provide functional information of disease, despite
474 many concerted efforts[10]. Combining CLE with optical agents offers the prospect of
475 diagnosing pneumonia faster and less invasively compared to current standard practice.
476
477 The critical steps to this protocol are in the sample preparation and setup of the CLE
478 platform. Obtaining human tissue relevant to the final clinical application is also important,
479 such as human lung tissue as demonstrated within this study. It is necessary to use human
480 tissue because the extent of tissue autofluorescence shows large inter-species variation, and
481 may therefore mislead the sensitivity of the bacteria-probe being imaged. Additionally,
482 obtaining ethics for retrieval and use of human lung tissue is essential. From a technical
483 level, correct cleaning, attachment of the imaging fiber to the imaging LSU platform, and
484 calibration is essential for good resolution and consistent imaging, as is ensuring equivalent
485 numbers of bacteria are added to each lung tissue sample. To further expand the utility of
486 this method for screening panels of probes, repeating the procedure with a range of
487 pathogens, such as those likely to be causative agents of pneumonia is necessary.
488
489 The largest limitation of this technique is that the clinically approved CLE device has only
490 one laser (488 nm). Therefore, currently, the selection of fluorophore for probe design is
491 limited for use with this system, though clinically approved single color devices do exist with
492 excitation wavelengths of 660 nm and near-infrared. It is highly desirable to have a second
493 laser line implemented within the same device to enable a probe to be developed with a
494 spectrally distinct fluorophore to improve bacteria-probe sensitivity over the level of tissue
495 autofluorescence. Whilst dual-color CLE devices are under development, they are either not
496 clinically approved and/or their cost is significant[16].
497
498 CLE *in vitro* using pathogenic bacteria and *ex vivo* human lung tissue to screen potential
499 probes bridges the gap between conventional *in vitro* techniques such as flow cytometry
500 and CLSM, and clinical utility. This step offers confidence when selecting promising
501 compounds to carry forward to be coupled with clinical CLE imaging; and will provide
502 indication as to whether the tested probe maintains target specificity, or demonstrates any
503 off-target labeling, such as binding directly to tissue, or shows instability with host
504 proteolytic enzymes. It would also be pertinent to add each of the activatable probes
505 directly to samples of human lung tissue plus bacteria, to fully characterize the speed of
506 probe binding and activation in real-time.
507
508 We believe that our pipeline for rapidly screening novel bacteria-specific probes to assess
509 their potential for imaging within the distal lung of patients will result in much faster
510 translation to the clinic. This is largely because the bacteria-specific probe could be
511 delivered locally within the lung through a catheter inserted down the working channel of a
512 bronchoscope, meaning that microdose (< 100 μg) amounts could be delivered. Therefore,
513 systemic delivery and biodistribution of the compound is not a concern, as is the case for

514 many other infection targets within the body, or with nuclear imaging. Moreover, delivering
515 the imaging probe in such a small dose reduces the risk of toxicity related complications
516 (although toxicity screening would be required for translation). Following instillation of the
517 probe, the catheter could then be replaced by the FCFM fiber and the same region of the
518 lung interrogated by CLE, much the same way we have performed within this method.
519 Imaging should be performed rapidly following installation of the probe before the probe
520 washes away to undetectable concentrations.
521
522 It is also important to note that screening of disease-identifying probes by this technique
523 should not be limited to bacterial-imaging agents, but could also extend to the validation of
524 probes with alternative targets, such as inflammation. This approach should also be
525 adaptable to other disease locations within the body where imaging via FCFM is permissible.
526
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532
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537
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