Enhanced avidity from a multivalent fluorescent antimicrobial peptide enables pathogen detection in a human lung model

Ahsan R. Akram1, Nicolaos Avlonitis2, Emma Scholefield1, Marc Vendrell1, Neil McDonald1, Tashfeen Aslam2, Thomas H. Craven1, Calum Gray3, David S. Collie4, Andrew J. Fisher5, Paul A. Corris5, Timothy Walsh1, Christopher Haslett1, Mark Bradley1,2 & Kevin Dhaliwal1

Rapid in situ detection of pathogens coupled with high resolution imaging in the distal human lung has the potential to provide new insights and diagnostic utility in patients in whom pneumonia is suspected. We have previously described an antimicrobial peptide (AMP) Ubiquicidin (fragment UBI29–41) labelled with an environmentally sensitive fluorophore that optically detected bacteria in vitro but not ex vivo. Here, we describe further chemical development of this compound and demonstrate that altering the secondary structure of the AMP to generate a tri-branched dendrimeric scaffold provides enhanced signal in vitro and ex vivo and consequently allows the rapid detection of pathogens in situ in an explanted human lung. This compound (NBD-UBI dend) demonstrates bacterial labelling specificity for a broad panel of pathogenic bacteria and Aspergillus fumigatus. NBD-UBI dend demonstrated high signal-to-noise fluorescence amplification upon target engagement, did not label host mammalian cells and was non-toxic and chemically robust within the inflamed biological environment. Intrapulmonary delivery of NBD-UBI dend coupled with optical endomicroscopy demonstrated real-time, in situ detection of bacteria in explanted whole human Cystic Fibrosis lungs.

Pulmonary bacterial and fungal infection is a common and often fatal event1,2 and pneumonia is a foremost differential diagnosis in ventilated and immunocompromised patients who develop rapid respiratory deterioration3. Severe pneumonia, the development of ventilator associated pneumonia (VAP) and adult respiratory distress syndrome (ARDS) all cause significant morbidity and mortality and are responsible for a large proportion of healthcare costs4–6. Current management algorithms7,8 include the retrieval of bronchoalveolar lavage (BAL) or endotracheal sampling prior to empirical broad-spectrum antimicrobials, but all methods have variable sensitivity, are prone to contamination and confer significant time delays for microbial results.

Optical endomicroscopy (OEM) is a promising and attractive diagnostic platform9–12 and our work has demonstrated the utility to delineate gram-negative bacteria in mechanically ventilated patients in situ13 through an approach involving topically administered microdosed compounds with fiber based OEM. Furthermore, we have previously reported14 on the antimicrobial peptide (AMP) Ubiquicidin (UBI29–41) demonstrating that the
linear fragment coupled with a fluorophore is insufficient for bacterial imaging in *ex vivo* lungs due to rapid degradation, despite being utilised as a nuclear medicine imaging agent.  

Here, we describe further development of this compound, engineering a multivalent (tri-branched) form, while utilising a modified portion of the bacterial binding fragment (UBI 29–41) of the 59 amino acid sequence in which the metabolically labile methionine residue in replaced with a norleucine analogue. The arms of the trivalent scaffold were capped with the environmentally sensitive fluorophore, 7-nitrobenz-2-oxa-1,3-diazole (NBD) that self-quenches any residual fluorescence within the aqueous environment and then generates excellent signal-to-noise ratios when dispersed in the distal human lung, with fluorescent amplification only upon entry into the hydrophobic environment of the bacterial membrane. When compared to its' monovalent equivalent, NBD-UBI selectively labelled a diverse panel of pathogenic bacteria and *Aspergillus fumigatus*, and remained chemically stable in the complex environment of the inflamed and injured lung. It detected bacteria in an *ex vivo* large animal model of bacterial burden and in infected explanted cystic fibrosis (CF) human lungs.

**Results**

**NBD-UBI** specifically labels bacteria and fungi with high signal-to-noise ratio over inflammatory cells. The multivalent peptide (NBD-UBI) comprising three modified monomeric UBI peptides, coupled to the environmentally responsive fluorophore NBD (NBD-UBI) (Fig. 1A) was synthesised by solid-phase peptide synthesis (Fig. 1B).

NBD-UBI demonstrated fluorescent amplification in *vitro* with increasing environmental hydrophobicity (Fig. 1C). Bacterial labelling was demonstrated in a concentration dependent manner (Fig. 1D) and of a panel of clinically relevant lung pathogenic bacteria, including both Gram-positive and Gram-negative species (Fig. 1E) with varying labelling intensity (Fig. 1F).

Furthermore, to ensure the compound retains specificity and does not label the inflammatory cell infiltrate observed with pneumonia, NBD-UBI was co-cultured with freshly isolated primary human neutrophils, monocytes, lymphocytes and human alveolar macrophages (Fig. 2A–D) with no labelling seen. Confocal imaging was undertaken in the continued presence of the NBD-UBI demonstrating the fluorescent amplification in bacteria. To further demonstrate selectivity in human lung tissue, bacterial labelling was demonstrated...
Figure 2. NBD-UBI\textsubscript{dend} demonstrates selectivity for bacteria and \textit{A. fumigatus} over human cells. (A–C) Co-culture of (A) freshly isolated human neutrophils, (B) freshly isolated human mononuclear cells and (C) human alveolar macrophages retrieved from bronchoalveolar lavage with \textit{P. aeruginosa} imaged with NBD-UBI\textsubscript{dend} 5 µM. Plot profiles quantify fluorescence across the dashed line in the NBD channel and counterstain (Syto-82) channel. Each panel shows representative images of at least three independent experiments for each cell type, scale bars represent 10 µm. (D) Quantification of benchtop confocal data demonstrating significantly lower fluorescence on neutrophils, mononuclear cells and BALF macrophages compared to bacteria. Bars show mean fluorescence (+/- SEM) from three independent experiments, where at least three fields of view were assessed. Analysis by Students t-test, *p < 0.05, **p < 0.01. (E,F) 3D reconstruction of benchtop confocal microscope imaging of (E) human alveolar tissue, incubated with \textit{P. aeruginosa} and labelled with NBD-UBI\textsubscript{dend} 5µM and (F) human alveolar tissue, incubated with \textit{A. fumigatus} and labelled with NBD-UBI\textsubscript{dend} 10µM. White arrows indicate specific labelling of bacteria or germinating fungal hyphae, whereas lung epithelial cells are not labelled (blue arrows). Yellow arrows indicate elastin autofluorescence present in the human lung. Images are representative of three independent experiments. Scale bars represent 10 µm.
Figure 3. NBD-UBI<sub>dend</sub> has greater affinity and demonstrates improved signal-to-noise when labelling bacteria in pulmonary surfactant than the linear NBD-UBI and shows stability in ARDS BALF. (A) Quantification of retained fluorescence following a wash and re-imaging on a benchtop confocal microscope. Each bacterial strain and compound were normalised to their pre-wash fluorescence intensities (bacteria were incubated with NBD-UBI at 15 μM or equimolar concentration of NBD-UBI<sub>dend</sub> at 5 μM). Bars represent means (+/− SEM) of independent experiments where at least three fields of view were assessed, and analysed by a Student’s t-test, *p < 0.05, **p < 0.01, ***p < 0.001. (B) Representative images of *P. aeruginosa* imaged in the presence of synthetic pulmonary surfactant. Green panels show either NBD-UBI 10 μM or NBD-UBI<sub>dend</sub> 3.3 μM with red panels showing Syto82 counterstaining, purple panels showing synthetic surfactant and phase contrast images demonstrating surfactant vesicles. (C) Fluorescence quantification of *P. aeruginosa* imaged with NBD-UBI at 10 μM or NBD-UBI<sub>dend</sub> at 3.3 μM in the presence of fluorescently labelled high-density surfactant vesicles. Data represents the mean fluorescence of the NBD channel on bacteria compared to surfactant and show a significantly higher bacteria:surfactant fluorescence intensity for NBD-UBI<sub>dend</sub> than NBD-UBI. Bars represent means (+/− SEM) of three independent experiments where at least three fields of view were assessed, analyses by Student’s t-test, **p < 0.01. (D) Stability of compounds in saline and ARDS BALF demonstrating breakdown of NBD-UBI in ARDS BALF when analysed by MALDI-TOF MS (left panel) the presence of NBD-UBI<sub>dend</sub> in ARDS BALF when assessed by FTMS (right panel). (E) Representative flow histograms for bacteria when incubated with NBD-UBI (grey histograms) at 15 μM or NBD-UBI<sub>dend</sub> 5 μM (dotted line) demonstrating a
greater fluorescence intensity at eqimolar dye concentrations. (F) Quantification of flow cytometry data for the monomeric form (white bars, normalised) and dendrimeric form (grey bars) demonstrating between a 2–10 fold increase in fluorescence at eqimolar dye equivalents. Bars represent means (+/− SEM) from three independent experiments, analysis is by students t-test, **p < 0.01, ***p < 0.001.

by confocal microscopy of human alveolar tissue17. Three-dimensional optical reconstructions confirmed bacterial-specific labelling within this complex environment (Fig. 2E).

NBD-UBI<sub>den</sub> also labelled the pulmonary pathogenic fungus Aspergillus fumigatus in human lung tissue (Fig. 2F). The pattern of labelling of Aspergillus fumigatus, imaged with OEM (Supplementary Fig. S1) was clearly discrete from bacteria, supporting the utility of NBD-UBI<sub>den</sub> and OEM to discriminate bacteria from Aspergillus fumigatus.

NBD-UBI<sub>den</sub> demonstrates high avidity, stability when compared to its linear fragment and is non-toxic. NBD-UBI<sub>den</sub> demonstrated high avidity for bacteria as evidenced by retention of bacterial labelling despite washing steps in vitro. Compared to 15 μM of the analogous linear moiety (NBD-UBI), 5 μM of NBD-UBI<sub>den</sub> retained bacterial labelling after washing (Fig. 3A). Hydrophobic phospholipoprotein components of pulmonary surfactant have the potential to generate off-target fluorescence. In order to investigate this, synthetic surfactant was co-cultured with bacteria and imaged. NBD-UBI<sub>den</sub> labelled bacteria in the presence of synthetic surfactant with good signal-to-noise ratios (Fig. 3B,C). Chemical stability was assessed by incubating NBD-UBI<sub>den</sub> and NBD-UBI with bronchoalveolar lavage fluid (BALF) from ARDS patients in ICU and performing mass spectroscopic analysis. NBD-UBI was rapidly degraded in ARDS BALF, while NBD-UBI<sub>den</sub> remained intact (Fig. 3D). Furthermore, the multivalent peptide provided enhanced fluorescent labelling when compared to equimolar levels of the analogous monomeric construct NBD-UBI (Fig. 3E,F).

NBD-UBI<sub>den</sub> rapidly detects bacteria with topical microdosed endobronchial delivery and OEM in a large animal in vivo lung model. Using a previously reported model13 we evaluated NBD-UBI<sub>den</sub> against a diverse panel of clinically relevant pathogenic bacteria (Fig. 4). NBD-UBI<sub>den</sub> was able to label bacterial segments, over control segments and the linear NBD-UBI compound within a clinically relevant limit of detection (LoD) of 1 × 10<sup>3</sup>CFU/mL bacteria retrieved on BALF (Fig. 4A–F and Videos 1–4). Monomeric NBD-UBI failed to detect bacteria (Fig. 4D) in this model.

NBD-UBI<sub>den</sub> detects bacteria in situ in whole explanted human lungs. To further support clinical utility, we performed bronchoscopy and delivery of NBD-UBI<sub>den</sub> in freshly-explanted whole lungs from patients with cystic fibrosis (CF) undergoing transplantation (Fig. 5A). The CF lungs were extremely damaged with large amounts of mucopurulent secretions. Discrete distal bronchopulmonary segments were instilled with NBD-UBI<sub>den</sub> or equimolar monomeric NBD-UBI (negative control). The characteristic bacterial signal of punctate twinkling speckles and also distinct and intense colonies were detected in the alveoli in the NBD-UBI<sub>den</sub> instilled segments, but not the monomeric instilled segments (Fig. 5B, C and Video 5). BAL confirmed the presence of pathogenic bacteria in all segments of the CF lung (Fig. 5D). This confirmed the ability of NBD-UBI<sub>den</sub> to rapidly detect bacteria in situ in the human lung using a microdosing regime and OEM.

Discussion

Immediate-point-of-care bedside methodologies to enable clinicians to determine the presence or absence of bacteria or pathogenic fungi in the distal lung are required. Clinicians are faced with significant uncertainty in relation to triggers to commence antibiotic treatment, the choice of agents and especially when to de-escalate therapy. These issues are barriers to effective antibiotic stewardship because of the association between delayed and inadequate antibiotic therapy and adverse clinical outcomes.

Optical imaging of bacteria is an emerging and attractive modality and utilising OEM with localised microdosed delivery of SmartProbes in distal bronchopulmonary segments potentially offers an immediate method for bacterial detection. This approach shows particular promise for the future as it can be multiplexed using fibre based systems capable of multirspectral imaging, used in combination with other SmartProbes for Gram-negative bacteria or inflammatory cells and compounds can be delivered directly into the imaging field of view. Furthermore, with ongoing refinements to the image analysis algorithms through multiple methods, automated readouts of the signals generated may help decision making and advance our understanding of the pathophysiology during suspected pneumonia.

UBI<sub>39,41</sub>, in its monomeric form, has been applied in human infection imaging in the form of radionuclide imaging with technetium<sup>99</sup>Tc. We demonstrated that this monomeric scaffold was rapidly degraded in the inflamed bronchoalveolar compartment, and have demonstrated that a monomeric ligand remains ineffective for bacterial labelling in assays using fragments of human lung tissue. In contrast, the trivalent dendrimeric scaffold afforded enhanced proteolytic stability, partitioning in surfactant and high avidity in the whole human lung.

Although this compound demonstrates promise for clinical utility, it now requires clinical translation to determine utility alongside other SmartProbes in patients with suspected pneumonia.
In summary, we have synthesized a multivalent fluorescent UBI analogue that demonstrates excellent optical properties for the detection by optical imaging platforms already in clinical use and with microdosed delivery selectively labels a range of pathogens in vitro and in an ex vivo ovine model. Importantly, we have also shown...
that this novel technology can specifically detect bacteria in the harsh environment of freshly explanted human lungs from patients with CF.

**Materials and Methods**

**Ethics statement.** All experiments using human samples were performed following approval of the appropriate regional ethics committee (REC), with informed consent of the patients and in accordance with the relevant guidelines and regulations at the University of Edinburgh. BALF for alveolar macrophages: (East of Scotland Research Ethics Service REC no: 07/S1102/20), blood for isolation of neutrophils and mononuclear cells: (Lothian REC no: 08/S1103/38), human lung tissue: (East of Scotland Research Ethics Service REC no: 13/ES/0126) and explant Cystic Fibrosis Lung assessment: (NRES Committee North East-Newcastle & North Tyneside REC no: 13/NE/0353).
lavage fluid (BALF) was harvested via 3-point). Airway resistance was assessed via whole body plethysmography. Following necroscopy, bronchoalveolar

toxylin and eosin. Lung, liver and kidney were removed, fixed and paraffin-embedded. Slides were prepared and stained with hema-

stained with Diff-Quick (Thermo Fisher Scientific). BALF cell types were quantified using a light microscope. Chemical probes is described in the Supplementary Information.

potato dextrose broth and grown overnight in a shaking incubator at 37 °C. Germinating hyphae were washed

1 of Animals (Scientific Procedures) Act 1986. In accordance with the guidelines and regulations at the University of

11/NE/0291). Animal experiments were performed under UK Home Office Animals Scientific Procedures Act 1986 (Project License Number 60/4434) in accordace with the guidelines and regulations at the University of Edinburgh and following approval of experimental protocols by the Animal Welfare and Ethical Review Body at the University of Edinburgh. Ovine lungs were from ewes destined for cull and were euthanized under Schedule 1 of Animals (Scientific Procedures) Act 1986.

for confocal assays.

Analytical, Gillingham, UK) and conidia were counted on a haemocytometer. 1 bacterial strains were used: Methicillin Resistant

S. aureus (ATCC 25923), Methicillin Sensitive S. aureus (ATCC 252), S. pneumoniae (D39 NCTC 7466), H. influenzae (Clinical Isolate), K. pneumoniae (ATCC BAA1706), E. coli (ATCC 25922), A. baumannii (J3433 Clinical Isolate), S. maltophilia (J3270 Clinical Isolate), P. aeruginosa (PA01-ATCC 47085 and J3284-clinical isolate) and GFP fluorescent S. aureus (RN6390-Gfp-EryR).

Bacterial culture. Bacterial strains were grown and counterstained as previously described13,14. The following bacterial strains were used: Methicillin Resistant S. aureus (ATCC 25923), Methicillin Sensitive S. aureus (ATCC 252), S. pneumoniae (D39 NCTC 7466), H. influenzae (Clinical Isolate), K. pneumoniae (ATCC BAA1706), E. coli (ATCC 25922), A. baumannii (J3433 Clinical Isolate), S. maltophilia (J3270 Clinical Isolate), P. aeruginosa (PA01-ATCC 47085 and J3284-clinical isolate) and GFP fluorescent S. aureus (RN6390-Gfp-EryR).

Fungal culture. Aspergillus fumigatus (clinical isolate) was stored in potato glucose agar (Fluka Analytical, Gillingham, UK) at 4 °C. A wet harvest of conidia was performed into 1 mL of potato dextrose broth (Fluka Analytical, Gillingham, UK) and conidia were counted on a haemocytometer. 1 × 10⁶ conidia were added to 9 mL potato dextrose broth and grown overnight in a shaking incubator at 37 °C. Germinating hyphae were washed for confocal assays.

Neutrophil and mononuclear cell isolation. Neutrophils and mononuclear cells were isolated from the peripheral blood of healthy human volunteers using dextran sedimentation and discontinuous Percoll gradients, as previously described15.

MALDI-TOF and FTMS. NBD-UBI or NBD-UBI_dend were added to saline or pooled BALF from three patients with ARDS incubated for 30 min. ARDS BALF was retrieved from patients in ICU, as previously described34. Samples were passed through ZipTip (C-18, 0.2 µL) conditioned with 5 µL MeCN (with 0.1% TFA as an additive) followed by 20 µL of H₂O. The ZipTip was loaded with the sample, washed and eluted with 5 µL of 80% aq. MeCN (with 0.1% TFA as an additive). Samples were analysed on a MALDI-TOF (PerSeptive Biosystems Voyager DE™STR MALDI-TOF) mass spectrometer, Applied Biosystems, Foster City, CA) or FTMS (Bruker Daltonics 12T SolarisX Fourier Transform Ion Cyclotron Resonance Mass Spectrometer (FT-ICR MS)).

Emission spectra. The fluorescence emission of NBD-UBI_dend solutions were measured in increasing concentrations of t-butanol on a Synergy H1 Multi-Mode Spectrophotometer (BioTek, VT, US) upon excitation at 450 nm.

Surfactant constituent synthesis. Surfactant vesicles were synthesised by dissolving 5 µg 1,2-Dipalmityl-sn-glycero-3-phosphocholine (DPPC) and 2.5 µg L-α-Phosphatidyl-DL-glycerol sodium salt (from egg yolk lecithin; PG) in 500 µL chloroform. The solution was evaporated under nitrogen to form a thin lipid film in a round bottom flask and subsequently rehydrated with PBS at 48 °C for 1 hour with continuous agitation at 750 rpm to generate multilamellar vesicles (MLV). MLVs were stained with Cellvue Claret dye (Sigma-Aldrich, St Louis, MO, USA) and diluted 1:4 for use in confocal experiments.

Haemolysis assay. Performed as previously decribed13. Briefly, erythrocytes (resuspended to 20 vol % in PBS) were cultured with varying concentrations of NBD-UBI_dend PBS (negative control) or 0.2% Triton X-100 (positive control) and incubated at 37 °C for 1 h. Wells were diluted with 150 µL of PBS, centrifuged and absorbance of supernatant read at 350 nm.

Single dose intra-tracheal rodent study. Adult male CD-1 mice aged 8–12 weeks were given direct intra-tracheal administration of a single dose of NBD-UBI_dend (100 µg) in 50 µL PBS (equivalent to 300 µM) or PBS vehicle control. Animals were monitored and then sacrificed at 48 hours or 14 days (n = 3 per group per time point). Airway resistance was assessed via whole body plethysmography. Following necropsy, bronchoalveolar lavage fluid (BALF) was harvested via 3 × 0.8 ml PBS flushes of the lung and cytospin slides were prepared and stained with Diff-Quick (Thermo Fisher Scientific). BALF cell types were quantified using a light microscope. Lung, liver and kidney were removed, fixed and paraffin-embedded. Slides were prepared and stained with hema-toxylin and eosin.

Confocal imaging and analysis. Imaging and analysis was conducted as previously reported13,14. ‘Green’ fluorescence (for NBD) was excited with a dedicated 488 nm line, Syto nuclear acid dyes were excited with a dedicated 543 nm line and Cellvue Claret labelled surfactant were excited with a dedicated 633 nm line. For affinity assays, following initial imaging, the fluid was aspirated from wells and two gentle washes with PBS were performed. The chamber was re-imaged on benchtop confocal.

Flow cytometry. NBD-UBI or NBD-UBI_dend were incubated with bacteria for 5 minutes at 37 °C prior to analysis using BD FACSCalibur (Becton Dickenson, San Jose, CA, USA) flow cytometer capturing 50,000 of counterstained gated events. Analysis performed with FlowJo version 7.6.5 (TreeStar Inc., Ashland, OR).

Ovine lung model and image analysis. The ovine model and analysis algorithm have been reported previously35. Briefly, surplus stock animals, which were destined for cull, had their lungs removed, which were ventilated and kept at 37 °C, humidity of 65% and ventilated using a Pressure Controlled Ventilator (Vivo PV.
403, Breas Medical, Sweden). Segments were bronchoscopically identified and bacteria (or PBS control) instilled. After >1 hour segments were re-identified with subsequent SmartProbe instillation and OEM imaging at 488 nm excitation (Cellvizio Lab, Mauna Kea Technologies) using a 1.5 mm diameter S-1500 fiber (Mauna Kea Technologies), followed by BALP retrieval. Frame-by-frame analysis was undertaken after removal of redundant frames; frames were considered positive if there were >80 dots per frame. Data is presented as the proportion of frames over a video sequence containing >80 dots per frame.

**Ex vivo cystic fibrosis human lung.** Whole explant CF lungs were received fresh from theatre (<1 hour) and main the bronchi were identified. Bronchoscopy and suction of the Airways was performed prior to instilling NBD-UBI/NBD-UBI$_{end}$ into anatomically distinct distal bronchopulmonary segments. The bronchoscope was proximally wedged and the S-1500 fiber passed into distal segments. Following imaging in the distal lung (alveolar regions) as described above, a BAL was undertaken, sample was centrifuged at 200 g for 5 minutes to remove erythrocytes and cellular material and was plated for bacterial quantification.

**Statistical analysis.** All experiments were performed at least three times unless otherwise stated and results expressed as mean ± SEM. Data was analysed by Student's t-test or ANOVA, significance was determined as p < 0.05 (GraphPad Prism version 5.01 for Windows, GraphPad Software, San Diego California USA).

**Data Availability**

The data that support the findings of this study are presented in the published article and any additional data is available from the corresponding author upon reasonable request.

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Acknowledgements

The authors would like to thank the NHS Lothian SAHSC Bioresource for facilitating the studies using ex vivo human lung tissue and the flow cytometry facility at the Queen’s Medical Research Institute. Funding from Wellcome Trust and Department of Health HICF (HICF-0510-069), The Engineering and Physical Sciences Research Council (EP/K03197X/1) and EU Commission (FP7 CIG333847).

Author Contributions

N.A., M.V., T.A. and M.B. performed chemical design, synthesis and characterisation. A.A., N.M., E.S., C.H., M.B. and K.D. designed and performed the in vitro biological assays. E.S. and K.D. designed and performed the murine work. A.A., N.M., T.C., E.S., D.C. and K.D. designed, set-up and performed the ex vivo ovine lung experiments. A.A., T.C., A.F. and K.D. designed and undertook the ex vivo CF lung experiments. A.A., N.M. and K.D. undertook data analysis. C.G. and T.M. designed the image analysis algorithm with input from A.A., N.M. and K.D. C.H., M.B., T.W. and K.D. conceived and supervised the project. A.A. and K.D. wrote the manuscript. All authors approved the manuscript.

Additional Information

Supplementary information accompanies this paper at https://doi.org/10.1038/s41598-019-44804-0.

Competing Interests: C.H., M.B. and K.D. have a shareholding in Edinburgh Molecular Imaging Ltd. A.R.A., T.H.C., N.M. and K.D. have received travel fees for attendance at educational conferences supported by an unrestricted educational grant from Mauna Kea Technologies. K.D. has received travel and attendance fees from Mauna Kea Technologies as a consultant for an advisory board. T.H.C. has received travel fees for attendance at an educational conference from Edinburgh Molecular Imaging Ltd. K.D., M.B., M.V., C.H., T.A., N.M. and N.A. are inventors on a patent (WO 2016/075484 A1) held by the University Court of the University of Edinburgh that covers the probe and method of use. The other authors declare that they have no competing interests.

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