Non-invasive transcutaneous spectroscopy for the assessment of gut permeability

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GLOSSARY OF ABBREVIATIONS

AE	Adverse event
EED	Environmental enteric dysfunction
FITC	Fluorescein isothiocyanate
GI	Gastro-intestinal
GER	Gastric emptying rate
HIV	Human immunodeficiency virus
IBD	Inflammatory bowel disease
ICG	Indocyanine green
IMP	Investigational medicinal product
L:M	Lactulose:Mannitol
LED	Light emitting diode
MHRA	Medicines and Healthcare products Regulatory Agency
NRES	National Research Ethics Service
PD	Photodiode
PEG	Polyethylene glycol
REC	Research Ethics Committee
SAE	Serious adverse event

KEYWORDS

Fluorescence, spectroscopy, gut permeability, non-invasive, low-cost

STUDY SUMMARY

Title
Non-invasive transcutaneous spectroscopy for the assessment of gut permeability

Design
Basic science study involving procedures with human participants

Research questions
Is it possible to use transcutaneous optical spectroscopy to non-invasively assess the permeability of the intestine via measurements of the concentration of orally administered contrast agents in the blood?

Outcome measures
Spectroscopic measurements of gut permeability (and gastric emptying rate, GER) will be assessed via comparison against a range of traditional markers:

1. Where the blood concentration is known – for example, in ophthalmology patients who have received a direct intravenous dose of contrast agent – a direct comparison will be made without the need for further measurements.
2. In subjects receiving an oral dose of contrast agent, blood/urine samples will be taken alongside the spectroscopic measurements in order to permit accurate *ex vivo* quantification of the serum/urine concentration in the laboratory.
3. In control experiments aimed at determining the impact of GER, the rate of uptake of fluorescent dye will be compared against the rate of uptake of paracetamol (which is used clinically to assess GER).
4. In patients who are also undergoing polyethylene glycol (PEG)-based permeability assays, spectroscopic permeability measurements will be compared to the results of the more traditional approach.
5. Finally, in patients for whom intestinal biopsy and histology data is available, spectroscopic permeability measurements will be compared against histological measures of epithelial damage and permeability.

Investigational medical devices
The devices under investigation in this study are optical spectrometers designed for the transcutaneous measurement of the concentration of a fluorescent contrast agent in the blood. Two devices will be tested – a bench-top system that provides high level spectral information ('mark I') and a miniaturised wearable system suitable for wider clinical application ('mark II'). The 'mark II' system will be designed during the study, based on results collected with the 'mark I' device. As such, at this stage this research represents an investigation of a technique for assessment of gut permeability (transcutaneous fluorescence spectroscopy) rather than an investigation of a particular medical device.

Contrast agents
A variety of contrast agents will be tested in this study in order to find the most suitable agent(s) for transcutaneous quantification of gut permeability. The agents tested will include fluorescein, indocyanine green (ICG), fluorescein isothiocyanate (FITC) conjugated dextran (FITC-dextran), and FITC-conjugated polyethylene glycol (FITC-PEG). For the FITC-dextran and FITC-PEG studies, contrast agents with varying molecular weights will be investigated. For GER experiments, the only contrast agent used will be fluorescein.

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Population
Three groups will be studied:

Group 1   **Ophthalmology patients** who are receiving an intravenous dose of either fluorescein or ICG as part of their routine ophthalmic care (e.g. as part of a fluorescence angiography examination). These patients will take part in preliminary studies aimed at determining whether it is possible to detect fluorescein and ICG in the blood using transcutaneous fluorescence measurements.

Group 2   **Healthy subjects** will be split into two groups (2a and 2b) dependent on the aspect of the study they participate in.

                 2a: Healthy subjects with no known issues of increased gut permeability. These subjects will act as negative controls in all gut permeability studies. A subset of these volunteers will be asked to take two spectroscopic permeability tests – one with and one without a supplementary dose of a hyperosmotic solution, which acts to transiently increase intestinal permeability. This subset will provide proof-of-concept data prior to recruitment of patients in Group 3.

                 2b: Healthy subjects with no known issues of increased gut permeability or gut motility. These subjects will participate in GER experiments, which will provide complementary data to aid in the design of transcutaneous optical spectroscopy as a non-invasive tool to assess gut permeability. Separate "healthy volunteer" sub-group will prevent the possibility of build-up of contrast agent.

Group 3   **Gastro-intestinal (GI) and non-GI patients** who are expected to exhibit increased gut permeability (e.g. patients with coeliac disease, inflammatory bowel disease (IBD), liver disease, HIV or another condition in which increased intestinal permeability is common). The more extreme cases in this group will act as positive controls.

Eligibility
Aged 18 and over; able to give informed consent; not pregnant or breastfeeding; no prior adverse reactions to fluorescein, ICG, dextran, PEG or iodine.

BACKGROUND

Increased gut permeability – often referred to as "leaky gut" – occurs when the intestinal barrier becomes compromised and involves increased transmission of certain intestinal constituents (such as endotoxins) into lymph and blood vessels [1, 2]. The condition is associated with many widespread diseases including coeliac disease [1, 3], inflammatory bowel disease (IBD) [3-5], human immunodeficiency virus (HIV) [6], fatty liver disease [7-9] and environmental enteric dysfunction (EED) [10, 11]. These diseases affect millions of patients worldwide and represent a huge cost burden to national healthcare systems. Importantly, in many of them, the role and impact of intestinal barrier function is not yet completely understood. Moreover, the methods used to assess permeability are cumbersome. As a result, increased permeability of the intestine has not yet been conclusively linked to outcomes in these illnesses [1].

The issue of increased intestinal permeability is particularly acute in the case of EED, a chronic inflammatory condition of the small intestine that impairs both nutrient absorption and barrier function [10, 11]. EED is endemic in many developing countries – where it manifests in children at a very early age (< 6 months) – and can lead to malnutrition, poor response to vaccines, and severely restricted physical and cognitive development [12-17]. As such, it is strongly associated with poor developmental and socio-economic outcomes including growth stunting, poor performance in education, reduced income in adulthood, and increased fertility [18-20].

Increased gut permeability is believed to play a pivotal role in EED [21, 22], where the leakage of pathogens (including endotoxins and, in some extreme cases, whole bacteria) from the intestine causes repeated infections, which lead to additional inflammation in the gut. In turn, this further damages the absorptive and barrier functions of the intestinal epithelium, trapping those who suffer with this condition in a cycle of infection, inflammation and malnutrition.

At present, both EED itself and the role of increased gut permeability in EED (and in many of the other diseases highlighted above) are not well understood [1, 23, 24]. For this reason, the development of tools that can accurately monitor gut permeability and provide further information on its role in all of the above conditions is vitally important. Ideally, such devices should be minimally invasive and suitable for widespread deployment to allow large scale studies. Furthermore, if they are to be applicable to ailments that are prevalent in the developing world such as HIV and EED, these tools will also need to be low in cost. Importantly, systems of this sort could be useful not only in the investigation of the diseases above but also in providing a method for diagnosis, screening, and assessment of the efficacy of interventions.

The most common technique that is currently used to directly assess gut permeability is the lactulose:mannitol (L:M) test [25]. Using this approach, patients receive an oral dose of the two sugars lactulose and mannitol, and urine samples are subsequently collected for up to six hours. The concentrations of the two sugars in urine are determined via liquid chromatography or mass spectrometry, and the L:M ratio provides an indication of the permeability of the intestine. A low L:M ratio indicates normal permeability, as lactulose is a large disaccharide molecule that is not absorbed by the healthy intestine (and, hence, is not found in urine) while mannitol is a smaller monosaccharide that passes the healthy intestinal barrier. An increase in the L:M ratio then indicates either increased permeability, impaired absorption, or a combination of the two. While the L:M test has been widely used to measure permeability, results are dependent on the proficiency of those carrying out the protocol as well as the level of diligence observed in dietary fasting (which is required prior to the test) and regular urine collection [25]. These problems are particularly acute when performing tests in infants, which

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is desirable in the case of EED and, in some cases, coeliac disease. Moreover, in all cases the protocol is time consuming for both the patient and the care provider, and post-processing of urine samples is required in a laboratory, which can be challenging in developing world settings (where suitable infrastructure may not be available).

Some alternative approaches based on similar methodologies are also available (see [2]), with one example involving the use of an orally administered polyethylene glycol (PEG) solution containing PEG molecules of varying molecular weights [1, 26]. Concentrations in urine are assessed in the same manner as described above, but more detailed permeability information is provided due to the wider range of molecular sizes being investigated (compared to just two in the L:M test). Nonetheless, this approach is prone to the same problems as L:M tests, suggesting that improved protocols will still be desirable.

Endoscopic Biopsy and histology can also be used to assess the fragility of the intestinal epithelial barrier. However, this approach only provides an indirect, highly localised measure of the permeability and also represents an invasive procedure that is particularly undesirable in children and infants.

Fluorescence spectroscopy may offer an alternative approach for the assessment of gut permeability, as it has previously been used in animal studies to quantify both intestinal closure and barrier function [27, 28]. In these investigations, intestinal permeability was assessed by feeding animals (rats and pigs) doses of fluorescent dextrans of varying molecular weights and then measuring the relative concentrations of these markers in the blood some time later. This was achieved using *ex vivo* measurements of blood samples and demonstrated that the passage of the fluorescent dextrans from the digestive system into the blood stream was dependent on both the state of intestinal closure and the size of the markers [27, 28].

Interestingly, it may be possible to translate this approach to non-invasive, *in vivo* use in humans through the use of transcutaneous detection of fluorescence (i.e. measurement of the fluorescence of the blood-borne markers through the skin). This method would entail a patient receiving an oral dose of a fluorescent contrast agent, with the concentration of that contrast agent in the bloodstream being measured in a transcutaneous arrangement using fluorescence spectroscopy. This approach – which would not require collection of urine or blood samples – would provide a direct measure of permeability and would be considerably less time consuming and labour intensive than the traditional L:M and PEG tests. Thus, it could be expected to offer considerable improvements in terms of both the reliability of the test and the experience of the patients. In addition, the devices used for fluorescence detection could be both low in cost and small in size, and only minimal post-processing would be required, making it possible to more quickly return test results to patients.

As an aside, it is noteworthy here that a rate-limiting step for the entrance of substances into the intestine, and thus a factor in the quantification of gut permeability, is gastric emptying rate (GER). Gastric emptying is rarely considered, however its measurement would be highly complementary to that of gut permeability. In addition, it may have an effect on the ability to measure intestinal permeability using transcutaneous fluorescence spectroscopy. As such, control experiments will be performed here to determine the impact of GER on transcutaneous optical spectroscopy as a non-invasive tool for assessment of gut permeability. Interestingly, these control experiments may also serve to demonstrate the potential of transcutaneous fluorescence spectroscopy for non-invasive quantification of GER.

In the experiments proposed here we aim to test and validate transcutaneous fluorescence spectroscopy as a tool for the non-invasive quantification of gut permeability (and GER). This will entail testing two devices – a 'mark I' bench-top system and a miniaturised 'mark II' device suitable for larger scale deployment – along with four fluorescent contrast agents (fluorescein; indocyanine green, ICG; fluorescein isothiocyanate conjugated dextran, FITC-dextran; and FITC-conjugated PEG, FITC-PEG). These devices and contrast agents will be applied to patient studies with the key aims of validating transcutaneous spectroscopy, developing and optimising a fluorescence-based gut permeability assay (in terms of both the device and the protocol), and applying the optimised system to wide-ranging disease studies. The diseases investigated will include (but will not necessarily be limited to) coeliac disease, IBD, liver disease and HIV, as patients with these conditions will be accessible through Imperial College Healthcare NHS Trust. A longer term aim of this research (which is beyond the scope of this protocol) is to apply the fluorescence gut permeability assay to widespread studies of EED in low and middle income countries. Importantly, the successful development of this novel gut permeability sensor is likely to have a considerable impact in the broad field of gut health due to the opportunities it will offer for both non-invasive diagnostics and large-scale investigational studies.

AIMS & OBJECTIVES

This study has three overarching aims:

1. to determine whether it is possible to monitor the permeability of the human gut using transcutaneous fluorescence spectroscopy of fluorescein, ICG, FITC-dextran and/or FITC-PEG;
2. to develop, optimise and validate fluorescence-based permeability sensing via comparison to traditional gut permeability measurements (including PEG-based permeability assays and histological assessments) in healthy volunteers and positive controls (i.e. subjects with highly increased permeability such as untreated coeliac or IBD patients);
3. to investigate a wide spectrum of gut permeabilities and to correlate transcutaneous permeability measurements to disease state in some or all of the conditions studied (which will include IBD, coeliac disease, HIV and fatty liver disease).

MATERIALS & METHODS

Instrumentation
Two separate optical systems will be developed and used in this study. The first is a bench-top device consisting of two laser sources for excitation of fluorescence, a spectrometer for detection, and a bifurcated optical fibre probe to allow interrogation of the fluorescence signal at the subjects' skin (see Figure 1). This system is described in detail in Appendix A and is similar in design to the spectrometers reported in [29, 30]. Briefly, it entails two laser sources – at wavelengths of 488 nm and 785 nm – which permit excitation of fluorescence from fluorescein/FITC and ICG respectively. The fibre probe acts to deliver laser light to the subjects' skin and to direct the excited fluorescence signal to the spectrometer. This allows for detection of the fluorescent dyes in the blood stream of the subjects. In order to ensure laser safety, the laser power at the distal end of the fibre probe will be limited. The allowable power levels will vary with the skin-probe distance, which will be tested in early experiments to determine the

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optimum probe position. Thus, the optical powers will range from 63 µW (skin contact) to 565 µW (maximum skin-probe separation of 2 mm – see Appendix A) at 488 nm and from 93 µW (skin contact) to 836 µW (2 mm) at 785 nm. At these power levels the light output will be below the maximum permissible exposure for the skin (at the appropriate probe-skin separation) and will be eye-safe when the probe is held at a distance of >10 cm [31-33]. As the optical system is also contained within an interlocked, light-tight container, it can therefore be used by non-expert users in a clinical environment without the use of laser safety goggles and without risk of injury. To mitigate this risk even further, patients will be notified that they should not look directly at the tip of the fibre probe and will be supervised at all times during the experiments.
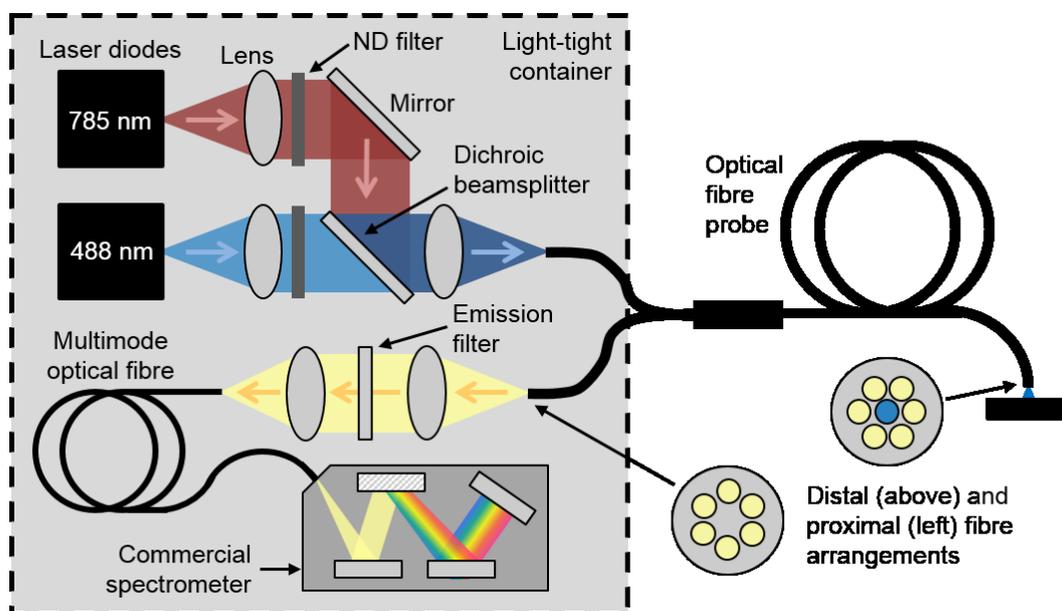
Figure 1. Schematic diagram of the 'Mark I' bench-top optical system. All optical components are contained within a light-tight container that is interlocked to ensure the laser beam can only be manipulated by expert users. The optical power is attenuated using neutral density (ND) filters such that the output at the distal tip is below the maximum permissible exposure for skin at all times and is eye-safe when held at a distance of >10 cm. In this configuration the device can be classified as a Class 1 laser system and, hence, can be used by non-expert laser users in a clinical environment. The fibre probe consists of seven 200 µm diameter optical fibres, one for excitation (blue) and six for collection of fluorescence (yellow) – see insets at bottom right. At the distal tip of the probe the central excitation fibre is surrounded by the six collection fibres such that the 'active area' of the probe spans a diameter of approximately 600 µm.

The second device used in this study will be a miniaturised version of the system described above. This device will use light emitting diodes (LEDs) for excitation and photodiodes (PDs) for detection of fluorescence (and for detection of the directly scattered laser light). It will entail a wireless, wearable tool that is secured in contact with the subjects' skin during use. As above, the optical excitation powers will be limited in order that the system is eye-safe and skin-safe during use. The exact design of the 'mark II' device will be guided by the results collected with the bench-top ('mark I') system and the first stage of experimentation with the miniaturised system will entail validation against the bench-top version. All optical and electrical safety concerns will be factored into the design process, which is discussed in more detail in Appendix B.

As discussed in the Study Summary section, this research is inherently an investigation of a novel *technique* for the assessment of gut permeability rather than a study of a particular medical device. Indeed, the devices used in the study will be designed, amended and optimised based on the data collected throughout the investigation, with the goal of designing and validating an optimal approach for studies/measurements of gut permeability in large cohorts.

Contrast agents

As discussed above, four contrast agents will be used in these studies: fluorescein, ICG, FITC-dextran and FITC-PEG. In Stage 1 of the experiments (see 'Experimental protocols' section below), either fluorescein or ICG will be administered to patients via intravenous injection. In Stages 2 and 3, combinations of fluorescein, ICG, FITC-dextran and/or FITC-PEG will be administered to patients orally (as a solution) with a variety of combinations tested (e.g. fluorescein + ICG, FITC-dextran/FITC-PEG + ICG, and fluorescein followed by FITC-dextran/FITC-PEG). The order in which dye combinations will be tested in Stage 2 is described in the 'Experimental Protocols' section below.

There are risks of side effects with all four of the proposed contrast agents, ranging from mild nausea in the least serious case through to anaphylaxis in the most serious. Importantly, however, patients participating in Stage 1 will only receive doses of contrast agent that have been prescribed clinically, meaning that these experiments will bring no additional risks (related to contrast agents). Furthermore, in Stages 2 and 3 all contrast agents will be delivered orally, which considerably reduces the risk of adverse events relative to intravenous use [34]. Nonetheless, in all cases, the dyes will be administered to subjects by trained healthcare professionals and all measurements will be carried out within a hospital environment. Thus, patients/volunteers will be monitored for signs of side effects at all times allowing rapid responses to any serious adverse events that occur. Doses will be limited to minimise the risk of complications while still allowing acceptable data to be collected (see summary of maximum dose levels in Table 1). The specific safety issues relating to each of the individual contrast agents are described below, and these are also explained in the participant information sheets so that subjects can make an informed choice about whether to take part in the study.

Table 1. Maximum proposed doses for each of the fluorescent dyes to be used in this study. Maximal doses (for individual experiments) and the chosen administration routes are shown for each dye. In addition, relevant citations are given where further safety information can be found. Note that the total cumulative dose may be higher than the values shown in the Table in some instances, as some volunteers will be recruited for more than one spectroscopic gut permeability test. ICG – indocyanine green; FITC – fluorescein isothiocyanate; PEG – polyethylene glycol; IV – intravenous.

Contrast agent	Maximum dose	Dose route	Relevant citations
Fluorescein	500 mg (IV); 5 g (oral)	IV and oral	34-36, 38, 41, 44-47
ICG	5 mg/kg	IV and oral	50, 52, 54, 55
FITC-dextran	1 g	Oral only	56, 57, 60-62
FITC-PEG	1 g	Oral only	56, 57, 72-76

Fluorescein   Fluorescein is approved for medical use with intravenous delivery for ophthalmic angiography [35-37]. This is true despite the fact that adverse events – ranging from mild nausea through to anaphylaxis – are known to occur with varying frequencies. Mild adverse reactions (including mild nausea) are the most common, occurring in approximately 1/10 patients [35]. The frequency of

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severe adverse events (including anaphylaxis) is much lower, at approximately 1/2000 [34, 35]. In extremely rare cases, fatal allergic reactions have also been reported, accounting for approximately 1/200,000 patients [35]. Fluorescein is also often used 'off-label' with oral delivery – either as a solution or in the form of a tablet/capsule – for angiography [35, 36, 38-40], and this has been shown to be both safe and effective [34, 38, 41-44]. Indeed, it has been observed that minor adverse events are less common when administering fluorescein orally rather than intravenously [34, 44, 45] and reports of severe adverse events are extremely rare with oral fluorescein [34, 44] (two examples of anaphylaxis reported in response to oral fluorescein can be found in references [46, 47]). In addition, fluorescein has been further used 'off-label' for a variety of surgical and endoscopic imaging applications (e.g. [48, 49]).

In the experiments proposed here, subjects will receive either an intravenous or oral dose of fluorescein depending on the experiment in which they are taking part. The maximal intravenous dose will not exceed 500 mg (typically administered as a 5 ml solution with a concentration of 100 mg/ml) as per the Medicines and Healthcare products Regulatory Agency (MHRA) guidelines [35, 36]. Furthermore, the intravenous doses used will be those prescribed as part of the patients' ophthalmic care and will not be increased or amended in any way as part of this study. In the case of oral delivery, a maximal dose of 5 g will be used. This is approximately double the maximal dose typically used in oral fluorescein angiography, however, it can be considered safe as complications are extremely rare with oral fluorescein delivery [34, 38, 41, 44, 45]. Furthermore, it will be necessary to test higher doses than those used for retinal imaging in order to determine the minimum oral doses that are detectable using a transcutaneous measurement protocol (i.e. lower doses will be detectable in the eye – due to its transparent nature – than through the skin). Once suitable minimum concentrations have been established, these will be used in all further experiments.

ICG   ICG is approved for medical use in solution using intravenous delivery for a variety of applications [50-53] and safe human use with oral delivery has also been reported (e.g. [54]). Complications are less common with ICG than they are with fluorescein (for intravenous delivery), with serious adverse events occurring with a rate of less than 1/10,000 and fatal allergic reactions having a frequency of below 1/330,000 [50]. In this investigation, subjects will receive either an intravenous or oral dose depending on the experiment in which they are taking part. The total dose delivered to a given patient will not exceed 5 mg of ICG per kg of body weight (mg/kg) for both intravenous and oral delivery. No significant toxic effects have been observed at this dose [52, 55] and it corresponds to the MHRA guidelines for intravenous use in adults [50]. Hence, it can also be expected to be a safe dose when administered orally. In addition, individual intravenous doses (i.e. those administered for a single experiment) will not typically exceed 0.5 mg/kg body weight, as per the MHRA guidelines [50]. Importantly, these dosage levels are also in agreement with doses used in investigational studies with oral dye delivery, in which this method of ICG administration was found to be safe (e.g. [54]).

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FITC-dextran FITC-dextran is not yet approved for medical use, however, it has been applied to a limited number of *in vivo* clinical studies in humans using both intravenous [56] and oral [57] delivery. In addition, oral delivery of FITC-dextran has been used widely in animal experiments (e.g. [27, 28]). In human studies, total oral and intravenous doses of up to 1 g have been used (delivered as a 100 ml solution at 10 mg/ml orally and as a 10 ml solution at 100 mg/ml intravenously) [56, 57], and FITC-dextran has been shown to be non-toxic at up to 18 times this dose in rats [56]. Comparable oral doses have been used in animals, corresponding to 20-250 mg/kg in piglets [27, 28] and up to 2.5 g/kg in rats [28]. FITC-dextran is stable in solution and shows no evidence of degradation into smaller molecular weight species [56, 58]. This has been demonstrated both *in vitro* in solution [56, 58] and *in vivo* in rabbits after intravenous injection [58]. Furthermore, when applied orally in animals, FITC-dextran is detected in tact in the blood stream and the uptake into blood is dependent on both the molecular size and the state of intestinal closure [27, 28]. Similarly, after oral ingestion in humans, FITC-dextran is observed in tact in urine, with negligible free fluorescein detected [57]. Together, these results indicate that any degradation of FITC-dextran in the stomach and small intestine is minimal (although some breakdown of dextran into smaller molecular weight carbohydrates is expected to occur in the large intestine [59]).

It is also noteworthy that dextran itself is approved for clinical use with intravenous delivery as a plasma expander [60]. However, serious anaphylactic reactions have been reported in response to dextran. These occur most frequently with dextran 70 (the largest molecular weight dextran used clinically) with rates estimated at between 1/2000 and 1/2700 patients [61, 62]. This corresponds to approximately twice the "background rate" of anaphylaxis reported in hospitalised patients [63]. Importantly, however, the doses of dextran used clinically are much higher than those proposed here – as a plasma expander, 15 g of dextran 70 are delivered in a 250 ml solution [60] and it is not uncommon that multiple sequential doses are used [61]. In addition, in these experiments, FITC-dextran will be delivered orally rather than intravenously, which will further mitigate the risk in three ways. Firstly, the rate of uptake into the blood stream will be much slower than with intravenous delivery (uptake is likely to occur over a timescale of several hours rather the 2-5 minutes recommended for intravenous delivery of dextran as a plasma expander [60]). Secondly, it is expected that there will be some digestion of large dextrans into smaller molecular weight carbohydrates by the bacteria in the large intestine [59], and it has been shown that smaller dextrans are less likely to induce anaphylaxis [61] (also see the use of dextran 1 to reduce allergic reactions to dextran 40 and 70: https://www.nps.org.au/medical-info/medicine-finder/promit-injection). Lastly, as discussed above for the specific case of fluorescein, serious adverse reactions to any potential allergens are much less likely with oral rather than intravenous delivery [34].

In the experiments proposed here FITC-dextran will be administered orally. In order to mitigate the risk, maximal doses of 1 g will be used. This corresponds to a dose that has previously been used safely in humans (using both intravenous and oral delivery) [56, 57] and is below the levels set out for oral delivery of fluorescein above. This dose is also well below the maximum

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allowable dose of dextran, which is approved for use at considerably higher concentrations under certain circumstances [60] (see discussion above). Prior to administration, FITC-dextran solutions will be sterilised for clinical use via filtration through 0.2 μm filters, in accordance with the preclinical protocol reported in reference [56]. This will ensure that any heavy metal constituents or endotoxins are only present in very low concentrations that are suitable for human use [56]. As such, the risks presented by the oral delivery of FITC-dextran have been minimised as far as possible.
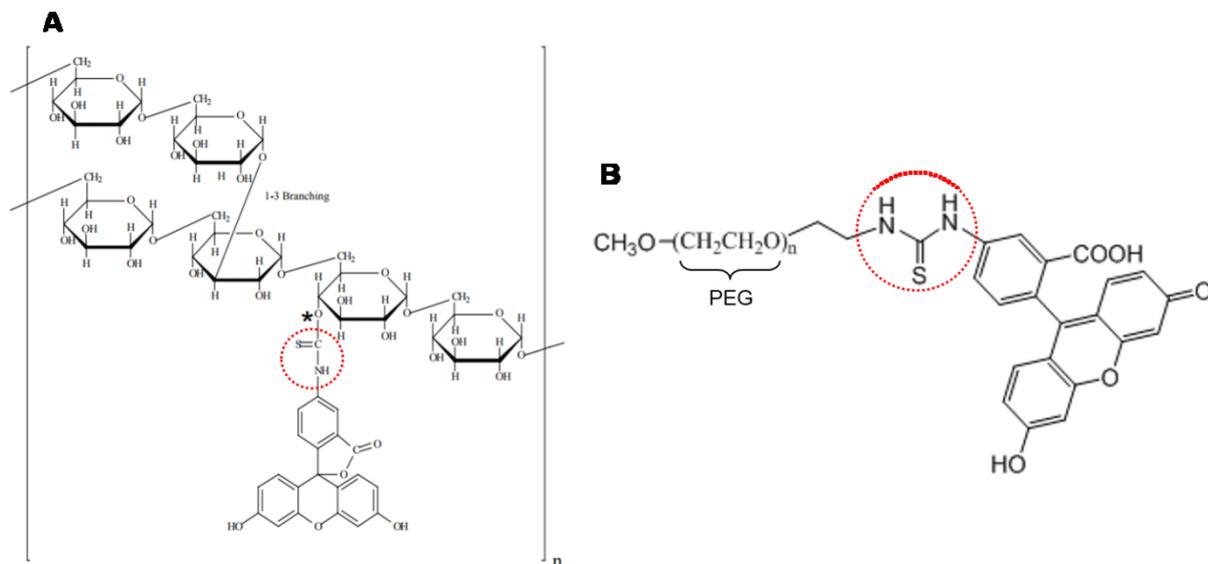
Figure 2. Molecular structures of FITC-dextran and FITC-PEG. (A) FITC-dextran. Asterisk (*) indicates that the location of the FITC binding is random and can occur at any hydroxyl group. **(B)** FITC-PEG. Dotted red circles highlight the binding between the FITC moiety and the dextran/PEG moiety. The binding is similar in both cases indicating that the stability of the two molecules (at the highlighted binding site) in any given environment is likely to be comparable.

FITC-PEG   At present, FITC-PEG is not approved for clinical use and it has not yet been used *in vivo* in humans. Despite this, it has been used widely in research studies for both cellular (e.g. [64-69]) and animal imaging (e.g. [70]) experiments, where the PEG molecule is often used as a linker such that fluorescein-based fluorescence imaging can be combined with an additional functionality (e.g. thermal tumour ablation, selective binding, etc.) using a single molecule. In these studies, FITC-PEG has been shown to have a low toxicity in cells (in cases where it is not linked to an additional toxic moiety for therapeutic or other purposes) [64, 68-70]. Furthermore, PEG itself is used clinically [71] – for both intestinal permeability measurements [72, 73] and as a laxative [74-76] – and is known to be inert, non-toxic and stable (i.e. it is not broken down by gut bacteria, nor it is metabolised by GI tissues) [73]. Despite this, there are limited reports of serious adverse events (including anaphylaxis) being induced by PEG (even with oral delivery) [77-80], however, such reactions are extremely rare. In FITC-PEG, the binding between the FITC moiety and the PEG moiety is almost identical to the binding between FITC and dextran in FITC-dextran (see Figure 2). Thus, it can be expected that FITC-PEG will be stable within the GI tract in the same manner that FITC-dextran is (see above). As such, FITC-

PEG can be considered safe for *in vivo* human use assuming that the concentrations of FITC and PEG are respectively kept below their maximum allowable doses. For this reason, we will use a maximum FITC-PEG dose of 1 g, and this will be administered orally as a solution. This corresponds to the level set out for FITC-dextran above – which has previously been used safely in humans with both oral and intravenous administration – and is also below the maximum allowable dose of fluorescein. In addition, it is well below the maximum allowable dose of PEG, which is approved for use at considerably higher levels (e.g. 10-20 g as a laxative) [72-76]. As with FITC-dextran, prior to administration FITC-PEG solutions will be sterilised via filtration through 0.2 μm filters to allow safe clinical use.

In experiments where two dyes (ICG + fluorescein or ICG + FITC-dextran/FITC-PEG) are administered simultaneously, the doses will be limited as described above. The aim of these experiments will be to determine whether both dyes can be detected simultaneously (through their differing excitation and emission profiles) and whether ratios of the fluorescence intensities of the two dyes can be used to provide a readout of permeability.

In experiments where fluorescein and FITC-dextran/FITC-PEG are administered in sequence, the FITC-dextran/FITC-PEG dose will be taken into account when calculating the maximum fluorescein dose. In this way, both the total fluorescein dose and the FITC-dextran/FITC-PEG dose will be kept within the limits described above. These experiments will serve to investigate whether ratios of the fluorescein and FITC-dextran/FITC-PEG fluorescence intensities can provide a readout of permeability. In this case, administration of the dyes will be performed in sequence (rather than simultaneously) as the spectral properties of the contrast agents are very similar.

Patient/subject selection
We aim to recruit patients/subjects for this study from three groups as detailed below.

Group 1   **Ophthalmology patients** who are receiving an intravenous dose of either fluorescein or ICG as part of their routine ophthalmic care (e.g. as part of a fluorescence angiography examination). These patients will take part in early tests (Stage 1) determining whether it is possible to detect intravenous fluorescent dyes at known concentrations using a transcutaneous measurement protocol.

Group 2   **Healthy subjects** will be split into two groups (2a and 2b) dependent on the aspect of the study they participate in.

2a: Healthy subjects with no known issues of increased gut permeability. These subjects will act as negative controls in all gut permeability studies. A subset of these volunteers will be asked to take two spectroscopic permeability tests – one with and one without a supplementary dose of a hyperosmotic solution, which acts to transiently increase intestinal permeability. This subset will provide proof-of-concept data prior to recruitment of patients in Group 3.

2b: Healthy subjects with no known issues of increased gut permeability or gut motility. These subjects will participate in GER experiments, which will provide complementary data to aid in the design of transcutaneous optical spectroscopy

as a non-invasive tool to assess gut permeability. Separate "healthy volunteer" sub-group will prevent the possibility of build-up of contrast agent.

Group 3   **GI and non-GI patients** who are expected to exhibit increased gut permeability (including, but not limited to: coeliac, IBD, HIV and liver disease patients). This group will represent the cases for the gut permeability study. Participants in this group who exhibit especially high intestinal permeabilities (as measured using control techniques such as PEG permeability assays or histology) will serve as positive controls.

Eligible participants will be identified by the regular clinical teams in the relevant departments (HIV and Hepatology outpatient clinic, GI outpatient clinic, ophthalmology clinic). If a patient agrees to participate in the study their details will be passed on to the research fellow directly involved in the study. Participants will be asked to provide written informed consent. Healthy volunteers will be recruited from Imperial and hospital staff.

Experimental protocols
The experimental protocols will entail giving patients a dose of contrast agent as described above and then making transcutaneous fluorescence measurements for up to three hours by placing the optical probe in contact with the skin. In the case of the 'mark I' bench-top spectrometer, the tip of the fibre probe will be secured using a 3D-printed clamp such that it is either in contact with the skin or held up to 2 mm above the skin's surface. A variety of measurement locations will be tested with this system in order to determine the most suitable for widespread deployment. The data from these studies will be used to guide the development of the casing for the miniaturised 'mark II' device (as well as the optical/mechanical/electrical design) such that it can be secured in the most appropriate body location. When using the miniaturised device, the entire system will be secured in place on the subject's skin using a custom-designed 3D-printed casing (e.g. on the fingertip, arm, earlobe or other location, as determined by the bench-top measurements). In all cases, the skin at the measurement location will be cleaned/sterilised using 70% alcohol swabs and the probe/sensor will be cleaned in the same manner both before and after use. The exact experimental protocols will vary depending on the phase of the investigation and the subjects will also be asked to take some supplementary tests to provide data for validation of the spectroscopic gut permeability assay. Thus, the protocols are broken down into three stages (each with its own specific aims), which are described below and summarised in the flowchart presented in Figure 3.

Stage 1   In the first stage of the experiments we will recruit ophthalmology patients who are due to receive intravenous doses of fluorescein or ICG as part of scheduled angiography examinations. Measurements in these patients will serve to test whether it is possible to detect fluorescein/ICG in the blood using transcutaneous fluorescence spectroscopy at the known concentrations used for ophthalmic angiography. Experiments will be performed at the same time as the patient's angiography meaning that they will require no additional hospital visits and no additional doses of fluorescent contrast agents (beyond that used for their eye examination). Before the fluorescent dye used for the angiography is administered (in accordance with the procedure recommended by the patient's ophthalmologist), the spectrometer will be attached to the patient's skin as described above. Background fluorescence data will be collected for five minutes before the patient receives their dose of fluorescein/ICG. Measurements will then continue for the duration of the angiography and for

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some time afterwards (up to a maximum total experimental time of three hours, depending on the availability of the patient). Throughout the experiment fluorescence spectra will be recorded at regular intervals (e.g. every 30 s). These measurements will be carried out using the 'mark I' device in the first instance and they may also be repeated using the 'mark II' device as part of its validation. With the 'mark I' device, the probe-skin distance will be varied and a range of body locations will be tested as measurement sites in order to determine the optimum probe position/orientation. These preliminary tests will serve to optimise the protocols used in Stages 2 and 3. In the event that it is not possible to recruit a sufficient number of ophthalmology patients, we will also consider recruiting healthy volunteers for the Stage 1 intravenous injection protocol. If the transcutaneous detection of dyes is unsuccessful in the first instance then we will explore the use of more sensitive detectors as well as alternative fibre-optic probe designs. We will also consider increasing the concentration of contrast agents given to the subjects, but this will of course be conditional on further REC/HRA approval.

Stage 2   The aim of the second phase of the experiments will be to validate transcutaneous fluorescence spectroscopy as a tool for the assessment of gut permeability. This requires two studies, and each will use different participants to prevent the possibility of build-up of contrast agent. Within the first study, we will recruit healthy volunteers (Group 2a) to take two spectroscopic gut permeability tests, the first under normal circumstances and the second with an additional dose of a hyperosmotic solution (which acts to transiently increase intestinal permeability). This will allow self-contained validation experiments to be performed in individuals (i.e. one healthy volunteer participating in two sequential fluorescence gut permeability tests, one with a dose of hyperosmotic solution and one without). In the first instance these volunteers will be given a combined oral dose of fluorescein and ICG as part of their spectroscopic gut permeability tests. This will permit measurements investigating the potential of each dye alone and the two dyes in combination to provide a readout of permeability (because the differing spectral properties will allow fluorescence to be recorded from both dyes simultaneously). If the fluorescein/ICG combination does not provide a suitable readout of permeability in the self-contained experiments in healthy volunteers, then we will recruit further healthy subjects and will investigate the use of alternative dye combinations and protocols (e.g. fluorescein followed by FITC-dextran/PEG or ICG combined with FITC-dextran/PEG). The order in which dye combinations will be tested is illustrated in Figure 4, and we note that new subjects will be recruited for tests with new dye combinations. Hence, no subjects will be asked to take more than two spectroscopic gut permeability tests (in Stage 2) and, therefore, no subjects will receive doses of more than two of the four fluorescent contrast agents (in any Stage).

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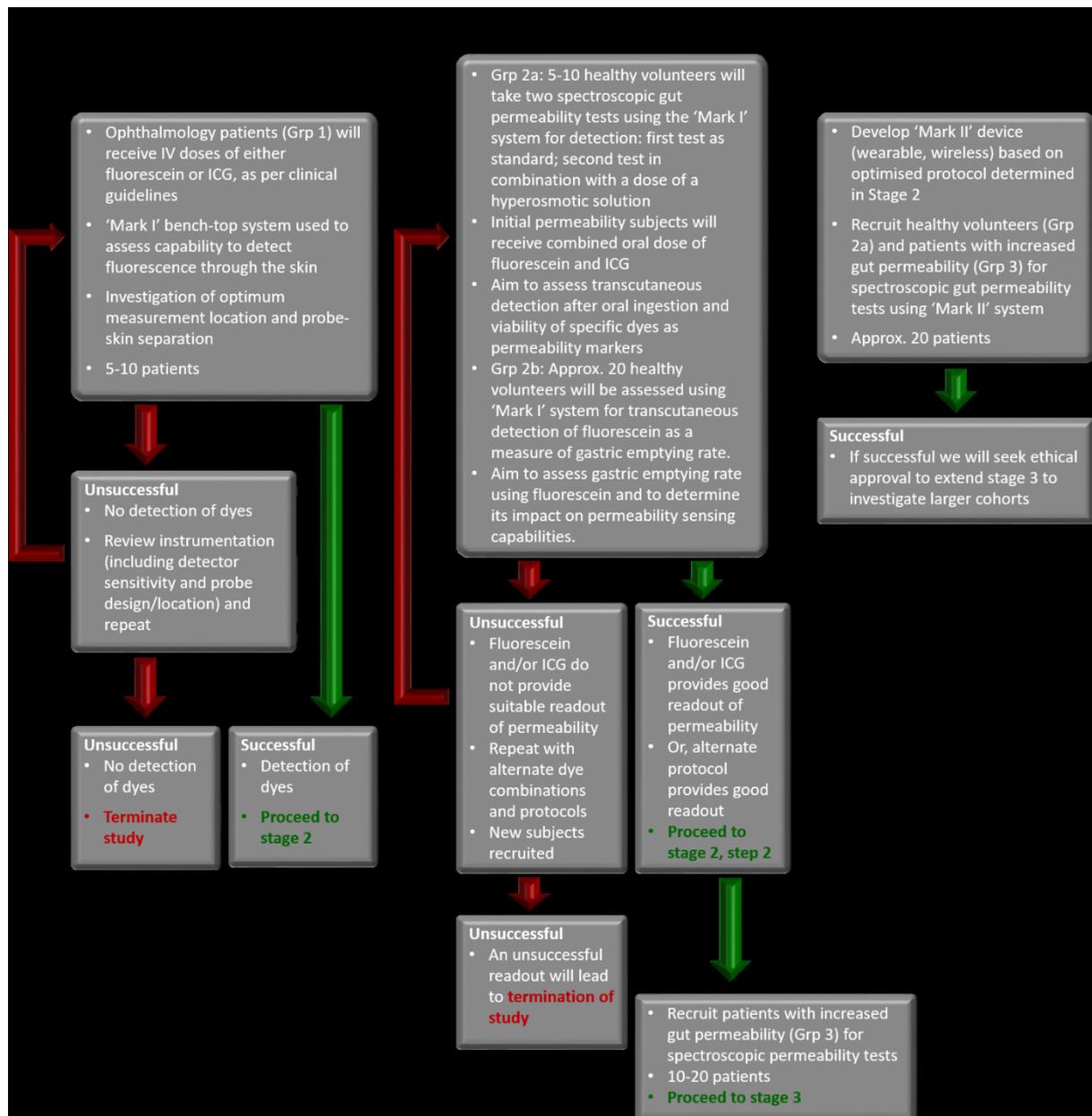
Figure 3. Flowchart describing experimental protocols and explaining the choice of contrast agents delivered at each stage of the investigation. The expected levels of recruitment are also given for each experimental step. Green arrows indicate progression through the protocol after successful experimental steps, red arrows indicate progression in the event of unsuccessful tests. Grp - group; IV - intravenous; ICG - indocyanine green.

During the above validation experiments in healthy volunteers (Group 2a) it will also be necessary to test and optimise the concentrations of the fluorescent contrast agents used. To do this while also minimising each individual volunteer's exposure to the dyes, we will begin with low doses of each dye and will only increase these doses if necessary (i.e. if the detected transcutaneous signals are too low to permit permeability sensing). For example, the first volunteer recruited to the study will receive a dose consisting of 500 mg

fluorescein and 1 mg/kg ICG (which are at the lower end of the values proposed in Table 1 and fall within the approved clinical limits for fluorescein and ICG). This volunteer will participate in two spectroscopic gut permeability tests (one without and one with a dose of hyperosmotic solution, as described above), even if no signal is detected in the initial experiment without the hyperosmotic solution. This is because increased permeability may have an effect on the dose required – i.e. at higher permeability it is likely that it will be possible to detect lower concentrations using the transcutaneous sensing setup (as more dye will leak from the gut into the blood stream). Based on the results from the first volunteer, the dose given to the second volunteer will be amended if necessary. If no (or very low) signal was observed (and if it cannot be reasonably expected that signal detection could be improved through changes to the spectrometer hardware) then the doses of fluorescein and/or ICG will be increased accordingly in a stepwise manner. If no signal was observed from either dye then the respective doses will be increased to 1 g fluorescein and/or 2 mg/kg ICG. The experiments in which the second volunteer participates will then be performed in an identical manner to those for the first volunteer. If these experiments also demonstrate a requirement for higher dye concentrations then in the following volunteers we will continue to increase the doses of fluorescein and/or ICG as necessary in steps of 500 mg and 1mg/kg respectively. This will continue until suitable concentrations are determined or until the maximum proposed dose levels are reached (see Table 1). Importantly, these dose escalation experiments will be performed in separate volunteers (i.e. they will be performed in a cohort not in an individual) so that individuals are not exposed to doses above those quoted in Table 1. Thus, in Stage 2, the maximum cumulative dose for any given dye will be twice that shown in Table 1, as volunteers will take no more than two spectroscopic gut permeability tests (Table 1 lists the maximum dose levels for individual tests). Group 2a and 2b will also be made up of different volunteers to prevent the possibility of contrast agent accumulation. Overall, these initial experiments will allow us to minimise the doses given to all future participants, thereby minimising the total exposure required throughout the study.

Once the optimal dose of fluorescein has be determined, we will recruit healthy volunteers (Group 2b) to take a spectroscopic gut permeability test, using fluorescein as the contrast agent, and a paracetamol absorption test (standardised protocol for GER assessment of liquids). This will allow self-contained validation experiments to be performed in individuals for GER (i.e. one healthy volunteer participating in two concurrent tests, one being a standardised method for GER measurement, and one being the transcutaneous fluorescence spectroscopy method). The paracetamol absorption method is a standardised protocol for GER assessment of liquids. As paracetamol is readily absorbed in the small intestine, and as GER is a rate-limiting step in substances entering the small intestine, the appearance of paracetamol in the blood provides an indirect measure of GER. Within this study, volunteers will arrive fasted having not consumed any food or drink (but water) in the last 12 hours. They will be given a combined oral dose of fluorescein (dose determined by the experiments discussed above) and paracetamol (1.5 g) within a 300-500 ml milk-based drink. Importantly, at these concentrations, all solutions are safe for human consumption. 1.5 g paracetamol is the recommend dose for

standardised GER measurements [81], and it represents 1.5x the usual dose used in home use. For each volunteer, blood samples (5 ml per sample) will be taken at regular intervals to allow subsequent quantification of the concentration of paracetamol in the blood (which will be used to determine GER). Samples will be taken over 4 hours. First, a baseline sample will be taken prior to consumption of the fluorescein/paracetamol dose. Then, for the first hour, blood samples will be taken every 10 minutes. Finally, for the remaining three hours, samples will be taken every 15 minutes. At the same time, measurements will also be made with the transcutaneous fluorescence spectrometer. The fibre probe will be attached to the arm of the participant and data will be collected at regular intervals (e.g. every 30 s). During the study, participants will be sat down and made comfortable.

Measurement of GER effects will be achieved by comparing the fluorescence data to the standardised measure of GER (paracetamol absorption test). Specifically, data showing the rate of uptake of paracetamol into the blood will be compared against the transcutaneous fluorescence signal (rate of uptake of fluorescein). This will provide insight into the rate of substance entrance into the intestine, which will aid in the understanding and design of transcutaneous fluorescence spectroscopy as a measure of gut permeability. In addition, it will demonstrate whether transcutaneous fluorescence spectroscopy will also be suitable for non-invasive assessment of GER.
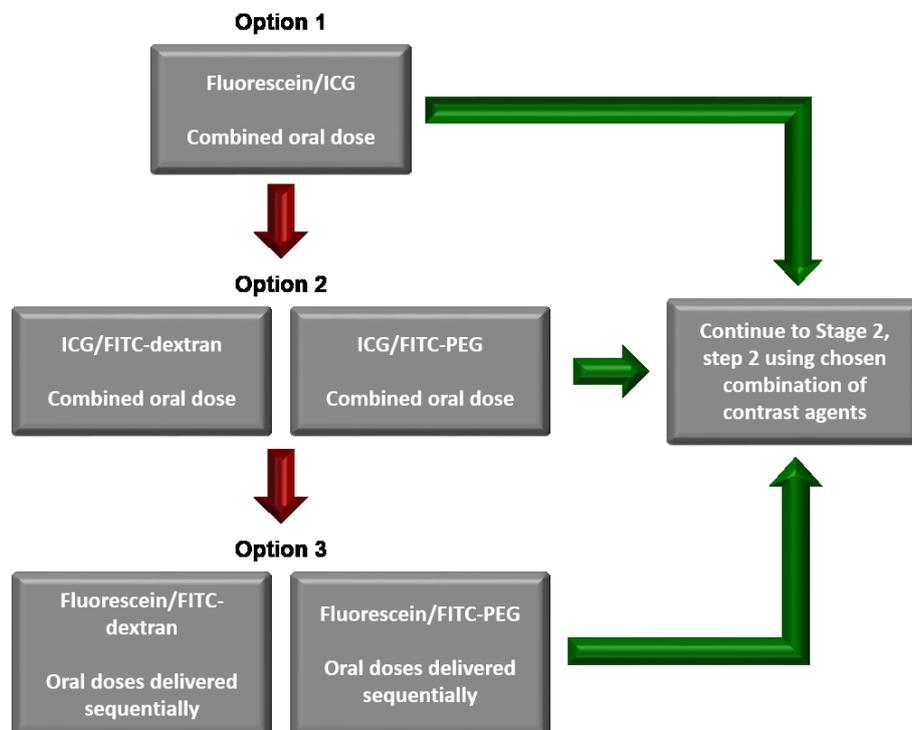
Figure 4. Flowchart illustrating the order in which combinations of fluorescent contrast agents will be tested in the first step of Stage 2 (i.e. in healthy volunteers taking spectroscopic gut permeability tests both with and without a dose of hyperosmotic solution). Green arrows indicate experimental progression after succesful tests of a dye combination, red arrows indicate progression through the experiment in the event of unsuccessful tests. ICG – indocyanine green; FITC – fluorescein isothiocyanate; PEG – polyethylene glycol.

In the permeability study (Group 2a), if it is necessary to test FITC-dextran and/or FITC-PEG (i.e. as described above and in Figures 3 and 4), then the doses of these agents will also be minimised as far as possible. The initial doses will be determined based on the results obtained with fluorescein and the maximum proposed doses for FITC-dextran/PEG (1 g). If it is possible to detect fluorescein after oral ingestion at doses of below 1 g, then the initial dose of FITC-dextran/PEG will be set at the level determined for fluorescein. If the minimum detectable dose of fluorescein is greater than or equal to 1 g then all FITC-dextran/PEG experiments will be performed using a dose level of 1 g (which is the maximum proposed dose for these dyes, as set out in Table 1). In the case that an initial dose of below the maximum value of 1 g is used, then this will be increased in a stepwise manner (as described above for fluorescein and ICG) if necessary. For FITC-dextran/PEG, doses will be increased in steps of 100 mg. As above, these dose escalation experiments will be performed in a cohort and not in individual volunteers. Once suitable minimum doses have been established these will be used in all further experiments, thereby minimising the overall exposure.

For all experiments (in all Stages of the protocol) and for all dye combinations/concentrations, measurements will be separated by a minimum of 24 hours so that it is possible to detect any adverse events that occur, even if they are delayed (by up to 24 hours). In the case that any serious adverse event occurs then a meeting of the Study Management Group will be triggered and all safety concerns will be discussed before any further experiments are performed (see Safety Monitoring section below for further details).

Once we have determined a suitable measurement protocol and combination (and concentration) of contrast agents in healthy volunteers (Group 2a), we will seek to recruit patients with GI (or other) conditions who are expected to exhibit particularly high intestinal permeabilities (e.g. untreated coeliac or IBD patients). These patients (Group 3) will serve as positive controls, and the technique will be further validated by comparing the data collected in patients with that collected in healthy volunteers (without doses of hyperosmotic solutions).

Throughout Stage 2, all subjects will be given oral doses of the fluorescent contrast agents and transcutaneous fluorescence measurements will be made using the 'mark I' bench-top device.

In all cases, once patients/volunteers have agreed to take part in the study, a time will be arranged for them to come into the hospital for measurements to be taken. On the day of their study visit, the subjects will first be asked a series of questions regarding their general details, medical history and current diet (including details of any medication they are taking and their current alcohol intake). Measurements will also be made of the subjects' height, weight and waist circumference, and we will ask for consent to access their medical records. At this point we will also ask female participants to take a pregnancy test and if this returns a positive result then they will be excluded from the study. Once the preliminary questions and measurements are complete, the optical

probe will be secured in contact with the skin and the subject will receive an oral dose of fluorescein, ICG, FITC-dextran, FITC-PEG or a combination thereof (with the doses limited as described in the 'Contrast agents' section). Where GER is measured (Group 2b), only fluorescein will be used as the contrast agent. Transcutaneous fluorescence measurements will be recorded at regular intervals (e.g. every 30 seconds) for up to 3 hours (although longer experiments may be considered in patients who are expected to exhibit slow intestinal transit and/or slow gastric emptying), beginning five minutes before the contrast agent is administered in order to allow collection of a baseline (for Group 2b, the time-course detailed above will be used). During the experiment, two 5 ml blood samples will also be taken – one prior to administration of the contrast agent and one at the point at which the peak fluorescence intensity is detected – in order to allow correlation of the fluorescence measurements with the blood concentration of the fluorophores. For the same purpose, two urine samples will also be collected – one before administration of the contrast agent and one at the end of the experiment. The blood/urine concentrations will be measured using a laboratory-based spectrofluorometer (as described in [27]) after the transcutaneous fluorescence procedure is complete. Some of the collected blood and urine may also be used to perform other tests, for example to assess the degree of bacterial translocation.

During the GER experiments (Group 2b), the blood sample regime detailed above (page 22) will be used, which is in line with standardised methods for paracetamol absorption testing. These blood samples will be analysed for paracetamol concentration by North West London Pathology (Imperial College Healthcare NHS trust), and no additional blood (or urine) samples will be taken for the purposes of assessing concentration of fluorescent dyes or bacterial translocation.

Following the spectroscopic gut permeability tests, Group 2a/3 subjects will be asked to make two further contributions to the study. Firstly, they will be asked to provide one or more fresh stool samples within approximately 48 hours of their study visit. These will be analysed to determine the concentration of fluorescent dye in the stool (which will provide an indication of intestinal transit time) and may also be assessed with the aim of identifying and quantifying the microbiota. Secondly, Group 2a/3 subjects will be asked to take a PEG permeability test, which will act as a 'gold standard' measurement against which we will compare our spectroscopic permeability readings. Subjects will be given a specific collection kit that will allow them to take the PEG permeability test at home at a convenient time. The test entails drinking a 250 ml aqueous solution of PEG molecules of varying molecular masses and then collecting urine for the following six hours. The urine sample is then shipped to a laboratory for analysis using prepaid packaging provided by the research team.

In patients who have had or are due to have an intestinal biopsy as part of their clinical care, we will ask for consent to use any tissue from the biopsy that is surplus to diagnostic requirements for further analysis in our study. This will allow correlation of the spectroscopic gut permeability test with histological measurements of epithelial damage or permeability (e.g. quantification of the efficacy of the tight junctions via Ussing Chamber measurements).

Finally, as discussed above, in the subset of Group 2a (healthy subjects) recruited at the beginning of Stage 2, we will ask the volunteers to take the spectroscopic gut permeability test twice. In this group, the first fluorescence gut permeability test will be performed as described above. In the second test, however, the volunteers will receive a dose of the chosen fluorescent contrast agent(s) in combination with a hyperosmotic solution that will act to transiently increase their gut permeability (i.e. in a similar manner to that described in references [57, 82]). Thus, by comparing the results in these subjects with and without the hyperosmotic solution, we will be able to validate the capability of the spectroscopic test to monitor changes in intestinal permeability. Interestingly, this validation will be possible without the requirement for additional 'gold standard' measurements (such as PEG permeability tests or histopathology, as discussed above) as each subject will act as their own internal control. A range of hyperosmotic solutions will be investigated, including sucrose, glucose, glycerol and sodium chloride, with maximum doses of 60 g, 30 g, 15 g and 5 g (in 100 ml of water) respectively. Importantly, at these concentrations, all solutions are safe for human consumption (all are widely used in the food industry – for example, a 500 ml bottle of Coke contains approximately 50 g of sugar) and can be expected to instigate temporary alterations in intestinal permeability [57, 82]. These self-contained experiments in individual healthy volunteers will be performed before measurements in patients in order to provide a preliminary validation of the technique (and to determine the most valuable experimental protocol and combination of contrast agents).

Overall, the aim of Stage 2 is to develop, optimise and validate the spectroscopic assay proposed in this protocol as a readout of gut permeability. This will involve determining the optimum combination and dose of contrast agents, controlling for GER effects, and investigating data analysis/processing approaches to ascertain which offer the optimal permeability readouts. This will be achieved by testing a range of contrast agent combinations and analysis protocols, and assessment of GER effects will be achieved by comparing fluorescein in relation to a standardised measure of GER (paracetamol absorption test). Measurements will first be performed in healthy volunteers taking two spectroscopic gut permeability tests (one with and one without a dose of hyperosmotic solution) and later in patients expected to exhibit increased intestinal permeability. In both cases, the data acquired will be compared to one or more of the 'gold standard' measurements discussed above.

Stage 3   Based on the data collected in Stage 2, we will design and develop an optimised, wearable device ('mark II') – along with an optimised experimental protocol – suitable for larger scale studies. This device and protocol will then be applied to disease studies aimed at: (i) demonstrating the capability of the optimised spectroscopic gut permeability assay to accurately monitor a wide range of permeabilities; and (ii) correlating spectroscopic measures of permeability to disease state (via comparison to the 'gold standard' markers discussed above). The experimental protocol used will be identical to that described for Stage 2, but the use of the 'mark II' device and the optimised

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procedure (which will most likely entail a much shorter measurement duration) will allow for measurements in considerably larger cohorts. We will aim to recruit patients expected to exhibit increased gut permeability with a variety of diseases. This will include (but will not necessarily be limited to) inflammatory bowel diseases (such as Crohn's disease and colitis), coeliac disease, fatty liver disease, and HIV. In cases where patients receive a treatment/therapy that begins after their planned spectroscopic gut permeability test, we will also seek consent to make multiple longitudinal measurements to allow us to determine whether this approach can be used to monitor the impacts of interventions. At this stage of the experimental program it is possible that we will have the opportunity to extend the level of recruitment beyond that set out in the Sample Size & Statistical Analysis section. If this is the case then we will seek further ethical approval – via an amendment to this protocol – before recruiting additional participants. Overall, the experiments performed in Stage 3 will serve to demonstrate the utility of the spectroscopic gut permeability assay in a wide array of prevalent disorders.

Schedule of procedures

As described above, participants will take part in a series of procedures as part of this study, with some carried out in the hospital by the study team and others undertaken at home by the subjects/patients themselves. The procedures will vary depending on the stage of the investigation and the patient/subject group. Thus, the table below shows and describes the procedures that will be performed for each patient group.

Table 2. Description of study procedures. The column labelled 'Group' indicates which patient groups (see 'Patient/subject selection' above) will participate in each procedure. 1 – Ophthalmology patients; 2 – healthy volunteers; 3 – GI and non-GI patients with expected increased gut permeability.

PROCEDURE	DESCRIPTION	VISIT	GROUP
Informed consent	The Study Doctor will discuss the Patient Information Sheet and Informed Consent Form with the patient and will answer any questions. Once the patient feels satisfied that their questions have been answered and feels certain that they want to join the study, the doctor will ask them to sign the Informed Consent Form. At this point we will also arrange a time for the patient to come in and participate in the study (the study visit).	Consultation visit (prior to study)	1, 2a, 2b, 3
Inclusion/exclusion criteria	The Study Doctor will go through the inclusion and exclusion criteria to ensure that the patient is eligible to join the study by asking questions regarding medical history.	Consultation visit (prior to study)	1, 2a, 2b, 3
Pregnancy test	Before taking any further part in the study, female volunteers will be asked to take a pregnancy test. If this returns a positive result then they will be excluded from the study. This step will only be performed in Stages 2 and 3. In Stage 1, inclusion of pregnant women will be at the discretion of the patient's ophthalmologist.	Study visit	2a, 2b, 3
General, medical and dietary questions;	Subjects will be asked a few questions about their general details and medical history, including any medication they are taking. Height, weight and waist circumference will be	Study visit	1, 2a, 2b, 3

PROCEDURE	DESCRIPTION	VISIT	GROUP
measurements of height, weight, etc.	measured. Subjects will also be asked some questions about their diet, including current alcohol intake.		
Consent to use tissue from intestinal biopsy	If patients in group 3 have had or are due to have an intestinal biopsy as part of their clinical care, we will ask for their consent to use any surplus tissue from the biopsy in our study. This will allow us to correlate the results of the spectroscopic gut permeability test with histological markers of tissue damage or increased intestinal permeability.	Study visit	3
Intravenous injection of contrast agent: fluorescein or ICG	Ophthalmology patients will receive an injection of either fluorescein or ICG, as prescribed by their ophthalmologist. The dye and dose used will be exactly the same as for their normal angiography examination.	Study visit	1
Oral administration of contrast agent: fluorescein, ICG, FITC-dextran and/or FITC-PEG	Group 2a and 3 volunteers will receive an oral dose of one or more fluorescent dyes (fluorescein, ICG, FITC-dextran and/or FITC-PEG). This will be administered as a single solution of approximately 300 ml (similar to the size of a can of soft drink) for the subject to drink. Group 2b volunteers will receive an oral dose of fluorescein. This will be administered as a single solution in a 300-500 ml milk-based drink that also contains 1.5 g paracetamol.	Study visit	2a, 2b, 3
Spectroscopic gut permeability test	We will attach a small device (spectrometer) to the arm or fingertip of the subject. This device will be used to make measurements of the amount of contrast agent in the blood stream, through the skin. The device is comfortable to wear and will not break, damage or hurt the skin in any way.	Study visit	1, 2a, 2b, 3
Oral dose of hyperosmotic solution	A subset of the healthy volunteers will receive an oral dose of a hyperosmotic solution (which will act to temporarily increase their gut permeability) as part of a second spectroscopic gut permeability test. This will take place at a second study visit. Other than the dose of the hyperosmotic solution (which will consist of sucrose, glucose, glycerol or sodium chloride), the procedure will be identical to the volunteers' first spectroscopic gut permeability test.	Second study visit	2a
Blood samples	Group 2a: We will take two 5 ml blood samples during the tests (equivalent to about one teaspoon of blood for each sample). The first will be taken before the measurements begin and the second will be taken during the measurements. Group 2b: For each participant, we will take nineteen blood samples (up to 5 ml) over the course of 4 hours. One sample will be taken at baseline, followed by one every 10 minutes for the first hour, then one every 15 minutes for the final 3 hours.	Study visit	2a, 2b, 3

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PROCEDURE	DESCRIPTION	VISIT	GROUP
Urine samples	We will ask participants to provide two urine samples. The first will be collected before the measurements begin and the second will be collected at the end of the measurements.	Study visit	2a, 3
Adverse events	Participants will be asked if they are undergoing or have experienced any adverse events (such as any problems, symptoms or discomforts).	During and after study visit	1, 2a, 2b, 3
Stool sample	Within 48 hours of the Spectroscopic gut permeability test we will ask subjects to provide one or more fresh stool samples, which will be used for further analysis.	2 days after study visit	2a, 3
PEG gut permeability test	Finally, subjects will be asked to take a PEG permeability test sometime in the week following their study visit. This can be carried out at home at the convenience of the participant, and they can administer the test themselves. We will provide a collection kit to perform the PEG permeability test, which will involve drinking a solution and then collecting urine for the following 6 hours. Subjects will then post the collected urine sample to a laboratory for analysis using prepaid packaging. We will ask that subjects try to perform the PEG permeability test within 1 week of their study visit.	2+ days after study visit	2a, 3

Safety Monitoring

The Study Management Group will also act as a Safety Monitoring Committee for all experiments proposed in this protocol. The group is well suited for this purpose as it contains experts in intestinal health, liver health, ocular health (where fluorescein and ICG are widely used) and laser safety. The group will meet on a monthly basis to review experimental results and, when necessary, to take decisions as to whether to advance to the next experimental stage/step (as described in Figures 3 and 4). In these meetings, any and all safety concerns will also be discussed. If any non-serious adverse events (see definitions in Adverse Events section below) have been reported then these will be discussed in detail and decisions will be taken with regards to the specific experimental procedures used in the case of the adverse events – i.e. the committee will decide whether a particular procedure (e.g. a particular dye combination/concentration or a particular laser power / measurement location) presents an unexpected and unacceptable risk. In this situation the committee will discuss whether it is sensible to move on to an alternative experimental protocol, to perform further measurements using the same protocol, or to terminate the study completely (i.e. as per the flowcharts shown in Figures 3 and 4).

In the case that a serious adverse event is reported, then all measurements/experiments will be temporarily postponed with immediate effect and an exceptional meeting of the Study Management Group will be triggered. At this meeting the committee will discuss the serious adverse event in detail (as well as any other safety concerns that have arisen since the previous meeting) and will determine whether it is safe to proceed with further experiments or whether termination of the study is necessary.

Patient recruitment

Potential participants will be approached by members of their healthcare/clinical team. This will either be in the outpatient department or prior to their appointment by telephone. For those

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patients approached on the phone, patient information sheets will be sent to them in the post prior to their outpatient appointment. All patients who agree to see the study team will then be provided with an information sheet (if they were not already sent one) and given an opportunity to discuss their involvement in the study after their clinic appointment. Informed consent will be taken by trained (GCP accredited) clinical members of the study team after a minimum of 24 hours has elapsed from the time at which the study information was provided.

Healthy volunteers will be recruited from Imperial College and St. Mary's Hospital staff. Potential healthy volunteers will be approached in person by members of the research team or recruited via advertisements, which will be displayed around Imperial College campuses and circulated to staff/students via e-mail (attached separately). Study information will then be provided as described above and informed consent will be taken by trained (GCP accredited) clinical members of the study team (a minimum of 24 hours after the study information was provided).

Subjects taking part in Stages 2 and 3 of this research program will be offered a fixed financial incentive of £20 to encourage participation in the study. Subjects will receive this fee upon completion of all aspects of the study in which they agreed to take part. Transport costs will not be reimbursed due to the limited level of funding. Volunteers participating in Stage 1 of the study will not be offered financial incentives as the impact on subjects is much smaller in Stage 1 than it is in Stages 2 and 3. This information will be made available to potential participants in the participant information sheets.

INCLUSION CRITERIA

- Ability to give informed consent
- Aged 18 years or above
- No evidence of prior adverse reactions to fluorescein, ICG, dextran or PEG (dependent on experiment)
- No evidence of prior adverse reactions to iodine (for ICG experiments only)
- *For healthy volunteers*: healthy with no active GI/liver disease (or other condition in which increased gut permeability is expected, e.g. HIV) and no antibiotics taken within the previous four weeks.
- *For cases*: exhibiting symptoms of GI, liver or other diseases (e.g. HIV) in which increased intestinal permeability is expected.
- *For ophthalmology patients recruited in Stage 1*: healthy (i.e. as described above for healthy volunteers) and prescribed to have an ophthalmic angiography with an intravenous injection of either fluorescein or ICG.

EXCLUSION CRITERIA

- Unable to give informed consent
- Aged <18 years
- Previous adverse reaction to fluorescein, ICG, dextran or PEG (Group 1, 2a, 3)
- Previous adverse reaction to fluorescein (Group 2b)
- Known allergy to iodine (for ICG experiments only)
- Pregnancy (in Stage 1 this will be at the discretion of the patient's ophthalmologist)

- Breastfeeding (in Stage 1 this will be at the discretion of the ophthalmologist)
- Known allergy to paracetamol (Group 2b only)
- Known allergy to ingredients in milk-based drink (i.e. dairy, nuts, gluten) used in gastric emptying experiments (Group 2b only)

WITHDRAWAL OF SUBJECTS

Subjects are free to withdraw consent for inclusion in the study at any time and, where requested, their samples/data will be removed from further study and destroyed.

ADVERSE EVENTS

Definitions

Adverse event (AE): any untoward medical occurrence in a patient or clinical study subject.

Serious adverse event (SAE): any untoward and unexpected medical occurrence or effect that:

- **Results in death**
- **Is life-threatening** – *refers to an event in which the subject was at risk of death at the time of the event; it does not refer to an event which hypothetically might have caused death if it were more severe*
- **Requires hospitalisation, or prolongation of existing inpatients' hospitalisation**
- **Results in persistent or significant disability or incapacity**
- **Is a congenital anomaly or birth defect**

Medical judgement should be exercised in deciding whether an AE is serious in other situations. Important AEs that are not immediately life threatening or do not result in death or hospitalisation but may jeopardise the subject or may require intervention to prevent one of the other outcomes listed in the definition above, should also be considered serious.

Reporting procedures
All AEs should be reported. Depending on the nature of the event the reporting procedures below should be followed. Any questions concerning adverse event reporting should be directed to the Chief Investigator in the first instance.

Non-serious AEs
All such events, whether expected or not, should be recorded.

Serious AEs
An SAE form should be completed and sent to the Chief Investigator within 24 hours. However, relapse and death due to a pre-existing condition, and hospitalisations for elective treatment of a pre-existing condition do not need reporting as SAEs.

All SAEs should be reported to the London – Bromley REC where in the opinion of the Chief Investigator, the event was:

- 'related', i.e. resulted from the administration of any of the research procedures; and
- 'unexpected', i.e. an event that is not listed in the protocol as an expected occurrence.

Reports of related and unexpected SAEs should be submitted within 15 days of the Chief Investigator becoming aware of the event, using the NRES (National Research Ethics Service) SAE form for non-IMP (investigational medicinal product) studies. The Chief Investigator must also notify the Sponsor of all SAEs.

Local investigators should report any SAEs as required by their Local Research Ethics Committee, Sponsor and/or Research & Development Office.

Contact details for reporting SAEs

Sponsor   Imperial College Joint Research Compliance Office – jrco@imperial.ac.uk.

Chief Investigator   Dr Alex Thompson – alex.thompson08@imperial.ac.uk, +44 (0) 20 3312 5035.

SAMPLE SIZE & STATISTICAL ANALYSIS

We aim to recruit 70 subjects to this study. We expect this to be an achievable number as we will have access to patients through the Endoscopy unit and the Hepatology and HIV clinics at St Mary's Hospital as well as the Ophthalmology clinic at Western Eye Hospital.

This will include a total of 40 subjects with no suspected intestinal health issues. This subset will be made up of ophthalmology patients undergoing fluorescein or ICG angiography and healthy volunteers. We will aim for 5 of these patients to receive intravenous doses of fluorescent contrast agents (ophthalmology patients) and for the remaining 35 (healthy volunteers) to be given oral doses (Group 2a – 15 volunteers; Group 2b – 20 volunteers). The measurements on subjects receiving intravenous dyes will serve to allow a simple test as to whether the fluorescence signal can be detected in a transcutaneous arrangement. The measurements on subjects receiving oral doses will act as the healthy control measurements in which intestinal permeability is expected to be normal. As discussed above, some healthy volunteers in Group 2a will take the test twice in order to assess repeatability or to validate the ability to detect changes in permeability (with doses of hyperosmotic solutions used to induce temporary increased permeability).

A further 30 patients will be recruited who are expected to show increased intestinal permeability. These patients will have either GI conditions or non-GI conditions such as HIV and liver disease for which increased intestinal permeability is expected.

The study will serve to answer a number of clinical questions including whether transcutaneous fluorescent signals can be detected after intravenous and oral administration of contrast agents and whether these signals correlate to traditional (either direct or indirect) measures of gut permeability (i.e. PEG-based permeability assays or histological assessments). The most important clinical question, however, is whether the fluorescence-based permeability assay

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can be used to differentiate between subjects with normal intestinal permeability and those with increased permeability. Therefore, we estimated the number of patients required to answer this question with a statistical power of over 80% and an alpha value of below 0.05. This calculation requires knowledge of the mean values and standard deviations that will be obtained using the fluorescence permeability assay. As these are currently unknown (because this is a new test), we used previously reported L:M (lactulose:mannitol) ratios to estimate the power and alpha values. L:M ratios are likely to provide a similar readout to the fluorescence permeability assay as we will be investigating differences (ratios) between the fluorescence intensities of small (fluorescein/ICG) and large (FITC-dextran/FITC-PEG) fluorophores. Thus, the L:M ratio (which measures the relative urinary recovery of one large and one small sugar molecule) can be considered analogous to the fluorescence assay, and previously reported L:M ratios should serve as a suitable substitute in these calculations.

In healthy patients with normal intestinal permeability, L:M ratios are typically on the order of 0.01-0.02 [83, 84]. In patients with coeliac disease and Crohn's disease, increased L:M ratios have been reported, corresponding to 0.105 [84] and 0.085 [83] respectively. Considerable intra- and inter-patient variation is observed in both healthy volunteers and patients with increased intestinal permeability, with standard deviations of up to approximately 100% of the mean values having been reported [83, 84].

Using the patient numbers and L:M ratios above, we can calculate the statistical power for a comparison of the means of two samples (i.e. healthy volunteers vs. patients with increased permeability). With 45 patients (15 healthy, 30 increased permeability), following the calculation described at http://powerandsamplesize.com/Calculators/Compare-2-Means/2-Sample-Equality, we find that an alpha value of 0.05 and a statistical power of above approximately 80% can be obtained even when using the highest observed standard deviations. For example, using a standard deviation of 0.085 for both the normal permeability and increased permeability cohorts, with L:M ratios of 0.021 and 0.085 respectively (i.e. as reported by Andre et al. [83], with standard deviations for both cohorts estimated as 100% of the increased permeability mean), we obtain an alpha value of 0.05 and a statistical power of 79.2%. Using lower standard deviations or greater differences between the two means provides even higher statistical powers. This represents acceptable statistical significance and indicates that with 45 patients (assuming the means and standard deviations above) we would be able to confidently reject the null hypothesis that the mean permeabilities of the healthy and increased permeability cohorts are in agreement (H$_0$: $m_a - m_b = 0$) – i.e. we would be able to confirm that the fluorescence-based gut permeability assay was capable of detecting changes in intestinal permeability.

Of course, the actual mean values and standard deviations observed when using the fluorescence-based permeability assay will only be determined once the experiments commence. However, the above estimates represent acceptable statistical parameters, suggesting that the study will be capable of producing statistically significant results based on the proposed level of recruitment (assuming that the mean values and standard deviations obtained with the fluorescence-based assay are comparable to those reported in L:M tests). Furthermore, the patients recruited to this study as cases will naturally exhibit a range of permeabilities and it may be possible to use the fluorescence assay to differentiate between subgroups within the increased permeability cohort based on a variety of factors (e.g. disease type, disease state, treatment duration, etc.). While these are important research questions, we have based the above calculation of statistical power solely on the experiments designed to provide initial validation of the technique, as this is the key aim at this stage of the research

program. If differences are observed between subgroups that are not statistically significant at this level of recruitment then we will later seek to recruit additional patients (through an amendment to this protocol) to examine these phenomena in detail. Similarly, if it is not possible to obtain statistically significant results at the proposed level of recruitment because it is necessary to test multiple different dye combinations (i.e. as shown in Figure 4) before a suitable permeability readout is obtained, then we will also seek ethical approval to recruit additional subjects through an amendment to this protocol. In the first instance, however, 50 subjects represents a level of recruitment that we believe is feasible within the timescale of the funding for this research program and that has the potential to provide statistically significant findings as described above.

For GER experiments, the aim of the measurements is to assess the impact of GER on the ability to measure intestinal permeability and to perform a preliminary validation of transcutaneous fluorescence spectroscopy as a tool to quantify GER (by comparing collected fluorescence data against information from paracetamol absorption tests). As above, the mean values and standard deviations that will be obtained are unknown (as this is a new test), which means that suitable data are unavailable for power calculations. Therefore, a sample size of 20 healthy volunteers for the GER experiments (Group 2b) was chosen based on typical samples sizes used in previous validation studies for alternative techniques (e.g. see the review of paracetamol absorption test validation studies presented by Willems *et al.* [81]).

Statistical analysis will be performed using Matlab, and professional statistical advice will be sought where necessary.

DATA COLLECTION, DATA HANDLING & RECORD KEEPING

Explicit consent for access to medical records by members of the research team will be gained. Transfer on magnetic/optical media or networks will only be in encrypted form, according to local NHS ICT protocols. Where data is stored on NHS or Imperial College computers, appropriate access controls will be in place to ensure that access to confidential research information is restricted to those who need it. Paper records (consent forms etc.) will be stored securely on NHS premises. This will be within a locked filing cabinet or cupboard in a locked office to which only the senior research team has access.

The Data Protection Act and Caldicott principles will be adhered to at all times.

Data will be pseudo-anonymised as soon as possible. Data recorded on the case record form will be identified by a unique reference number. This will only be linked to the individual patient separately, in a secure database held by the recruiting clinicians. Samples, case record forms and other trial documentation will be labelled only with this unique identifier.

Identifiable patient data will only be stored on secure computers which may only be accessed by the clinicians involved in the patients' clinical care. A unique identifying numerical code – which is distinct from the NHS number or hospital record number – will be assigned to each record. This unique identifier will be used for all research data stored on investigators' computers. This pseudo-anonymised data will be kept on NHS and University computers. Such data will be encrypted to the local ICT requirements.

Only members of the research team who hold a relevant NHS Trust contract will have access to the medical records of those who agree to participate. Explicit consent for this access will

be sought from each participant on the consent form. Information gleaned from such access will remain entirely confidential, and will only be recorded anonymously in study records.

It is Imperial College policy that all data relating to research, including consent forms, are kept for 10 years.

SAMPLE STORAGE

Any samples collected from patients will be handled and processed in line with relevant biosafety regulations. In the case of samples collected from HIV-infected patients, all processing will be undertaken within a category 3 laboratory.

All samples (except those from Group 2b, where blood samples will be processed by an NHS pathology laboratory and then destroyed) will be registered and stored as a sub-collection of Imperial's Tissue Bank. This Tissue Bank fully conforms to HTA regulation. All freezers in the unit are constantly monitored by T-Scan alarm systems and are fully secured. Access to samples is by permission of the study CI. Freezers are locked and are kept within rooms with pin code entry systems.

At the end of the research, samples where consent allows for future research use will be transferred into the Biobank, which operates under the licence of Imperial College.

STOPPING/DISCONTINUATION RULES

The study is due to continue for four years. It is anticipated that healthy subject and ophthalmology patient measurements will be undertaken in the first year, while measurements on GI patients and other non-GI patients with increased gut permeability will continue for the duration of the study. There are no specific stopping rules as this is primarily a feasibility study investigating the novel gut permeability assay.

RESEARCH GOVERNANCE, MONITORING, ETHICS AND R&D APPROVAL

The study will be conducted in compliance with the Research Governance Framework for Health and Social Care and Good Clinical Practice. The study will be conducted in accordance with the approvals of the Research Ethics Committee and the Joint Research Compliance Office of Imperial College London & Imperial College Healthcare NHS Trust.

Indemnity
Imperial College London holds negligent harm and non-negligent harm insurance policies, which apply to the experiments outlined in this protocol.

Sponsor
Imperial College London will act as the main Sponsor for this study. Delegated responsibilities will be assigned to the NHS trusts taking part in this study.

Funding

PUBLICATION POLICY

Results of the study will be disseminated by conference presentation and peer reviewed journal publication. This will be in open access formats wherever possible.

Imperial College London

Imperial College Healthcare NHS
NHS Trust

APPENDIX A

Optical setup and laser safety considerations for bench-top fluorescence spectrometer ('mark I')

The optical setup for the bench-top fluorescence spectrometer ('mark I') used in the first stages of these experiments is shown in Figure 1. The two laser sources have wavelengths of 488 nm and 785 nm, which permit excitation of fluorescence from fluorescein/FITC and ICG respectively. The output from the two lasers are combined using a dichroic beamsplitter and coupled into the excitation channel of the fibre probe. This delivers the excitation light to the measurement site and the fluorescence is then collected by the detection channel of the probe and routed back to the spectrometer via an emission filter (in order to reject any directly scattered excitation light). The fibre probe is electrically insulating to avoid the risk of electric shock to the subject or user. All optical components are securely mounted on an optical breadboard and are contained within a light-tight box such that the only point at which laser radiation is emitted is at the tip of the fibre probe. Neutral density filters are positioned directly in front of the two lasers to limit the optical power to a level at which the maximum intensity that can be achieved at the distal end of the probe (i.e. when light is optimally coupled into the optical fibre) is below the maximum permissible exposure for the skin [31-33]. For both excitation wavelengths, this exposure limit will vary depending on the distance of the probe from the skin. A range of probe-skin distances will be tested in initial validation experiments to determine the optimal distance for spectral permeability measurements, and for each known distance the power will be limited accordingly. When the probe is in contact with the skin the power will be limited most stringently. In this case, the optical powers at the fibre probe output will be restricted to approximately 63 µW at 488 nm and 93 µW at 785 nm. The maximum distance from the skin at which the fibre probe will be secured will be approximately 2 mm. At this distance the excitation light will illuminate a circle of 600 µm diameter – i.e. it will fully illuminate the surface area from which the detection fibres collect light (see Figure 1). In this arrangement, as the light is spread over a larger area, the maximum allowable power at the distal tip of the probe increases to approximately 565 µW at 488 nm and 836 µW at 785 nm. For other fibre-skin separations the maximum permissible power will be calculated in the same manner and the laser outputs limited accordingly. Importantly, even with the highest power levels discussed above (i.e. at the maximum probe-skin separation), the light that is output from the fibre probe will also be below the maximum permissible exposure for the eye when the tip of the probe is held at a distance of >10 cm [31-33] (assuming a blink reflex of <0.5 s). Thus, in this configuration (with the light-tight box closed), the laser system is eye-safe and can be operated without the use of laser safety goggles. In addition, the system has an interlock that cuts out the laser emission if the lid is removed. This ensures that the system can be safely operated by non-expert users and allows it to be classified as a Class 1 laser device. Any re-alignment work is performed by expert laser users only and is carried out in a separate locked room with blacked out windows. In this case, the interlock is overridden by the expert user and alignment is performed while wearing appropriate laser safety goggles, in accordance with local risk assessments. For all clinical measurements, the interlock is engaged and the lid is closed such that the laser system is eye-safe, can be classified as a Class 1 laser product, and can be operated by non-expert laser users without risk of injury.

APPENDIX B

Discussion of design and safety considerations for the miniaturised ('mark II') fluorescence spectrometer

A miniaturised, wearable fluorescence spectrometer will be developed and validated as part of this study, with the aim of deploying this 'mark II' system to larger scale studies of gut permeability. This wearable monitor will use LEDs and PDs for excitation and detection of fluorescence. While the exact optical design of the device will be guided by the information collected with the 'mark I' bench-top system, a likely approach will involve the use of two LEDs for excitation of fluorescence (centre wavelengths of approximately 488 nm and 785 nm) and four PDs for detection. In this arrangement, optical filters will be placed in front of each of the PDs such that they specifically detect light over the following wavelength ranges: 485-495 nm (for measurement of the 488 nm excitation power); 505-600 nm (for detection of fluorescein fluorescence); 780-790 nm (for measurement of the 785 nm excitation power); and 800-900 nm (for detection of ICG fluorescence). This would allow detection of fluorescence signals from both fluorescein and ICG, as provided by the 'mark I' system. Crucially, as the 'mark II' device will be based on LEDs and PDs rather than lasers, optical fibres and a commercial spectrometer, it will be straightforward to develop a miniaturised system that can be deployed as a wearable sensor. Furthermore, due to the relative simplicity of the system, we hope to have manufactured the first 'mark II' prototype within a year of beginning measurements with the bench-top system (which are nonetheless required in order to guide the 'mark II' design).

The optical excitation power used with the miniature system will be limited to ensure optical safety (i.e. laser/LED safety), and the exact power limits will be determined once the final device has been designed/manufactured and the relevant dimensions are known (i.e. the illuminated area, the LED-skin distance, etc.). All components will be contained within a 3D-printed plastic case, which will be sealed to ensure that no electrical components come into contact with the skin of the subjects or the researchers. As such, it will be possible for the device to be safely operated by non-expert users. The case will also act to secure the device such that it is in contact with the patients' skin at all times during the measurements. The exact embodiment will be designed after data has been collected with the bench-top system that reveals the most suitable measurement location on the subject's body. This is likely to entail a 'fingerclip' type device, however skin patches, earlobe clips or other embodiments may also be considered, and this will be dependent on the results of measurements made with the 'mark I' device. Overall, the miniaturised device will be designed such that it is suitable for safe and widespread clinical deployment by non-expert users.

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Imperial College Healthcare NHS
NHS Trust

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