Methods and Applications in Fluorescence

TOPICAL REVIEW

Fluorescence Guided Surgery

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Abstract

Fluorescence guided surgery (FGS) is an imaging technique that allows the surgeon to visualise different structures and types of tissue during a surgical procedure that may not be as visible under white light conditions. Due to the many potential advantages of fluorescence guided surgery compared to more traditional clinical imaging techniques such as its higher contrast and sensitivity, less subjective use, and ease of instrument operation, the research interest in fluorescence guided surgery continues to grow over various key aspects such as fluorescent probe development and surgical system development as well as its potential clinical applications. This review looks to summarise some of the emerging opportunities and developments that have already been made in fluorescence guided surgery in recent years while highlighting its advantages as well as limitations that need to be overcome in order to utilise the full potential of fluorescence within the surgical environment.

1. Introduction

The term medical imaging covers many powerful techniques and processes which utilise wavelengths across the entire electromagnetic spectrum to visualise the human body for clinical purposes such as the diagnosis, monitoring and treatment of disease and medical conditions. Medical imaging can be dated back as far as the 1890s following the accidental discovery of x-rays by Wilhelm Röntgen, who took the first known x-ray picture of his wife’s hand in December 1895 [1]. Since then, medical imaging has progressed rapidly with many techniques being developed and utilised routinely in medicine. Based on a medical imaging market evaluation for 2015–2017 these techniques can be split into two main segments — optical and radiological imaging [2]. These segments can be broken down further into the main contributors to each segment. The major techniques making up the radiological imaging market include x-ray, computed tomography (CT), ultrasound, magnetic resonance imaging (MRI) and positron emission tomography (PET), while the techniques that contribute most significantly to the optical imaging market include endoscopy, microscopy, visual surgery, ophthalmic surgery, medical lasers and robotics-based surgery.

The biggest contributor out of the two segments to the global market is optical medical imaging (64% compared to 36% for radiological imaging). Optical medical imaging employs light in the ultraviolet, visible and near infrared regions of the electromagnetic spectrum, which offers advantages over other imaging techniques as they significantly reduce a patient’s exposure to more harmful ionising radiation, meaning the progress of disease or treatment can be monitored more frequently over time. In addition to this, optical medical imaging can be used to measure multiple properties of soft tissue due to the varying photon absorption and light scattering from different types of tissue, which can allow for the differentiation of suspicious tissue from healthy tissue as well as identify and monitor metabolic changes [3]. One of the earliest tools of optical medical imaging was developed in the 1930s to detect oxygen levels in blood by measuring the ratio of light absorbed at two different wavelengths, known today as the oximeter [4]. The introduction of light emitting diodes in the 1970s allowed for many more wavelengths of light to become available, developing this device into the crucial diagnostic
tool it has become today. Various optical medical imaging techniques exist within clinical practice today, with some of the most commonly used techniques including endoscopy, optical coherence tomography (OCT), photoacoustic imaging, fluorescence imaging and spectroscopy, Raman spectroscopy and diffuse optical tomography (DOT). Due to its many advantages and widespread applications, one area of optical medical imaging that has gained significant attention and research interest in the past few decades is fluorescence-based guided surgery, which will be the focus of this review.

1.1. Current image-guided techniques
The numerous image-guided techniques available today can provide a wealth of information to surgeons and doctors. Not only can imaging be used for diagnosis, certain techniques can be utilised preoperatively to assist surgeons in their decision making and surgical planning. Two of the most commonly utilised techniques for this include CT and MRI. CT scans are generated using x-ray sources that rotate circularly around a patient, where every rotation of the x-ray source creates a 2D image slice. These image slices can be viewed individually or compiled to form a 3D image of the patient, which can be used to identify any abnormalities in the body as well as their exact locations. The first commercially available CT scanner came in 1972 and was created by Godfrey Hounsfield, who was subsequently awarded the Nobel prize in Physiology and Medicine alongside Allan Cormack in 1979 [5]. MRI images are instead formed using powerful magnets that produce magnetic fields causing protons in the body to align with the field. A radio-frequency current pulses through the patient to stimulate the protons and when the current is switched off the MRI sensors can detect the energy release from the protons as they realign, where the time the protons take to realign can be used to build an image. The line-scanning technique used to generate MRI images was developed by Peter Mansfield [6], who generated the first MRI image of a human in 1978 and went on to share the Nobel prize with Paul Lauterbur in Physiology and Medicine in 2003 for their work on MRI [7]. While MRI and CT are the most commonly used preoperative imaging techniques, other techniques can also be used such as positron emission tomography and ultrasonography, among others depending on the application.

While preoperative imaging techniques are extremely important for surgical planning, they do not always provide the full picture in terms of what may be happening in the location of interest. Certain distortions in the images may be mistakenly identified as other structures, for example pockets of air or gas in the abdominal cavity can cause complications in the correlation of anatomical structures to the preoperative scans during surgery, and cause the surgeon to misinterpret the position of structures such as blood vessels and tumours [8]. In addition to this, the time interval between the preoperative imaging and surgery can have a negative impact on the surgical procedure. Examples of this can include when preoperative imaging is used for the assessment of aggressive forms of cancer which may change in that time frame, or when the area of interest shifts during surgery relative to the baseline preoperative images used for guidance, which is commonly employed during neurosurgery [9]. In an effort to aid the surgeon further, intraoperative imaging techniques have been developed, meaning they can be used during the surgical procedure to better visualise the location of interest, particularly areas that are not visible by eye. Intraoperative imaging techniques have different requirements in comparison to preoperative techniques. While there is more of a focus on image quality for preoperative techniques, the speed at which images can be obtained is often much more important for intraoperative techniques, as the ability for a surgeon to obtain the information from the images during surgery as quickly as possible is crucial. There is of course a balance that needs to be struck in the case of intraoperative imaging—image quality still needs to be good enough to provide sufficient information for positioning instruments relative to the site of interest or for monitoring a process, but not exceed unacceptable latencies, which can range from seconds to minutes depending on the procedure being undertaken [10].

Existing techniques have been developed to be used intraoperatively. Examples include MRI and CT, which can be used to produce newer up-to-date scans and then be synced with previously obtained scans; however, they require complex and expensive surgical suites in order to be utilised which may not always be practical, such as in developing countries. Fluoroscopy is also commonly used intraoperatively, where a contrast agent is administered to monitor the movement of a body part or instrument and can be used for procedures such as stent placement or catheter insertion. This technique however carries a risk of patients as well as surgeons receiving higher radiation doses compared to other techniques, depending on the procedure [11]. Ultrasound has also been developed for use intraoperatively, but it can be difficult to obtain high quality images without loss in other areas such as reduced penetration depth, as well as the appearance of acoustic enhancement artefacts which can cause difficulty for the surgeon in identifying the structures of interest [12]. To assist a surgeon further, these kinds of intraoperative techniques can be used as part of augmented reality visualisation, where the digital images obtained can be overlaid with what is seen in the surgical field in real-time, either with images obtained preoperatively or during surgery. Augmented reality can even be used in conjunction with virtual reality, which is a more immersive virtual simulation of the surgical environment to better enhance the field of view for
surgeons for applications such as neurosurgery, however uptake of this combination within clinical applications has been a slower process and is not implemented often [13].

It is clear that each of these methods have their own advantages but also their drawbacks, which is why there is a continuing need for the development and improvement of intraoperative imaging techniques. Fluorescence-based techniques provide a path for this improvement. One of the most significant advantages of fluorescence-based surgical techniques is the ability to provide real-time information to the surgeon that cannot be visualised by eye alone. This can be done by utilising either the fluorescence intensity of a probe, which may fluoresce in wavelength regions outside the visible spectrum and require specialist cameras, as well as utilising the fluorescence lifetime of a probe, which can provide additional contrast as well as environmental information on the location of interest. Fluorescence systems are relatively low cost compared to the previously described intraoperative imaging techniques, do not require specialist surgical suites and can have greater portability allowing the system to be brought into and removed from the surgical environment when required. Many fluorescence imaging techniques are also relatively intuitive and do not require extensive training, therefore reducing the need for special personnel for operating the equipment. These advantages make fluorescence-based techniques ideal for their use in the surgical environment.

1.2. Basic principles of fluorescence guided surgery

The use of fluorescence in surgery has found many clinical applications over the years, but can be first dated back to 1947 where it was used for the identification of brain tumours during neurosurgery using the dye fluorescein [14, 15]. Since then, many fluorescence visualisation techniques and fluorescent probes have been developed and used in various applications within the clinical environment, and research into their future applications continues to grow. Fluorescence-based techniques remain popular for their use in surgical applications due to the advantages listed in section 1.1 as well as having high contrast, intuitive operation, ease of image acquisition and high molecular selectivity in certain applications such as identifying cancer cells [16, 17]. A simplified diagram of the fluorescence guided surgery (FGS) configuration is shown in figure 1.

In its simplest form, a FGS system will consist of a light source with accompanying filters for excitation of the fluorescence contrast agent, which is often administered prior to surgery. The emitted fluorescence signal from the probe is then collected by removing unwanted signal such as excitation light and autofluorescence by first having the light pass through appropriate emission filters, followed by collection optics to focus the signal on the detector, where the signal will be transferred to an attached computer for visualisation. The choice of every component in an imaging system will have an influence on the final image quality in many ways and to varying degrees, and so careful consideration is required depending on what the application of the system will be. While the instrumentation emphasis of commercial systems for FGS has hitherto been on fluorescence intensity measurements, there is rapidly growing interest in bringing the advantages of fluorescence lifetime to bear, most notably in macroscopic adaptations of fluorescence lifetime imaging microscopy (FLIM) [18, 19].

This review looks to provide a comprehensive overview of the two main elements required for FGS—fluorescent probes and fluorescence imaging systems, with a focus on their intraoperative clinical
Intrinsic probes refer to developments in each.

fluorophores such as structural proteins, amino acids, enzymes, coenzymes and pigments that are responsible for the fluorescence of various biological materials along with their known fluorescent properties. Note: (f) = free, (b) = bound.

| Fluorophore         | Abs. Max.(nm) | Em. Max.(nm) | Stokes Shift (nm) | Quantum Yield | Avg. Lifetime (ns) |
|---------------------|---------------|--------------|-------------------|---------------|-------------------|
| Collagen [24, 25]   | 325           | 400          | 75                | —             | 5.3               |
| Elastin [24, 25]    | 290, 325      | 340, 400     | ~75               | —             | 2.3               |
| Tryptophan [26]     | 280           | 350          | 70                | 0.13          | 3.1               |
| Tyrosine [26]       | 275           | 300          | 25                | 0.14          | 3.6               |
| Phenylalanine [26]  | 260           | 280          | 20                | 0.02          | 6.8               |
| FAD, flavins [24, 27]| 450           | 535          | 85                | —             | 2.3–4.7            |
| NAD(P)H [26]        | 340           | 450          | 110               | —             | 0.4 (f), 1–5 (b)   |
| Melanin [25]        | <300–>400     | 440, 520, 575| 40–175            | —             | Up to 8 ns        |
| Lipofuscin [25]     | 340–395       | 430–460, 540 | 90–200            | —             | 1.34              |
| Protoporphyrin IX [25]| 400–450      | 630, 710     | 230–310           | —             | Up to 15 ns       |

applications as well as current areas of research interest in each of these categories.

2. Fluorescent probes

Fluorescent probes are used to provide contrast in FGS between the target of interest and the surrounding tissue in the body. While many fluorescent probes have been researched and developed for use in FGS, very few are routinely used today. This section looks to discuss the two main categories for fluorescent probes—intrinsic and extrinsic probes—and their relative advantages and disadvantages as well as current developments in each.

2.1. Intrinsic probes

Intrinsic probes refer to fluorophores that occur naturally and fluoresce in their native form. This can include fluorophores that are found naturally in the body such as aromatic amino acids, flavins and porphyrins, as well as fluorophores in other natural environments such as the green pigment chlorophyll in plants and the green fluorescent protein (GFP) found in the jellyfish Aequorea Victoria [20–22]. Interest in such natural probes underwent a spectacular renaissance, with the award of the 2008 Nobel Prize in Chemistry going to Osamu Shimomura, Martin Chalfie and Roger Tsien for the discovery and development of GFP. One of the major advantages of using intrinsic fluorophores for biological applications is that the cells or structures do not have to be altered in any way to be studied—no external fluorophore is required to monitor them. This is useful as not only does it mean there are no external factors that will influence the biological processes, but there are also significantly less regulatory requirements that have to be met for their use in clinical applications in comparison to extrinsic probes [16]. Complications from intrinsic probes can arise however from the potentially shallow penetration depth into tissue due to the frequent requirement for excitation in the UV/visible spectrum, as well as the number of other endogenous fluorophores that may fluoresce under similar conditions to the fluorophore of interest. There are in fact several biological structures in the body that can fluoresce, where some of the more commonly studied are listed in table 1 along with their main fluorescent properties. The excitation and emission spectra of some of these commonly studied fluorophores is also shown in figure 2 [23]. Both table 1 and figure 2 highlight the potential complexity of using an intrinsic fluorophore as a probe due to the overlap of excitation and emission properties between the fluorophores.

The additional property of fluorophores that is their fluorescence lifetime can prove to be extremely beneficial when utilising intrinsic fluorescence, as excitation and emission spectra alone does not always provide sufficient contrast between intrinsic fluorophores. Not only do intrinsic fluorophores have the potential for use in contrast imaging, monitoring their properties such as the fluorescence lifetime can provide a better insight into the molecule’s role within the body and how it can change depending on environmental factors. An example of this is the increase in fluorescence lifetime of NAD(P)H that occurs when it binds to proteins, as well as this lifetime value being dependent on which protein it is bound to [28]. While the properties of intrinsic fluorophores contain a wealth of information and have several advantages over extrinsic probes, it remains very difficult for them to be utilised in FGS due to much of the biological mechanisms responsible for certain properties not being fully understood, and therefore none are currently used routinely in FGS today. Despite this, due to their numerous advantages autofluorescence imaging proves to still be a very popular research topic today, with over 1000 search results appearing as of April for 2021 alone on ScienceDirect.

One of the most commonly utilised intrinsic fluorophores in autofluorescence imaging research is the reduced form of nicotinamide adenine dinucleotide (NADH) and its phosphorylated derivative (NADPH), collectively termed NAD(P)H. NAD(P)H are coenzymes, where NADH is used in cell respiration for the production of energy and NADPH is used in the
Pentose Phosphate Pathway (PPP) for use in biosynthesis of macromolecules and quenching reactive oxygen species. While NADH and NADPH have very different roles in the body, they have very similar structures and identical fluorescence properties, with peak emission at ~450 nm due to the adenine moiety present in the molecular structure. Another commonly utilised intrinsic fluorophore is flavin adenine dinucleotide (FAD). Like NADH, FAD is also an electron carrier used in REDOX reactions in cell respiration for the production of energy. While NAD(P)fluoresces in its reduced form, FAD instead fluoresces in its oxidised form with peak emission at ~535 nm which is also due to the conformation of the adenine ring in its structure [29]. NAD(P)H and FAD are often used in combination with each other for autofluorescence-guided surgery (AFGS)-based research applications, and in particular have recently received a lot of interest for their potential in estimating tumour margins, with examples including using FLIM as a tool to measure NAD(P)H and FAD signals for rapid intraoperative tumour margin analysis in prostate cell lines [30], as well as in lung [31] and laryngeal tissue [32] studies.

Another popular intrinsic fluorophore combination for AFGS-based applications include collagen and elastin. Collagen and elastin are both extracellular matrix proteins, where collagen is a structural protein that is a main component of connective tissue [33], and elastin provides resistance and elasticity in tissues [34]. As these fluoresce at even lower wavelengths than NAD(P)H and FAD, it is useful when utilising these probes to target applications that do not require a large penetration depth or any imaging deep in the body. Examples of this include visualising autofluorescence of bone collagen for improving the removal of necrotic bone parts in patients with osteonecrosis of the jaw [35], as well as quantifying collagen and elastin autofluorescence for monitoring oral cavity lesions [36]. Other interesting examples of recent uses of intrinsic fluorophores in studies relating to future AFGS applications include using lipofuscin fluorescence for imaging the interior surface of the eye [37], as well as monitoring the redox ratio of porphyrins for detecting...
Table 2. FDA-approved probes for surgical use along with their known fluorescence properties.

| FDA-approved Probe | Abs. Max. (nm) | Em. Max. (nm) | Quantum Yield | Avg. Lifetime (ns) |
|-------------------|---------------|---------------|---------------|-------------------|
| Fluorescein sodium [40, 41] | 490 | 510 | 0.95 (in 0.1 M NaOH) | 4.0 (in pH 7.5 buffer) |
| Fluorescein isothiocyanate (FITC) [41, 42] | 495 | 520 | 0.92 | 4.1 (in pH 7.8 buffer) |
| 5-ALA [25] | 410 | 630, 700 | — | Up to 15 ns |
| Methylene Blue [43–45] | 665 | 685 | 0.22 (in water) | 0.60 (in MeOH) |
| IRDye700DX [42, 46] | 680 | 690 | 0.24 (in MeOH) | — |
| IRDye800CW [42, 47] | 775 | 795 | 0.10 (in MeOH) | — |
| Indocyanine Green [48–50] | 780 | 830 | 0.14 (in water) | 0.16 (in water) |

Bladder cancer [38]. Despite the drawbacks associated with intrinsic probes, the amount of research still ongoing into their use in FGS demonstrates they still hold great potential as fluorescence probes and may still be utilised more fully in the clinical environment in the years to come.

2.2. Extrinsic probes

Extrinsic probes are much more commonly applied and studied for use in FGS in comparison to intrinsic probes. Extrinsic probes are molecules which are designed to fluoresce under certain conditions in order to provide a fluorescent signal where one would otherwise not exist. Extrinsic fluorophores have their own advantages over intrinsic fluorophores for medical applications, such as there being more control in choosing the location at which the fluorescent label is attached, as well as being typically designed to have enhanced fluorescence properties that make them ideal for such applications [39]. As previously mentioned however, strict regulatory requirements have to be met in order to have a fluorescent dye approved for medical applications, and as such there remains very few fluorescent probes that meet these requirements and have FDA approval. Table 2 shows currently used FDA-approved fluorescent probes for surgical purposes, highlighting the limited number available for use.

One of the most well-known and first used FDA-approved dyes is fluorescein sodium, an organic dye with peak excitation and emission at ~490 nm and 510 nm respectively [40]. Its first application within surgery was in the identification of brain tumours in 1948 [14], and is in fact still used for the same purpose in neurosurgery today in identifying various types of brain tumour. Fluorescein sodium is also commonly used in ophthalmic angiography for visualising the retina, with new uses in surgery and clinical procedures also being investigated including its use in detecting oral dysplasia and oral cancer [51], as an aide in detecting spinal dural arteriovenous fistulas [52] as well as its feasibility in identifying peripheral nerve sheath tumours during microsurgical removal [53]. The relatively low cost of fluorescein sodium compared to other available fluorescent agents as well as the ability to observe its fluorescence with the naked eye make it an advantageous dye to use in certain surgical applications [54].

One limitation of fluorescein is its fluorescence is within the visible spectrum, which overlaps with the emission of a number of endogenous fluorophores (see table 1 for examples). A more desirable wavelength range to utilise in surgical applications is the near infrared (NIR) range, as light in this range is less readily absorbed by tissue and also allows for greater penetration depth compared to light of the visible spectrum [55]. Another well-known and the most commonly used FDA-approved dye is Indocyanine Green (ICG), which has been designed for this purpose in particular. ICG is a water-soluble tricarboxycyanine dye with peak excitation and emission at ~780 nm and ~830 nm respectively [48]. The difference in spectra of ICG compared to the commonly studied intrinsic fluorophore NAD(P)H is shown in figure 3. ICG was originally developed for NIR photography by Kodak in 1955, and was approved for clinical use a few years later for cardiac output monitoring [56, 57]. Since then ICG has been used in a wide range of medical applications such as imaging retinal blood vessels and assessing liver function, but for fluorescence-guided surgery in particular it has gained interest for its use as a fluorescence contrast agent after being shown to accumulate in certain cancers such as those in the liver [58], as well as for sentinel lymph node mapping of various cancers [59]. The use of ICG in fluorescence-guided surgery continues to receive a huge amount of research interest, with a lot of recent research focussing on applications in cancer surgery such as being used to reduce anastomotic leakage in laparoscopic surgery for colorectal cancer [60], during bowel resections for patients with gynaecologic malignancies [61], as well as detecting leakage in other types of surgery such as staple line leakage in bariatric surgery [62].

While fluorescein sodium and ICG remain the most commonly used dyes for surgical applications,
other FDA-approved dyes with more limited applications do exist. One such dye is Methylene Blue (MB), which also has beneficial spectral properties in the NIR window like ICG with peak excitation and emission at ∼665 nm and ∼685 nm respectively [43]. Current uses of MB include sentinel lymph node mapping of various cancers such as breast and cervical cancer as discussed in more detail in section 4, the staining of abnormal parathyroid glands during surgery [63] as well as in the treatment of urinary tract infections and the blood disorder methemoglobinemia [64] among other uses. Many research publications are still being produced on the use of MB in surgery, with recent studies of interest including its potential use as a photosensitiser in photodynamic therapy (PDT) for the treatment of infected wounds [65], as a quantitative marker of breast cancer at the cellular level [66], as well as looking at its ability in reducing biliary leaking incidence in liver transplants [67]. MB has its limitations, which include a low fluorescence yield as well as no functional groups for adding ligands [68], making it a less attractive fluorophore to use for medical applications in comparison to ICG.

Another FDA-approved molecule is 5-aminolevulinic acid (5-ALA), which only gained FDA approval in 2017 for use in imaging high-grade gliomas. 5-ALA is not itself a fluorophore, but is instead a precursor of Protoporphyrin IX (PpIX) which is used in the biosynthetic pathway for the production of heme [24]. PpIX is highly fluorescent, and when 5-ALA is administered orally it induces the accumulation of PpIX in tumour tissue to a greater extent than in healthy tissue, as well as resulting in an increase in the fluorescence lifetime of tissue [25]. While this induced fluorescence is just outside the NIR window and therefore less attractive for surgical applications (peak excitation and emission at ∼410 nm and ∼630/∼700 nm respectively), it presents minimal toxicity issues compared to other extrinsic probes as both 5-ALA and PpIX are naturally present in the body. In addition to this, it has better targeting abilities compared to other probes for discriminating between healthy and cancerous tissue, as PpIX accumulates greatly in malignant glioma tissue in particular due to an abnormality in porphyrin-heme metabolism [69]. Publications within the last few years reflect this being the main area of research interest in terms of 5-ALA induced fluorescence in FGS, as they focus mainly on glioma surgery applications. However, initial research into PpIX accumulation in other cell lines has shown it to accumulate in cells derived from tissue such as that of melanoma [70], breast cancer [71], and lung cancer [72], with more recent work exploring it’s combined use with laparoscopy for observing the spread of cancers such as gastrointestinal cancer [73], as well as its potential as a contrast agent in FGS for pancreatic cancer [74]. As 5-ALA is a relatively new probe as well as its fluorescence being outside the NIR region, its widespread use and current applications are limited and therefore considerably more research will be required for its long-term application to FGS.

One of the most obvious limitations of extrinsic probes for surgical applications currently is the limited number that are approved for use. There are a number of barriers to overcome for a probe to be first suitable for surgery, where very few probes have met enough of these requirements in order to reach approval. Not only should a new probe be preferably designed to have attractive fluorescent properties such as having excitation and emission in the NIR window and ideally having a fluorescence lifetime longer than most intrinsic fluorophores, another significant barrier to overcome in clinical trials is human toxicity. The toxicity of fluorescent probes is investigated in Phase I of clinical
trials before moving on to determine the minimum effective dose at which no adverse effects are observed. This highlights how the quantum yield may be another important property of a new fluorophore to consider for use in humans, as a greater quantum yield could mean a smaller and therefore more desirable dose is required for effectiveness, depending on the fluorophore’s uptake in the target area. In addition to this, the half-life of the probe is another factor to consider, where for example ICG has a very short half-life and is rapidly eliminated from the body, which can lead to repeated dosing being required. Even with a fluorophore meeting all or at least many of the requirements in early preclinical stages, success is not necessarily guaranteed, and so this complex blend of requirements along with the huge expenses associated with clinical trials can make this a difficult avenue of investigation.

Despite these barriers, there remains a huge amount of interest and research into new fluorophores for use in surgery, with a recent review by Barth et al finding a total of 39 novel contrast agents for use in FGS being studied over 85 clinical trials in the US alone [75]. Four probes have reached Phase III of clinical trials in the US, which include BLZ-100, LUM015, OTL38 and SGM-101. Phase III of clinical trials involves testing the probes on a much larger number of patients with the condition the probe was initially designed to investigate compared to the number of patients in Phase II. The properties of the four probes in Phase III of clinical trials along with the purpose of each trial are summarised in table 3.

In addition to the probes that have reached clinical trial stage, there is a continuing interest in the development of new probes for FGS, as well as using those already in trials for other applications. A family of probes that are an interesting example of this are zwitterionic NIR fluorophores. A zwitterion is a molecule that has a net charge of zero but consists of at least one positive and one negative functional group. These unique chemical structures give zwitterionic fluorophores attractive properties such as low nonspecific binding to normal tissue, the ability to be conjugated to target ligands [84] as well as a greater fluorescence yield than the more commonly used ICG [85]. Some recent investigations have shown the zwitterionic fluorophore ZW800–1 has potential for use during laparoscopic surgery for visualising the ureter [86], as well as using an analogue of ZW800–1 called ZW800-Cl as a theranostic agent in photothermal cancer therapy [87].

Other examples of fluorophores in their early research stages include monoclonal antibody-based fluorescent probes which have been used for targeting various cancers such as squamous cell carcinoma in the head and neck [88]. Probes such as these have the advantage of improved binding specificity; however they also have a much slower clearance rate from the body [89]. Another type of probe in early research stages include activatable probes that ‘switch on’ upon engagement with the target molecule. These are beneficial in comparison to probes that are ‘always on’ as they will not emit any signal when unbound, which therefore minimises background signal and improves sensitivity. These types of probes are also being developed into sprays which can be during the surgical procedure rather than having to be administered prior to surgery and have shown promise for the detection and imaging of various cancer cells and tissues [90–92].

A related potential approach concerns using gold rod nanoprobes to plasmonically enhance fluorescence of a DNA hairpin-bound dye upon base-pairing with cancer-specific mRNA [93]. The continued research interest in new types of fluorescent probes holds promise for the future of FGS, where improvement on current probes may also open up more applications for FGS in the years to come.

3. Clinical systems for fluorescence guided surgery

There is a continuing need for the development of fluorescence-based clinical imaging systems that can be used in conjunction with the various approved fluorophores as well as those fluorophores currently in development. Imaging systems can utilise the fluorescence intensity, lifetime, wavelength, polarization etc in a combination of these properties of a probe for their use in surgery. Fluorescence intensity is a more intuitive parameter to use from a clinician’s point of view, as it is a simple case of whether or not the target structures of interest fluoresce when they interact with or bind to a probe. However, as previously discussed there is often overlap in the fluorescence emission properties of a probe of interest and other fluorescing structures in the body and it can therefore be difficult to discriminate between the two. Non-uniform probe concentration can also lead to errors. Time-resolved fluorescence techniques instead utilise the fluorescence lifetime of a probe and not just its fluorescence intensity. Fluorescence lifetime measurements can provide additional information about a sample compared to intensity measurements alone, and offer advantages such as the ability to discriminate against scattered light and potentially endogenous fluorophores, as well as typically being independent of probe photobleaching, concentration and wavelength which can often affect analogue fluorescence intensity measurements [94]. Both phase/modulation and pulsed methods [28] have traditionally provided the main approaches to measuring fluorescence lifetime. Within the pulsed domain, gated optical intensifiers combined with charge-coupled device (CCD) detection [95] and gated microchannel plate (MCP) photomultipliers [94] have found applications within oncology. However, the research method of choice for most fluorescence lifetime measurements is time-
Table 3. Summary of fluorescent probes that have reached Phase III of clinical trials.

| Fluorescent Probe in Phase III of Clinical Trials | Abs. Max. (nm) | Em. Max. (nm) | Molecular Formula | Purpose of Clinical Trial |
|--------------------------------------------------|----------------|---------------|-------------------|--------------------------|
| BLZ-100 [76, 77]                                 | ~745           | ~820          | C_{203}H_{296}N_{58}O_{52}S_{12} | Visualising tumours in the central nervous system of paediatric patients. |
| LUM015 [78]                                      | 650            | 670           | C_{120}H_{155}N_{19}O_{26}S_{4} | Detection of residual breast cancer. |
| OTL38 [79, 80]                                   | 775            | 795           | C_{61}H_{67}N_{9}O_{17}S_{4}   | Intraoperative identification of pulmonary nodules. |
| SGM-101 [81–83]                                  | 685            | 705           | —                 | Intraoperative delineation of primary and recurrent tumours in rectal and colorectal cancer patients. |
correlated single-photon counting (TCSPC) [96], where a simplified diagram of the process is shown within a testbed configuration to assess implementation for phantom tumour margin estimation is described in figure 4 [97]. The setup shown in figure 4 incorporates liquid light guides (LLGs) as a means of sample excitation and collecting fluorescence emission. LLGs have been little if at all explored in surgical margin assessment and yet they offer a number of advantages when compared to conventional silica fibre bundles used in procedures such as endoscopy. One is that there is the ability to transmit more light due to the open cross-section, where fibre bundles lose light in the space between the individual fibres. LLGs are also often more flexible due to the polymer tubing compared to fibre bundles which are prone to breakage when frequently bent. Even with a ~7 mm diameter excitation area using LLGs, TCSPC fluorescence lifetime analysis has been shown to offer a workable 1 mm phantom tumour margin resolution [97].

TCSPC involves monitoring the detection and arrival time of photons after excitation of a sample, where typically an excitation source will produce a signal to start timing electronics in a system, followed by a second signal to stop the timing electronics that is produced by the detector upon detection of a single fluorescence photon. The time between these two signals can then be converted into a digital signal and then stored as a photon count event in a histogram. To avoid data pile-up, stop to start ratios have to be restricted to less than 10% [96]. This adds to electronic dead times which together have hitherto held back translation of the research advantages of fluorescence lifetime measurements into FGS. Notwithstanding its global popularity and potential advantages in research in terms of its ultimate digital sensitivity and time resolution, adapting TCSPC for FGS raises significant challenges. These largely relate to obtaining a high enough data collection rate compatible with the need for effectively real time imaging. Consequently, fluorescence intensity currently remains the measurement of choice for commercially developed systems. However, recent developments in single-photon avalanche diode (SPAD) arrays discussed in section 3.1 and other developments in section 3.3 promise to greatly enhanced the
capabilities of TCSPC for FGS. There are a number of components required for utilising either fluorescence intensity or fluorescence lifetime in imaging systems, where the main ones will be discussed in this section.

3.1. Key components in fluorescence-based surgical systems

The key instrumentation components for employing fluorescence in surgical systems are largely based on proven technology and methodologies that are already in widespread use for fluorescence spectroscopy and microscopy [28, 98–100] and namely comprise of an excitation source, a fluorescent probe, excitation and emission filters and the fluorescence detector. Since the details on fluorescent probes have already been discussed in section 2, this section will focus on the requirements of excitation sources, filters and detectors as well as developments being made in these areas if applicable.

3.1.1. Excitation sources

The choice of excitation sources for fluorescence imaging systems typically consists of broadband light sources such as xenon lamps, light emitting diodes (LEDs) as well as laser diodes. The broadband nature of lamp sources allows flexibility in terms of wavelength selection, as filters can be chosen or changed to produce the excitation wavelength required depending on the application. Despite this, lamps are not the most viable option when developing a fluorescence imaging system for the clinical environment, as they usually require a warm-up period, their brightness will often decay over time and with high usage, and they also produce a lot of heat.

LEDs have become a more commonly utilised excitation source for fluorescence applications. LEDs are a much more robust option and have many advantages in comparison to lamps, such as having significantly longer lifetimes, they do not require a warm-up period, they emit over a narrower bandwidth (typically 20–30 nm) meaning they can be chosen depending on their emission properties and which fluorophore is being worked with, and also have lower electricity consumption [101]. While the bandwidth of LEDs is narrower compared to lamps, having a 20–30 nm bandwidth means they may not be appropriate for use with fluorophores with a small Stokes shift, which will require the use of further excitation filtering. Laser diodes are another viable option for fluorophore excitation. Laser diodes are highly selective in terms of wavelength with a very narrow bandwidth, and typically are much higher in intensity in comparison to lamps and LEDs. The high intensity of these sources can often still produce a fluorescence signal even if the excitation wavelength does not exactly match that of the fluorophore in use. The most significant disadvantage of laser diodes is that they are relatively high in cost in comparison to LEDs, meaning LEDs remain often a more popular choice as an excitation source.

Ti:sapphire laser technology has held sway for many years in time-resolved fluorescence research but another possible source that has not been utilised so commonly is a white-light supercontinuum laser. As suggested in the name, these are laser excitation sources but instead have a broad spectral range from ~400 nm to 2 μm while still maintaining a very high intensity. In addition to this, supercontinuum lasers can produce laser pulses in the picosecond range making them suitable for lifetime measurements. Supercontinuum lasers are also very high in cost in comparison to LEDs and not as compact, but may still find themselves being used more in future FGS systems that look to utilise fluorescence lifetime. Supercontinuum lasers have been demonstrated for use in multiphoton excitation [102] though the femtosecond pulses afforded by Ti:Sapphire lasers are more the norm. Multiphoton excitation offers advantages in terms of increased excitation depth, localised focusing and additional selectivity in the therapeutic infra-red optical window in tissue [103]. Multiphoton excitation has been combined with TCSPC [104], applied to silica colloids [105] and demonstrated in clinical tomography [106], given more improvements in technology and ease of use there is potential here for macroscopic TCSPC FLIM imaging in FGS. The pros and cons associated with the types of light sources available for excitation need to be assessed depending on the system requirements, where factors such as associated costs, stability and ease of filtering will need to be considered.

3.1.2. Filters & collection optics

The choice of excitation and emission filters allows for the careful selection of wavelengths as well as the discrimination of unwanted signals from a sample. Excitation filters may be required when white light sources are utilised, whereas emission filters are used to limit the fluorescence signal collected to the spectral band of interest. A balance will always need to be met in terms of emission filter selection in particular—using emission filters with a larger band will allow for greater collection of fluorescence, but increases the risk of unwanted fluorescence signals such as autofluorescence being collected, reducing the contrast and therefore overall quality of the signal from the fluorophore of interest. Another factor that needs to be considered when choosing emission filters is the Stokes shift of the fluorophore that will be utilised with the system. Fluorophores such as ICG have a small Stokes shift, therefore the choice of emission filter will be important in ensuring minimal unwanted excitation or scattered light is collected. The precision required in terms of filter choices is often a limiting factor of the applications a system can be used for, as they more often than not have to be designed for use with one particular fluorophore only.
Collection optics are another aspect that may have to be carefully considered when designing a fluorescence imaging system. These optics are utilised for focussing the fluorescence signal collected from the system onto the detector. The choice in these optics will most likely depend on the required field of view from the system, which can be influenced by the size of the optics through which the fluorescence is being collected. For example, a 3 mm diameter liquid light guide with a 0.59 numerical aperture results in an acceptance cone of 72°, which can illuminate ~7 mm of a sample surface from a height of 5 mm [107]. The detector being used will also influence the choice in optics, as the type of detector will determine the level of focussing required of the fluorescence signal, where detectors with larger active areas require less precise focussing compared to those with smaller areas.

3.1.3. Fluorescence detectors

Many types of detectors are available and capable of collecting the fluorescence emission from a sample. In the case of fluorescence lifetime imaging, the detectors ideally bring the benefits of sensing photons at the single photon level. How well a detector can detect single photons is typically defined by two parameters: the quantum efficiency and internal gain. The quantum efficiency refers to the ability of a detector to convert incident light into electrons, typically expressed in a percentage form as the ratio of the number of photoelectrically generated electrons in the semi-transparent photocathode to the number of incident photons. If a detector had 100% efficiency, it would be able to produce one electron for every one photon detected. In practice this is not achievable and more typically 20% maximum efficiency is reached, with detectors often responding less efficiently depending on the wavelength region being observed. The internal gain of a detector refers to its ability to amplify the detected signal caused by an incident photon into a large enough electrical signal to be detected by external electronics. A higher internal gain of at least 10⁶ is often advantageous for measurements with lower intensity samples in order to discriminate the signal from amplifier noise and have a sufficient signal-to-noise ratio (SNR). Other factors that should also be considered in terms of a detector’s performance include the dark-count rate, the dead time and the timing jitter, where the lower these are the better the performance of the detector [108–110].

Photomultiplier tubes (PMTs) were the first devices used to detect photons at the single level [110] and indeed have been the traditional choice of detector for many medical and biological applications over the years. PMTs typically work using the photoelectric effect, where incident photons will enter the input window and are absorbed by a photocathode to then generate photoelectrons. These photoelectrons are focussed and accelerated onto the first dynode out of many, where enough energy is gained to release multiple secondary electrons. This emission effect is repeated several times on each of the dynodes in the detector to amplify the number of electrons until they reach the anode, where by this point there are enough electrons to generate a spike in current easily detected by the detection electronics [111]. An advantage of PMTs as single photon detectors include that they typically have a larger active area (~cm²) of the photocathode, meaning that the fluorescence emission does not require tight focusing for efficient detection in comparison to SPADs [28]. In addition to this, PMTs have an adequate response rate, timing jitter and low enough dark count rate for many applications [112].

The use of PMTs also comes with its disadvantages, such as the requirement of a high voltage power supply, shielding from exposure to light and magnetic fields, as well as detector cooling often being required for photon counting applications, particularly when working in the NIR therapeutic window using trialkali S1 and S20 photocathodes [112].

The demand for improvement for single photon detection was recognised decades ago and led to the development of more technologies for this application. One such technology includes microchannel plate photomultipliers (MCP-PMs), which also incorporate photocathodes [113]. MCP-PMs consists of a plate with many small holes, or channels, typically 4–12 microns in diameter, which are lined with a secondary emissive dynode material to give what is effectively a continuous dynode. Instead of the chain of dynodes in a PMT alone, the electrons are amplified as they travel through these channels and drop down the voltage gradient across the MCP [28]. As the electrons are much more limited in what path they can travel in an MCP compared to a dynode PMT, there is much less variation in the transit time of the electrons which in turn improves the time resolution of the detection system. While time resolution is improved, drawbacks of implementing an MCP-PM include a degradation in gain over the lifetime of the MCP-PM, as well as being more expensive than traditional PMTs [114].

The more recent introduction of hybrid photomultipliers [115] that incorporate an avalanche photodiode rather than dynodes as the electron amplification mode offers lower after pulsing than both conventional PMTs and MCP-PMs as well as improved time and pulse-height resolution, and are already finding their way into commercial systems [106]. However, despite all these developments in technology the conventional photocathode-dynode photomultiplier rightly still finds a place in many new designs of spectroscopy and microscopy systems and still offers distinct capabilities for FGS and endoscopy.

A more appropriate alternative to PMTs particularly for clinical applications is the use of charge-coupled devices (CCDs). CCDs consist of usually either a 1D or 2D array of pixels etched onto a silicon
substrate, where each pixel builds up a charge proportional to the brightness and/or the exposure time of incident light which can then be read by output electronics [116]. Traditional CCDs typically offer high sensitivity but with a slow readout rate. However, the development of electron multiplying CCD (EMCCD) cameras allows for on-chip amplification of the signal and bypassing of readout noise to maintain high sensitivity at high speeds. The most significant advantage of EMCCDs over PMTs is their suitability for live imaging, allowing a clinical user to potentially visualise structure based on fluorescence intensity and lifetime signals in real-time and are in fact one of the most commonly utilised sensors in commercially available fluorescence imaging systems [68]. One drawback of EMCCDs is the production of multiplicative noise produced by the amplification mechanism required, which can increase shot noise and increase pixel to pixel and frame to frame variability [117], but their advantages remain significant in comparison.

Looking towards emerging detector technology there have been some recent developments in single photon avalanche photodiode (SPAD) array technology, which transcend the restrictions on TCSPC data collection rates already mentioned and eliminate the slowness of sequential point-by-point measurements by means of a multiplexed pixel array. This data rate enhancement promises to bring the advantages of time to digital (TDC) technology to fluorescence lifetime imaging for real-time FGS. The principle of multiplexed photon timing was first established some years ago using discrete detectors and multi-anode MCP-PMs in a range of TCSPC implementations including time-resolved spectroscopy [118] and a macroscopic imaging camera [119]. However, the recent demonstration of nearly 25,000 simultaneous fluorescence decay measurements using a SPAD array is opening up new vistas.

A SPAD is a photodiode with a p-n junction that is reverse biased at a voltage exceeding its breakdown voltage with many of the early developments for photon-counting pioneered in Sergio Cova’s laboratory in Milan [120]. When a single photon reaches the active device, an electron-hole pair is created which then triggers a self-sustaining avalanche of carriers [121, 122]. The generated current rises quickly to a steady level in the order of milliamperes, where the leading edge of the pulse indicates the arrival time of the photon if the primary carrier is photon-generated. Appropriate electronics are required to detect the leading edge of the avalanche pulse, lower the SPAD bias to below the breakdown voltage to quench the avalanche and reset the photodiode to operating levels [122]. One of the main advantages of a SPAD for photon detection is its higher quantum efficiency, which typically sits at around 60% in the visible region and around 90% in the NIR region in comparison to an efficiency of ∼20% maximum for PMTs [28]. Other advantages of SPADs in comparison to PMTs and MCPs include that they do not require a high bias voltage, they are less bulky, are not so sensitive to magnetic fields [123], and can be incorporated into arrays to produce SPAD imagers, analogous to CCD cameras. However, their micron scale individual pixel area and ∼10% fill factor has often limited their applications to microscopy. An example of recent developments of SPAD arrays for FLIM is the QuantiCam developed by Robert Henderson’s team in Edinburgh—a complimentary metal-oxide semiconductor (CMOS) fabricated 192 × 128 pixel array where each individual SPAD has its own electronics-based TDC incorporated within the pixel [124], allowing for the rapid collection of fluorescence lifetime data in every one of the 24,576 pixels simultaneously. HORIBA Jobin Yvon IBH Ltd has recently developed the QuantCam in the form of its FLIMera camera and the first report on using this device for estimating phantom tumour margins has now appeared [97]. Figure 5 shows the FLIMera camera integrated in a microscope silica phantom margin rig and typical wide field images of ICG fluorescence, which were shown to afford ∼1 mm margin resolution [97] in effectively real-time. Related work will no doubt follow and similar SPAD technology has been developed into line sensors such as 512 × 16 SPAD arrays incorporated into confocal laser scanning systems for video rate FLIM in life science applications [125]. These SPAD arrays have also been utilised for time-gated Raman measurements [126], with potential biomedical applications of time-gated Raman including the chemical imaging of teeth [127] as well as the transcutaneous evaluation of bone compositions [128].

Despite the advantages and potential of SPADs for fluorescence lifetime detection, PMTs and CCDs are still in high demand for their use as detectors. It is clear there are advantages and drawbacks to each type of detector used, and so depending on the application one may be more appropriate than the others, which will most likely be affected by factors such as associated costs and required sensitivity of the detector. In addition to these available detectors, the use of more newly developed single photon detectors such as superconducting nanowire single-photon detectors (SNSPDs) and transition edge sensors remains to be explored. These devices have advantages in life science applications such as high detection efficiency, low dark count, small timing jitter and perhaps most importantly broadband sensitivity from the visible to mid-infrared region [129, 130] and could be utilised in future FGS applications and systems.

3.2. Currently available FGS systems

There are a number of commercially available fluorescence imaging systems for clinical applications currently on the market. As comprehensive lists of those available along with their features have already been created and well summarised in recent years...
[68, 75, 131], a brief summary of the more well-known available systems and their applications will be provided here.

There are approximately 20 fluorescence-guided clinical imaging systems that have FDA approval for use in the clinical environment [75], which typically fall into the categories of hand-held imaging systems, endoscopic-based systems and surgical microscopes. All of these are intensity-, not lifetime-based. One of the most well-developed FDA-approved systems is the Fluobeam, which has been utilised in research applications since 2009 [132]. Fluobeam is a hand-held imaging system that incorporates a Class 1 laser beam at 750 nm with emission collection from 800 nm onwards for use with ICG for applications such as thyroid and lymphatic surgery. Another well-established system is the da Vinci system, which is a more versatile system that integrates the Firefly fluorescence visualisation capability, also designed to work using ICG.

The majority of currently approved systems are in fact designed to work with ICG, with excitation occurring at ~805 nm and emission collected from ~820 onwards in most of these systems. These systems are often fully enclosed where access to the optics that would need to be changed is not possible. Fluobeam can also be used to utilise MB fluorescence, and Quest Medical Imaging have developed the Quest Spectrum which can also be used for applications utilising MB fluorescence as well as that of ICG by incorporating two imaging channels, demonstrating more versatility than some of the other systems available. A system with even more versatility is the Leica M530 surgical microscope, which has a number of accessory...
modules that can be incorporated into the system for visualisation of fluorescence at various wavelengths, including those required for ICG as well as for fluorophores with emission above $\sim 450$ nm and $\sim 510$ nm [133, 134]. Zeiss have also produced accessories for their surgical microscopes to operate in similar wavelength regions.

3.3. Future challenges & systems in development

While there are a number of fluorescence-based imaging systems available on the market, the area of research involving the development of new clinical fluorescence imaging systems continues to grow as there are a number of challenges that still need to be addressed in order to integrate FGS more fully into the clinical workspace. One of these challenges is the ability of a system to work in ambient light. Ambient light can contribute significantly to background noise in fluorescence imaging, and since it is important to obtain the best signal-to-background ratio as possible when performing FGS, most applications require room lights to be off which can become a potentially hazardous environment in which to be performing complex procedures [135]. An example of a system shown to have the ability to work under such conditions is a modified version of the Photodynamic Eye Camera (m-PDE). This system uses an LED illumination source which is pulsed in synchronisation with the frame rate of the CCD, meaning the detector receives both a fluorescence image with an ambient light background as well as an image of the background only, which can be subtracted from the fluorescence image in real-time [136]. In terms of continuous operation without synchronisation the SPAD array systems already mentioned are much more robust under ambient light than their PMT counterparts [97].

Another limitation common among systems that are currently available is that they are often designed for one very specific application and consequently designed to work in limited wavelength regions or with specific probes. An ideal system would have the ability to switch between different wavelength regions and probes, perhaps by means of switching out excitation and emission filters of the system. Incorporating this into a system may come with its own challenges, as this would require either more training for an end user to be able to perform these changes, or extra support from medical technicians. While it is unlikely that a system could be designed to be completely versatile across many applications and wavelengths, some freedom for change would be extremely advantageous in new systems. One system soon to be studied in a clinical trial setting which could in future address this need is the KronoScan imaging system. This incorporates a SPAD line sensor similar to those discussed previously for real-time FLIM techniques such as those used by KronoScan. Reproduced with permission of the © ERS 2021: European Respiratory Journal Mar 2021, 57 (3) 2002537; DOI: 10.1183/13993003.02537–2020; In Press [139].

![Figure 6. Basic overview of optical endomicroscopy procedure as utilised by KronoScan and Panoptes, where the Panoptes fibre can be delivered via the working channel of a bronchoscope to access and characterise components such as solitary pulmonary modules and normal and malignant tissue using real-time FLIM techniques such as those used by KronoScan. Reproduced with permission of the © ERS 2021: European Respiratory Journal Mar 2021, 57 (3) 2002537; DOI: 10.1183/13993003.02537–2020; In Press [139].]
another one of the challenges for future clinical systems.

In addition to these challenges, other areas that should be addressed when developing new systems include improving penetration depth, compensating for motion artefacts when the areas of the body that are the subject of the surgery may move during the procedure such as the brain, as well as better quantification of the fluorescence information being provided. Despite the challenges, there are a number of systems in development and many that have reached the clinical trial stage. One system in the research stage and not yet FDA-approved is the FLARE (Fluorescence-Assisted Resection and Exploration) by Curadel. FLARE is a cart-based imaging system that can simultaneously display videos in two channels of ~700 nm and ~800 nm emission in real-time in combination with its own optimised contrast agents, with resolution down to the single cell level. Another system currently in the research stage is the Solaris by Perkin Elmer. This is a system designed for open air surgery and for operation in ambient light conditions, which can operate over a broad spectral range, making it a potentially ideal system in terms of the current clinical challenges. Another interesting system in development that is quite different in operation in comparison to those previously discussed is an optical see-through GAINS—goggle augmented imaging and navigation system as shown in figure 7 [140, 141]. This prototype system has been used to visualise NIR fluorescence from ICG overlaid with the surgical field through wearable goggles for sentinel lymph node mapping in real time, demonstrating another interesting area that future FGS systems may increasingly be developed in.

As previously mentioned, fluorescence lifetime is a parameter that is not currently utilised in commercially available systems despite its potential to provide a lot more information than fluorescence intensity alone. As well as KronoScan, some newly developed systems are looking to utilise this parameter. An example of this includes a proof-of-concept fluorescence lifetime camera developed by Ingelberts et al, based on a current-assisted photonic sampler pixel-gated sensor that was successfully used for imaging animal models [142]. Another group looking to utilise fluorescence lifetime are the Marcu Lab in the University of California, Davis who have presented a number of interesting results evaluating fluorescence lifetime for clinical applications, including the development of a multispectral lifetime imaging system based on avalanche photodiodes [143], real-time augmentation of label-free fluorescence lifetime signatures [144], as well as using label-free FLIM for assessing brain tumour margins [145, 146]. It is clear that fluorescence lifetime offers many benefits for FGS and in particular could be used to improve auto-fluorescence imaging, and so it is very likely future systems should and will look to incorporate this imaging aspect into their design.

Another area being explored and recently been utilised in FGS is machine learning. Machine learning is a type of artificial intelligence where a system can continually learn and improve based on past experience without the need to be manually reprogrammed. This has been utilised for a number of FGS-based applications in recent years, where for example the potential of machine learning-based models has been demonstrated in identifying the main features in brain tumour margins as well as surpassing expert assessment of the same margins [147, 148]. Machine learning has been applied in determining other types of malignant tissue as well, where models were used to analyse changes in tissue over time to develop lesion classification with high specificity [149]. Machine learning is already being used in well-developed systems, with Quest Medical Imaging utilising machine learning to validate clinical decision making in their next system the Quest Spectrum 3.0. Its potential has also been demonstrated in combination with label-free FLIM as mentioned previously, where various machine learning classification methods were investigated to determine which gave the best results for distinguishing healthy and cancerous tissue from patients with oral and oropharyngeal cancer, with an example shown in figure 8 [146]. With the continuous and rapid development in machine learning and other artificial intelligence techniques in recent years, it is certain these will continue to be utilised in further FGS studies and clinical systems.
4. Applications of fluorescence guided surgery

As discussed throughout the previous sections, FGS has widespread clinical applications and continues to gain significant research interest for future applications. Some of the most significant applications of FGS as well as future applications are discussed here.

4.1. Sentinel lymph node mapping

One of the most common areas of surgery where fluorescence has been successfully applied is within oncology. Oncological applications of fluorescence in particular have been widely reviewed in recent years, and remains one of the most commonly researched applications of fluorescence-based surgical techniques. One such use of fluorescence in oncology is sentinel lymph node (SLN) mapping. The lymphatic system is used to remove unwanted toxins and waste from the body, where a SLN is the first lymph node on a lymphatic drainage pathway for cancer cells from a primary tumour site. The ability to accurately identify the drainage sites for tumours against SLNs not associated with the tumour before dissection occurs minimises the need to remove more lymph nodes than necessary during surgery, as well as assist in future therapeutic approaches such as chemotherapy and radiotherapy and also in surgical decisions in relation to the tumour. SLN mapping is included in standard treatment guidelines of many cancers, with the most common being breast, melanoma, vulvar and cervical cancer [150].

SLNs are most commonly identified and visualised using a combination of radioactive tracer and blue fluorescent dye, which is often either Iosulfan Blue (IB), Patent Blue V (PBV), or MB [151] (figure 9). While it is possible to perform SLN mapping with a radioactive tracer alone or a blue dye alone, it was found the combination of the two could reduce the false negative rate [152]. IB and PBV were the more commonly used dyes originally, however later studies revealed that MB had significant advantages over these dyes for this application. While the use of MB was able to provide results as reliable as those obtained with IB or PBV [153], there were the added benefits of a reduced risk of side effects such as anaphylaxis than both IB and PBV [154, 155], as well as it being more readily available and less expensive than its counterparts [156].

While the use of a blue dye in combination with a radioactive tracer does reduce the false negative rate, some recent studies have questioned whether this is improved enough to outweigh the disadvantages of using a blue dye [157, 158]. As well as causing anaphylaxis, the use of any of the blue dyes mentioned can also cause tattooing and discolouration of the skin [157, 159]. However even with these disadvantages, another avenue of research that is of interest is whether or not the use of a blue dye alone can provide comparable results to those achieved with the combination of radioactive tracer and dye for use in developing countries in particular. The use of a dye alone—in particular MB due to its previously mentioned advantages—is an attractive option for such countries as there is often limited or no access to nuclear medicine facilities [160]. While caution is advised in the use of MB alone due to potentially higher false negative rates [160], studies have shown that MB alone can produce identification rates comparable to those achieved with the combination of radioactive tracer and dye [161–163], making this a viable option in developing countries without access to the facilities required when using radioactive tracers.

Figure 8. Classification visualisation (using Random Forest method) and histological slice annotations for in vivo patient scans. Labels for homogeneous regions (healthy/cancer only) were employed for classification training. Clear contrast can be seen between the healthy and cancerous regions. Scale bar corresponds to 5 mm in all cases. Reproduced with permission from [146].
Alternative dyes are also being investigated. ICG has been shown to be a promising alternative due to less adverse effects being associated with its use, with studies comparing its use with that of a radioactive tracer with or without a blue dye showing that ICG performs comparably \[164, 165\] and in certain cases better \[166, 167\]. In addition to this, the combination of ICG and MB has also been investigated, where nodal detection rates in breast cancer patients have been shown to be highest with a combination of the two dyes \[168, 169\] compared to the radioactive tracer and MB. Research such as this shows that a move to using dyes only for this application may be possible in the near future, which will be of huge benefit.

4.2. Tumour identification
Another oncological application of fluorescence that is still in its early development stages is in tumour imaging for visualising cancerous tissue during surgery. During cancer surgery it is important that as much of the cancerous tissue is removed as possible while also preventing unnecessary damage to surrounding healthy tissue \[59\]. While surgeons primarily rely on visual inspection, palpation and touch in order to achieve this, this is only really effective for bulk tumour and lacks sensitivity at the cellular level \[170\], which can potentially lead to the patient requiring further surgery. The current gold standard for assessing the removed tissue is slide-based histology, however this is performed post-operatively and is often a time-consuming and laborious process \[171\]. There are certain techniques currently available for estimating tumour margins intraoperatively, such as frozen section histology \[16\], intraoperative MRI, CT and ultrasound. These techniques have their own drawbacks such as requiring highly complex and specialised surgical suites in the cases of intraoperative MRI and CT, as well as other techniques being limited in spatial resolution and contrast compared to post-operative histology \[171\]. New technologies for intraoperative margin assessment need to be able to match the diagnostic accuracy of post-operative histology and bring additional benefits such as reduced turnover times, practicality and reduced costs before they can be considered for routine clinical practice \[172\].

Fluorescence-based techniques have gained significant interest for intraoperative margin assessment in recent years. While not yet in routine clinical practice, the advantages discussed previously such as high contrast and sensitivity as well as ease of image acquisition and relatively low cost make them an attractive alternative to the current options. One of the most commonly utilised fluorescence dyes in intraoperative fluorescence imaging studies is ICG. One recent interesting study has shown that ICG NIR imaging can be used as a complementary technique to the standard visual inspection and palpation skills of a surgeon to assess both the surgical margin before resection as well as the margin status after tumour had been removed in patients with oral squamous cell carcinoma (figure 10) \[173\]. Other recent studies have also shown the potential of ICG for distinguishing between healthy tissue and tumour tissue from patients with lung cancer \[174\], gastrointestinal tumours \[175\] and peripheral nerve sheath tumours \[176\]. Intrinsic fluorophores are also being researched for this application, where an example was discussed earlier involving the use of

![Figure 9. Chemical structures of (a) Isosulfan Blue, (b) Patent Blue and (c) Methylene Blue.](image)
FLIM for visualising NAD(P)H and FAD fluorescence in cell and tissue studies for margin assessment [30, 31].

The development of new dyes as well as improving already approved dyes is also of interest for estimating tumour margins. Antibody-conjugated NIR dyes have already been discussed in section 2.2 as being researched for this application, with other examples of new and improved probes including coating silica shells with ICG to inhibit its rapid diffusion into tissue and improve image quality for colon tumour marking [177], using bevacizumab-800CW for back-table determination of tumour margins in patients with advanced rectal cancer [178] as well as cetuximab-800CW for patients with head and neck cancer [179]. Cancer is a leading cause of death worldwide with over 18 million cases diagnosed globally in 2018, which is set to rise to over 29 million diagnoses by 2040 [180]. The continuous development and improvement of cancer treatment therefore remains of high importance, and utilising fluorescence for estimating tumour margins during surgery could become a routine clinical method for margin assessment in the years to come.

4.3. Angiography

Angiography is an imaging technique for visualising blood vessels, most commonly used for monitoring the vessels around the heart, brain, lungs and kidneys. An angiogram typically involves the administration of a contrast agent—which is usually either barium sulphate or an iodine-based agent—via a catheter into an artery, where the contrast agent allows the clinician to visualise the blood vessels of interest on x-ray.

Figure 10. Monitoring of surgical margins in oral squamous cell carcinoma using ICG, showing (a) preoperative merged images of the surgical site, (b) intraoperative merged images of the tumour beds, (c) postoperative merged images of the tumour samples, (d) pathological results from the tumour sample and (e) a comparison of the fluorescence intensities of the different tissue types. The study showed ICG localisation and clear delineation of the tumour margin in all 20 patients. Reproduced from [173] CC BY 4.0.
images. The technique first came to realisation in the 1920s with the first demonstration of cerebral angiography by Egas Moniz, followed by cardiac catheterisation by Werner Forssman a few years later [181]. Angiography has since become routine in clinical practice.

In terms of utilising fluorescence in angiography, this is achieved using fluorescein sodium for imaging the blood vessels in the back of the eye and examining blood circulation, with the technique being known as fluorescence angiography (FA). The first publication on FA appeared in 1962, where Dollery et al used fluorescein for monitoring retinal circulation and identifying various abnormalities [182]. FA is now routinely used in clinical practice, and as well as monitoring blood circulation it can also be used for the diagnosis of various eye conditions such as macular degeneration, diabetic retinopathy and cancer of the eye. In addition to its routine clinical practice, FA is also the subject of several research studies in recent years with studies focusing on its potential for the diagnosis of other eye problems, such as its combined use with OCT for delineating the anatomy of retinal aneurysms [183] and evaluating inflammation of the middle layer of the eye in paediatric patients being treated with immunosuppressive therapies [184], as well as its combined use with endoscopy for the treatment of abnormal blood vessels on the spinal cord surface [185]. ICG is also becoming an increasingly popular dye to utilise in angiography studies (ICG-FA), where examples of this include its ability for visualising and assessing anastomotic perfusion [61], feasibility studies for ICG-FA in the surgical evaluation of abdominal trauma [186], as well as demonstrating improvement on current detection methods for cutaneous angiosarcomas, a rare type of tumour which show differentiation in the blood vessels [187]. Adopting a NIR dye such as ICG opens up the applications of fluorescence-based angiography to other areas of the body, which may see its future clinical demand increasing.

4.4. Fluorescence endoscopy

Endoscopy is a procedure that has many clinical applications, and is probably most commonly associated with being used to examine the digestive system. Endoscopes are long thin flexible tubes containing a fibre bundle that typically consists of approximately 50,000 optical fibres. The first practical prototype of such fibre bundles came in the 1950s when Basil Hirschowitz demonstrated the use of a flexible fibre optic endoscope [188], and since then has found many applications where it is often used to examine various systems in the body other than the digestive system, such as the respiratory, urinary or female reproductive tracts, as well as be used in the detection of various types of cancer such as bronchoscopy for the diagnosis of lung cancer [189], colonoscopy for detecting colorectal cancer [190] and laparoscopy for diagnosing liver, pancreatic and ovarian cancer [191].

While most endoscopy procedures predominantly involve the use of white light alone, the combination of fluorescence with fibre-based procedures can also be used. The potential for such a combination was first realised in the early 1990s when laser-induced fluorescence was used to identify gastrointestinal abnormalities from tissue spectra obtained in vivo [192, 193]. While this combination is not currently in routine clinical practice, some potential future applications include its use in tumour margin assessment in particular, where it has been shown to improve sensitivity and accuracy in the detection of vascular endothelial growth factors in residual tumour tissue from rectal cancer patients in comparison to MRI and white-light endoscopy [194]. It has also been used in label-free FLIM imaging of precancerous and cancerous oral lesions [195] and for distinguishing cancerous and normal tissues of the head and neck using acrilavine [196].

4.5. Other applications

While the previously described applications are the main uses of fluorescence in surgery, other less common applications do exist and various future applications of FGS are presently under consideration. One of the less common applications is structural imaging, where the use of fluorescence can allow for the visualisation of structures such as nerves, ureters and those in the bile duct and therefore minimise any unnecessary damage to these structures during surgical procedures. Some demonstrations of utilising fluorescence in this manner for these regions of the body include using ICG during laparoscopic cholecystectomy for visualising the cystic duct for preventing bile duct injury [197], using ICG in reducing the risk of ureteroenteric anastomotic strictures in bladder cancer patients receiving radical cystectomy and urinary diversion [198], as well as demonstrating the potential of a nerve-binding fluorophore for visualising nerves and reducing intraoperative nerve injury [199]. In addition to these, the potential of fluorescence has been demonstrated for visualising other anatomical structures in the body such as the parathyroid glands for assessing vascularisation using ICG [200] as well as using label-free NIR autofluorescence imaging [201], and also for visualising other areas of the lymphatic system such as the lymph vessels, where it has for example been used for improving the treatment of lymphedema [202]. This research shows that fluorescence can be exploited for visualising a large variety of structures in the human body for improving surgical outcomes for patients.

One newer surgical technique where fluorescence could prove to be extremely beneficial is robotic surgery. Robotic or robot-assisted surgery was first introduced to the operating theatre over 30 years ago and is
5. Summary & perspectives

There remains a huge amount of interest and potential in fluorescence-based techniques for a variety of surgical applications. The ability to use fluorescence in the detection of information at the molecular level is the clear advantage for adopting its use into routine clinical practice, as this cannot be achieved using white light alone. While there are a few FDA-approved fluorophores and some routine surgical applications that are currently in use, fluorescence-based techniques are very much in their infancy in terms of routine use in clinical practice. However, the promising amount of research into developing these techniques indicates that they may be exploited more and more in the years to come, with oncology-based applications looking to be the primary focus due to the high demand for improvement in this area. Fluorescence may be best exploited in the surgical environment when used as a complementary tool to already existing surgical techniques rather than fully replacing these techniques, for example when using new dyes with already available surgical instrumentation such as endoscopes, or exploiting information from intrinsic probes in conjunction with already approved fluorophores. The field continues to progress at an extremely fast pace, where the weaknesses previously discussed in the key areas of fluorophore and instrumentation development are those which need attention from future research. Bridging the gap between these two areas will also be important for the development of this field, where dedicated and collaborative input from engineers, clinicians, biologists, chemists as well as industrial partners will be crucial for addressing these needs.

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Data availability statement

No new data were created or analysed in this study.

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