Use of Fluorescent Dyes in Endoscopy and Diagnostic Investigation

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Keywords

Optical contrast techniques · Real-time optical diagnosis · Fluorescent tracers · Visualization of specific biochemical processes

Abstract

Background: The advancement of innovative endoscopic technology in terms of improving the visualization of the mucosa has been of significant benefit. **Summary:** Advancements in image resolution, software processing, and optical filter technology have resulted in several techniques complementary to traditional white light endoscopy. These new techniques provide a real-time optical diagnosis as well as virtual histology of detected lesions. Optical molecular imaging permits a functional assessment within cells. **Key Message:** Optical molecular imaging provides an understanding of cellular processes and permits validation of the specificity of fluorescent tracers and the possibility of quantifying the signal.

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Advancements in image resolution, software processing, and optical filter technology have resulted in the commercial availability of high-definition endoscopy as well as optical contrast techniques such as narrow-band imaging, flexible spectral imaging color enhancement, and i-scan.

Along with autofluorescence imaging and confocal laser endomicroscopy, these techniques complement traditional WLE. They have the potential to serve as red flag techniques in terms of improving the detection of mucosal abnormalities. The techniques provide a real-time optical diagnosis as well as virtual histology of detected lesions.

Optical molecular imaging (OMI) permits organelle staining in live cells as well as their functional assessment to confirm the identity of specific proteins or targets within cells. It also provides a better understanding of cellular processes and permits validation of the specificity of fluorescent tracers, including the possibility of quantifying the signal.

Introduction

White light endoscopy (WLE) has been the gold standard for the detection of lesions in the gastrointestinal tract. The rapid advancement of innovative endoscopic technology in terms of improving the visualization of the mucosa has been of significant benefit.

Multimodal Endoscopy

During WLE, broadband white light in the visible spectral region illuminates the tissue, and images are acquired in the reflectance mode. WLE detects lesions based on structural changes or discoloration of the epithelial surface and may be used to guide the acquisition of tissue biopsies [1, 2] (Fig. 1).

Fig. 1. White light of a flat colonic lesion.

Despite the efficacy of current endoscopy, small and inconspicuous lesions or serrated adenomas tend to be flat and do not exhibit detectable discoloration relative to normal tissue in the three reflectance color channels (red/green/blue, RGB) used in WLE [1]. This makes the detection of disease and the acquisition of targeted biopsies with conventional WLE a challenging issue [1].

Missed lesions are a main risk factor for advanced or interval cancers, particularly in high-risk patients such as those with the Lynch syndrome. Consequently, there is strong clinical interest in the development of new endoscopic screening methods that may enhance contrast for precancerous or inflammatory lesions, permitting more targeted biopsies and improving diagnostic performance [1–8].

Many clinical endoscopes acquire spectral data. However, the added value of methods such as narrow-band imaging (two illumination bands and RGB detection), autofluorescence imaging (two illumination bands and monochrome detection), or trimodal imaging (combination of narrow-band imaging and autofluorescence imaging with high-definition WLE) have been extensively investigated, but were not associated with any significant improvement in adenoma detection rates [4, 9–11].

On the other hand, modern multimodal endoscopy can potentially improve the diagnostic performance of endoscopy by simultaneously addressing numerous contrast mechanisms [12]. The application of a targeted fluorescent contrast agent, consisting of a fluorescent dye conjugated to a targeting moiety, designed to highlight a biological process that is dysregulated in the diseased area, is referred to as “optical molecular imaging” (OMI). OMI was found to be promising in terms of better endoscopic inspection of the gastrointestinal tract [1, 3, 13]. In clinical endoscopic studies, OMI has served as a “red flag

technique,” prompting targeted tissue biopsy in studies of colorectal and esophageal disease [4, 14] (Fig. 2).

Molecular imaging allows dynamic as well as quantitative visualization of specific biochemical processes. It has been useful for early-stage diagnosis. Furthermore, its curative effect in monitoring disease, drug development, gene therapy, and other fields has also been proven. Its limitations stem from a paucity of multifunctional molecular imaging agents, the limitations of imaging technology (such as poor sensitivity to complement contrast agents), and the lack of nonradiative molecular imaging that can provide both structural and functional information.

Principles of Fluorescence Imaging

Fluorescence diagnostic imaging is a relatively new technique that involves the use of a photosensitizing drug to visualize lesions through tissue fluorescence.

Fluorescence molecular endoscopy (FME) or fluorescence imaging is based on the molecular absorption of light: a region of interest containing the fluorophore is excited at a specific wavelength, and light at a different wavelength is emitted. Each fluorophore has a characteristic excitation spectrum that is identified by monitoring the fluorescence emission while the fluorophore is excited by a range of consecutive wavelengths [15, 16].

Fluorescence imaging is known for its high detection sensitivity, specificity, and spatial resolution [17].

When electrons go from an excited state to the ground state, there is loss of vibrational energy. The emission spectrum is shifted to longer wavelengths than the excitation spectrum (a phenomenon known as “Stoke’s shift”) [18, 19]. The emission intensity peak is lower than the excitation peak. In order to achieve maximum fluorescence intensity, the fluorophore is usually excited at the peak wavelength of the excitation curve, and the emission detection is typically the peak wavelength of the emission curve. The fluorescence method is regenerative and permits longitudinal imaging, unlike the radionuclide decay process in nuclear imaging [20].

Fluorescence imaging can use intrinsic tissue properties such as contrast (autofluorescence) and also image exogenous contrast agents. The three primary processes that typically govern the interaction of photons with tissues are light absorption, light scattering, and fluorescence emission. In short, light absorption and scattering decrease with increasing wavelength. Below 700 nm, tissue absorption results in a low penetration depth of light (a few millimeters), allowing no more than a superficial assessment of tissues in the visible wavelength. The light absorption is caused by oxy- and deoxyhemoglobin, melanin at wavelengths <700 nm, lipid, and water at wavelengths >1,000 nm [18, 19].

Fig. 2. White light (**A–C**) and hypericin fluorescence (**D–F**) endoscopic images of normal (**A, D**), hyperplastic (**B, E**), and SCC (**C, F**) tongues. The fluorescence images show a progressive increase in the red-to-blue (R/B) intensity ratios from normal (R/B ratio 0.3) to hyperplastic (R/B ratio 1.0) to SCC (R/B ratio 2.0) tissue. Copyright: Thong et al. [76].

Since the absorption coefficient of tissue is significantly lower in the near-infrared (NIR) region (700–900 nm) than in the visible region, NIR light can penetrate to a depth of several centimeters into tissue [21, 22]. A major goal of optical imaging has been the development of suitable targeted NIR fluorochromes with high molar extinction coefficients, good quantum yields, and specific tissue binding [21, 22].

OMI and Contrast Agents

The effective implementation of molecular imaging and treatment approaches requires the identification of target proteins that can detect aberrant cells with high sensitivity. DNA changes that occur in premalignant and malignant lesions must be addressed in order to identify relevant target proteins. The difference between these lesions and their healthy surrounding cells is that the former exhibit genomic alterations that might lead to downstream changes in gene expression levels. These changed gene expression levels influence the downstream cellular phenotype, and thus affect protein expression levels [23, 24].

Nevertheless, these genomically driven changes in gene expression levels are subtle and usually overshadowed by nongenetic processes, such as the circadian rhythm or postprandial metabolic influences [25].

Due to the minimal NIR fluorescence contrast generated by most tissues, *in vivo* studies require exogenous contrast agents. Such targeted molecular agents consist of a signal component and a targeting component. The con-

trast agents must also have adequate contact time with the target for the binding to occur, and be retained by the target while nonbound material is cleared. Unconjugated organic NIR fluorophores are typically less than 1.2 kDa, but have widely different bio-distributions and pharmacokinetics, depending on their charge and the properties of their conjugated targeted molecule. As the charge per molecule is increased, fluorophores remain extracellular and their plasma half-life increases proportionally [26]. Clearance is typically accomplished by a combination of renal filtration and excretion into bile.

Many OMI contrast agents are currently under pre-clinical and clinical investigation, and novel products of better quality are being developed [1, 4, 27, 28]. For efficient dye-based fluorescent tracing *in vivo*, the dye must efficiently and homogeneously stain the cells without affecting their viability or function, while also transferring to daughter cells. Additionally, the stain should not be transferred to adjacent resident cells, as this would render it useless for tracing purposes.

One important class of organic fluorophores is indocyanine. Indocyanine green (ICG), one of the most common of these, was approved by the FDA for human use in 1958. It is one of the least toxic agents administered to humans, with the only adverse reaction being rare anaphylaxis. Furthermore, ICG is tetra-sulfonated; this increases its solubility and aqueous quantum yield [29]. The excitation peak of this class lies between 760 and 800 nm, and its emission peak between 790 and 830 nm [30, 31].

Another important consideration for *in vivo* molecular probes is toxicity. Without charged groups, indocya-

nines can be quite toxic because of their intracellular accumulation [32]. However, disulfonated ICG has been used in humans for over 50 years and has an excellent safety profile. ICG has been employed for a variety of applications, including angiograms of the eye and brain, the determination of postresection tumor margins, and the identification of gastrointestinal tract lesions, to name a few [30, 33–35].

Although ICG provides a contrast mechanism, its enhanced permeability and retention create limitations. For this reason, and for the identification of specific pathological tissues, interest in the development of tumor-specific optical contrast agents has been growing rapidly [1, 4, 27, 28].

Today we have a plethora of targeting components, such as a small molecule, peptide, antibody, or aptamer, which are applied to ligand-directed imaging agents.

Overview of Ligand-Directed Imaging Agents

The success of a fluorescent tracer as a useful imaging tool is dependent on several characteristics; these include the different types of synthetic fluorophores and their administered quantities, as well as the targeting moiety and the type of “carrier molecule” [36]. Several types of molecules can be deployed as dye carriers. Monoclonal antibodies, but also peptides, lectins, antibody fragments, or nanoparticles can be used to form the “backbone” of the tracer. All of these carrier molecules have their specific pros and cons; monoclonal antibodies are highly specific and permit specific targeting, but their high molecular weight (approximately 150 kDa) leads to slow delivery, while their long half-life and slow wash-out cause a high background signal [37].

Peptide tracers possess favorable properties for *in vivo* use, as these are low-molecular-weight molecules providing high clonal diversity. Moreover, their immunogenicity – and their costs – are low. However, since small peptide tracers “wash out” relatively quickly, high doses of the tracer need to be administered in order to achieve adequate accumulation. Other disadvantages include their variable affinity, the consequent risk of their lack of efficacy, and uncertainty of appropriate targeting [37, 38]. As binding sites and targeting moieties can be affected by conjugation with dye molecules, monoclonal antibodies or lectins of a relatively large size (20–200 kDa) are less prone to these risks. This results in more dye attachment and a higher fluorescence intensity per unit. Last but certainly not least, fluorescent tracers differ in terms of their fluorescent “status.” A distinction can be made between “always-on-probes” and so-called “smart activatable probes.” The first type of tracer irradiates fluorescence nonselectively when excited by light from a specific wave-

length. In other words, its specificity and utility are bio-distribution dependent, and its affinity for binding to the target site compared to the accumulation of unbound or nonspecifically bound agent determines the tumor-to-background signal and its distinctiveness [37–39]. In contrast, “smart activatable probes” are unique because these agents first need to be activated by specific biomarkers located at the target site in order to become excited. In their original state their fluorescent activity is repressed by quenching of the dye molecules, rendering the fluorophore undetectable. After dequenching, for example due to cleavage by proteases, these probes show a significant increase in fluorescent activity [38–41]. This “activatable” feature creates improved target-to-background ratios, which is highly relevant for small lesions and for early detection of lesions. Therefore, this “activatable” feature makes these tracers interesting for future clinical application and further research, particularly in the field of endoscopic imaging. Furthermore, targeted fluorescent contrast agents are advantageous because they combine reflectance-based imaging in the visible wavelength range with exogenous targeted fluorescent contrast agents in the NIR wavelength, showing a relatively high signal-to-background ratio [1, 13, 42–44].

Fluorescent dyes excited by light in the NIR spectrum (665–900 nm), also known as the so-called “NIR window,” are considered optimal because they create minimum tissue autofluorescence and provide maximum tissue penetration.

Previous studies have employed fluorophores excited by light from the visible spectrum (350–550 nm; such as FITC), which produce a high autofluorescent signal and hence high background signals or “visual noise” [4, 27, 45]. The low autofluorescence of human tissue in the NIR light spectrum improves target-to-background ratios. Secondly, NIR wavelengths penetrate deeper into the target tissue because they are less prone to interference due to tissue absorption (such as hemoglobin) and tissue scattering [46]. Finally, NIR fluorescence enables the investigator to concurrently capture visible white light and thus superimpose fluorescent signals on morphologic images in real time.

Multi- or Hyperspectral Imaging

Molecular optical imaging may be defined as visualizing the molecular signature of cells *in vivo* and the biological response (such as upregulation of a particular cell surface receptor) that may precede visible structural changes. However, the limited spectral range of these methods provides no more than a qualitative picture of the epithelium. Methods with finer sampling of the spectral response, such as multi- or hyperspectral imaging (MSI/HSI) systems (≈ 10 and 100 spectral color channels,

Fig. 3. Images acquired during ex vivo molecular fluorescence colonoscopy of IgG-800CW (I) and bevacizumab-800CW (II) targeted tumors (3.3 mm in size). Endoscopy images were obtained with video endoscope and fiber bundle. White-light, fluorescence, and composite images of fiber bundle were real-time-projected. Copy-right: Tjalma et al. [28].

respectively) may permit quantitative assessment of functional tissue properties and more detailed statistical classification of the spectral changes that occur during disease [43, 44]. The use of reflectance-based MSI/HSI systems in biomedical applications has, for example, been shown to increase contrast in vascular investigations, wound healing, ophthalmology, cancer diagnosis, and for the determination of tumor resection margins [47–57].

These potential molecular targets and the promising approach of using molecular targeted tracers for advanced, noninvasive therapeutic purposes in the near future constitute a truly theranostic approach.

Molecular Targets

Cells contain subcellular structures and signaling substances responsible for carrying out essential functions for cell survival. Due to their specialized functions and well-defined nature, these structures should ideally be labeled in cell biology studies. More specifically, the use of specific dyes permits selective staining of specific organelles and signal substances without increasing cytotoxicity. Specific staining in live cells can help scientists to confirm the identity of specific proteins or targets within cells and thus achieve a better understanding of cellular processes.

Based on the established role of vascular endothelial growth factor A (VEGF-A) in tumor angiogenesis, it is an early target protein for molecular imaging of premalignant and malignant gastrointestinal lesions [58]. Since solid tumors cannot grow beyond 2–3 mm³ without adequate blood supply, most human cancer types express high levels of VEGF-A [58–63]. Therefore, elevated VEGF-A levels are present in early dysplastic and malignant gastrointestinal lesions. VEGF-A levels increase throughout the dysplasia-carcinoma sequence, also known as the so-called early angiogenic switch [64, 65]. VEGF-A is markedly overexpressed specifically in colorectal lesions that are easily missed during endoscopy, such as SSA/P lesions and Lynch adenomas.

The ability to visualize colonic lesions in the simulation model, in combination with the good manufacturing practice-based production of 800 CW-labeled antibodies, should facilitate rapid adoption of this technique in the clinical setting (Fig. 3).

Quantitative fluorescence endoscopy (QFE) is a new technique that can visualize and quantify fluorescently tagged tumor tissue [66]. In one study, patients with locally advanced rectal cancer received neoadjuvant chemoradiotherapy and then underwent surgery for local disease control (Fig. 4).

The identification of patients with complete clinical response before surgery is a subject of increasing interest because the nonsurgical management of these patients is associated with high survival rates, reduced morbidity, and improved functional outcomes [67–71]. However, assessing tumor response after neoadjuvant chemoradiotherapy is challenging. QFE can target VEGF-A to detect a residual tumor in the rectum after neoadjuvant chemoradiotherapy. Thus, VEGFA-targeted QFE can be of additional value in restaging patients with locally advanced rectal cancer.

QFE detects significantly higher fluorescence in tumor compared with normal rectal tissue and fibrosis, and improves the predictive power of the final pathology results in 16% of patients compared with standard MRI and WLE [72].

Interestingly, 15–27% of patients had a complete response on pathological investigation [2, 3]. In untreated patients, QFE clearly showed enhanced fluorescence in all rectal tumors compared with normal rectal tissue.

In conclusion, the results of preliminary pilot studies, even in a small group of patients, are encouraging and constitute a first step towards QFE for tumor response evaluation after neoadjuvant treatment [66].

Fluorescent imaging can be also used in for making decisions with regard to treatment response. Tumor necrosis factor (TNF) is a cell signaling protein (cytokine) in systemic inflammation, known to be involved in regulating immune cells and generally overexpressed in inflammatory bowel disease [73]. For this reason, TNF in-

Fig. 4. Representative images of the quantitative fluorescence endoscopy (QFE) procedure after neoadjuvant chemoradiotherapy of a patient with residual tumor (**A**), submucosal tumor (**B**), mucosal high-grade dysplasia (HGD) (**C**) and a pathological complete response (**D**). From left to right: a high-definition white-light video endoscope image of the rectal tumor; a white-light image from the QFE fiberoptic, followed by the corresponding near-infrared (NIR) fluorescence image captured with an exposure time of 100 ms and the composite image of both modalities. The maximum quantified fluorescence value is depicted on the NIR fluorescence image. The rightmost column depicts an HE staining of the surgical specimen in which the pathological TNM stage is indicated. Copyright: Tjalma et al. [66].

hibitors are commonly used therapeutic agents. However, some patients do not respond to these.

A fluorescent antibody directed against TNF has already been tested in a clinical trial in Crohn's disease patients to predict response to subsequent anti-TNF therapy. It could be shown that this diagnostic approach of performing TNF-targeted NIR-FME at baseline was able to predict clinical and endoscopic response to therapy based on the amount of mucosal TNF expression. Thus, patients who are likely to benefit from anti-TNF treatment regimens can be selected accordingly [74, 75]. Similar molecular endoscopy approaches have already been successfully tested ex vivo to predict therapeutic response to therapy with the anti $\alpha_4\beta_7$ integrin antibody vedolizumab in Crohn's disease patients [73].

Conclusion

One of the prime objectives of the study was to facilitate the use of FME in the clinical setting and illustrate the potential of FME as a means of improving the detection of gastrointestinal lesions. A further aim was to visualize the molecular signature of cells in vivo and their biological response.

FME is certainly a milestone in this field, but technical improvements will be needed before the procedure can be integrated into clinical routine. Further research in larger study populations will be required in order to verify the advantages of FME in terms of detection rates and surveillance strategies. Furthermore, current research has yielded promising molecular targets for future use. These

novel targets can help to improve imaging sensitivity and specificity. Last but not least, the theranostic application of molecular-targeted antibodies will permit selective cancer treatment.

The results of preliminary pilot studies are encouraging and potentially a first step towards QFE for the evaluation of disease response after treatment.

Disclosure Statement

A. Hoffman has no conflicts of interest to declare. M.F. Neurath has acted as a consultant for pharmaceutical, device, or biotechnology companies: Bionorica SE, e.Bavarian Health GmbH, Boehringer Ingelheim GmbH and Co. KG, F. Hoffmann La Roche GmbH, Genentech Inc., Hexal AG, Index Pharmaceuticals AB, Janssen-Cilag GmbH, MSD Sharp and Dohme GmbH, Pentax Eu-

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Author Contributions

A. Hoffman wrote the manuscript and was responsible for the literature research. R. Atreya, T. Rath, and M.F. Neurath gave advice, particularly in the molecular imaging section.

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