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### Advanced endoscopic imaging of esophageal neoplasia; old looks and new visions

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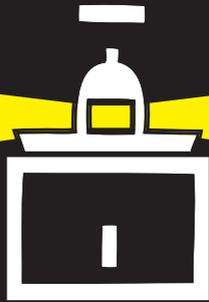
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## FLUORESCENCE IMAGING FOR THE DETECTION OF EARLY NEOPLASIA IN BARRETT'S ESOPHAGUS; OLD LOOKS OR NEW VISION?



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Submitted

## ABSTRACT

Early neoplasia arising from Barrett's esophagus is often small, focally distributed and endoscopically poorly visible, and random four-quadrant biopsies may easily miss early lesions. Advanced imaging techniques, such as (auto)fluorescence based modalities, aim to increase the detection rate of early lesions or the yield of random biopsies.

Fluorescence based light-tissue interaction has been successfully designed in point-probe differentiating spectroscopy systems or integrated into wide-field endoscopic systems such as autofluorescence imaging (AFI).

In this review, we discuss the most recent advances in fluorescence spectroscopy and imaging for detecting early Barrett's neoplasia. A spectroscopy probe, integrated into a regular biopsy forceps, was shown to offer decent discriminatory capabilities, while ensuring spot-on correlation between the measured area and the corresponding histology. With this tool, surveillance endoscopy with random biopsies may become more efficient and sensitive.

AFI was shown to increase the targeted detection of early neoplasia. However, random biopsies could compensate for this effect. The clinical impact of AFI on the diagnosis and treatment of early neoplasia is limited, yet AFI may offer a novel approach in biomarker-based risk-stratification models. Moreover, in combination with new, readily available contrast agents such as fluorescent lectins, fluorescence imaging may receive renewed interest.



## INTRODUCTION

The incidence of esophageal adenocarcinoma in the western world has increased sixfold over the past three decades<sup>1</sup>. Barrett's esophagus (BE) has been recognised as one of the major factors involved in its carcinogenesis, associated with an 30-100 fold increased risk for the development of adenocarcinoma<sup>2</sup>. In BE, the regular squamous epithelium of the esophagus is replaced by columnar lined epithelium as a result of long standing gastro-esophageal reflux disease (GERD). The development of adenocarcinoma in Barrett's esophagus occurs in a sequence of gradually evolving, histologically recognizable steps: intestinal metaplasia, low grade intraepithelial neoplasia (LGIN), high grade intraepithelial neoplasia (HGIN) and eventually early adenocarcinoma (EAC)<sup>3,4</sup>. These intermediate grades of dysplasia carry an increasing risk of progression to EAC. HGIN and mucosal cancer have a low risk of lymph node metastasis and can be treated endoscopically with an excellent prognosis<sup>5</sup>. Therefore, in patients with known BE, regular surveillance endoscopy with random biopsies according to the Seattle protocol is recommended to detect these early neoplastic lesions at a curable stage<sup>6</sup>.

However, early neoplasia in BE is often small, focally distributed and endoscopically poorly visible. Random four-quadrant biopsies may easily miss early lesions, since only about 5% of the Barrett's segment is sampled<sup>7</sup>.

## ADVANCED ENDOSCOPIC IMAGING

Recent technological advances have opened up possibilities of integrating more complex optical modalities in the endoscope. Influenced by differences in biochemical and structural characteristics, the interaction between light and tissue offers the ability to identify unique, tissue-dependant optical properties. The challenge is to uncouple nonspecific physiological optical differences from specific signals due to pathological processes, and capture these with a clinically applicable tool. In order to improve the detection of early BE neoplasia, many optical technologies have been studied, one of which is fluorescence imaging. In this overview we discuss the current role of fluorescence imaging for the endoscopic detection of neoplasia in BE.

### Autofluorescence imaging

Autofluorescence imaging is based on the principle that certain endogenous substances, such as NADH (nicotinamide adenine dinucleotide), FAD (flavin adenine dinucleotide) and collagen emit light of longer wavelengths when excited with short wavelengths of light (i.e. blue light). When an incoming photon excites an electron of these fluorophores to a higher energy state, the electron will subsequently relax back into its ground state and emit a photon of lower energy, thus of longer wavelength. Each fluorophore has a characteristic emission range of wavelengths as a function of a given excitation wavelength, called the fluorescence spectrum. The emitted fluorescence spectra are highly dependent on the concentration, distribution and biochemical status of the fluorophores. In addition, structural alterations due to changed tissue architecture – thickening of the mucosa, increased number of bloodvessels, enlarged nuclei – influence the optical characteristics. Fluorescence spectroscopy is the technique to

induce, measure and quantify the fluorescence characteristics of tissue. Spectroscopy studies using this autofluorescence principle have shown that neoplastic tissue in BE demonstrates decreased autofluorescence intensity in the green spectrum and increased intensity in the red spectrum, compared to non-neoplastic BE tissue<sup>8,9</sup>. However, spectroscopy studies and the clinical application in daily endoscopic practice have been hampered by the small field of investigation with the point probe spectroscopy techniques used. Most spectroscopy systems typically image only 1 mm<sup>2</sup> of the tissue of interest, whereas Barrett surveillance endoscopy would really benefit from a wide-field imaging technique that is a “red-flag” for areas of concern.

### **Endoscopic autofluorescence imaging**

Autofluorescence (AF) as an endoscopic wide-field detection tool was first introduced by Xillix Technologies Corp. The light-induced fluorescence endoscopy system (LIFE) was composed of a fiber optic endoscope with two light sources and two camera modules, to capture the white light and autofluorescence images. The AF camera contained two charge coupled devices (CCD); one for the detection of green AF (490-550nm) and one for red AF(>590nm). The two AF signals were subsequently processed into a real-time pseudocolor image, projected onto a monitor. The first feasibility studies with this system suggested that LIFE might improve the detection of early neoplasia in BE<sup>10,11</sup> but this was contradicted by a subsequent randomized crossover study with the LIFE endoscopy system<sup>12</sup>. This was the first study in the field of endoscopic imaging for Barrett's that used a randomized crossover design; patients were randomized for their first imaging endoscopy with either the LIFE system or standard video endoscopy. Six weeks later, a second imaging endoscopy with the other system was performed by a second endoscopist who was blinded for the results of the first endoscopy. With this design, each patient acts as his or her own control and possible bias in terms of information available to the endoscopist is effectively eliminated.

In this study, no additional value for the detection of early neoplasia was found for LIFE. This was due to a high number of false positive findings caused by a high sensitivity for background inflammation and the incorporation of LIFE in a fiberoptic system, with an inferior white light performance compared to video endoscopy.

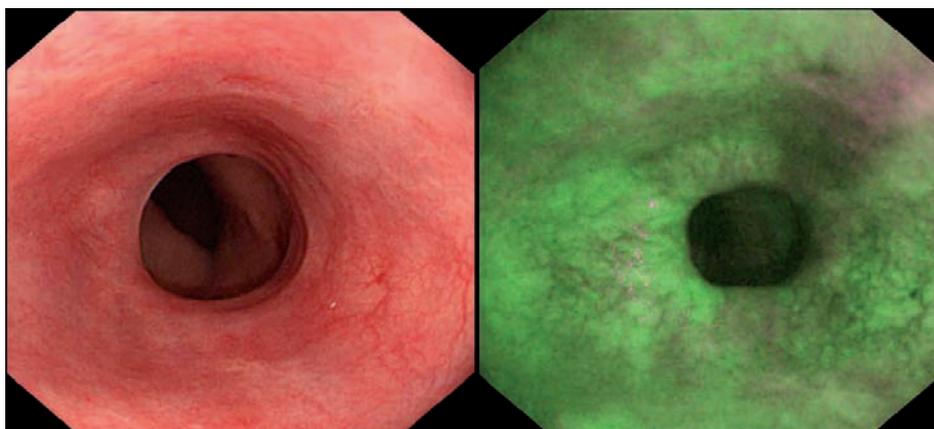
These limitations were taken into account in the development of the first video-autofluorescence imaging (AFI) system. This system represented a major step forward in image quality by combining autofluorescence endoscopy with high-resolution white light video-endoscopy (HR-WLE). Subtle mucosal abnormalities that were overlooked by fiberoptic endoscopy, could now be detected with white light imaging. The AFI mode of the endoscope used a combination of blue light excitation for autofluorescence, green and red reflectance, and a high resolution CCD for improved image quality. The first report on AFI showed promising results with an increased detection of HGIN/EAC by AFI over WLE from 63% (14/22) to 90% (20/22) in 60 patients<sup>13</sup>, however, with a false positive rate of 51%. AFI was therefore combined with narrow band imaging (NBI) as a differentiation tool. NBI uses superficially penetrating blue light to enhance the contrast of the mucosal and vascular patterns. A feasibility study on WLE and first generation AFI, followed by NBI for detailed inspection of lesions suggested that the false positive rate could be reduced to 10%, supporting the concept of combining these techniques<sup>14</sup>.



Subsequently, these three imaging modalities were combined into one “endoscopic trimodal imaging” (ETMI) system. In this system, high resolution WLE, second generation AFI and NBI can be rapidly alternated with a simple switch on the endoscope handle. In this second generation AFI algorithm, the image was composed of total emitted autofluorescence after blue light excitation (390-470nm) and green reflectance (540-560nm) (*figure 1*).

In a multicenter feasibility study, ETMI showed promising results: AFI improved the detection of early neoplasia with 45% and detailed inspection with NBI reduced the false-positive rate from 81% to 26%<sup>15</sup>. To investigate the true value of ETMI, two multicentre, randomized crossover trials were conducted, comparing ETMI to standard video endoscopy (SVE)<sup>16,17</sup>. The first trial was performed in tertiary referral centres, in a high risk population of patients who were referred for work-up of early BO neoplasia<sup>16</sup>. Although ETMI demonstrated a significantly increased targeted detection of HGIN and EAC, the overall yield of ETMI (targeted + random biopsies) was not significantly better compared to SVE (targeted + random biopsies). Moreover, the yield of targeted biopsies by ETMI was inferior to the overall yield of SVE plus random biopsies, meaning that one still cannot depart from the requirement to obtain random biopsies. Detailed inspection with NBI brought the false positive rate down from 71% to 48%, yet misclassified 17% of HGIN/EAC lesions as not suspicious.

To correct for the selection bias in the high risk population and the influences of highly experienced endoscopists, a second crossover trial was conducted in community hospitals, including only Barrett’s patients with an intermediate risk profile of confirmed LGIN<sup>17</sup>. Again, an increased *targeted* detection of HGIN/EAC was achieved with ETMI, but no difference in the overall histological yield (targeted and random biopsies) between ETMI and SVE was shown. In this study, both ETMI and SVE produced even higher false positive rates, reflecting the lower prevalence of early neoplasia in the study population and the limited exposure of the endoscopists to early Barrett’s neoplasia. Inspection with NBI reduced the high false positive rate



**Figure 1.** Image of an area with high grade intraepithelial neoplasia (HGIN), nearly invisible on WLE (left), which clearly stands out of AFI (right).

of ETMI to a lesser extent: from 90% to 52% and misclassified one neoplastic area as unsuspecting. These randomized crossover studies therefore demonstrated the limited clinical applicability of AFI and NBI for the detection and differentiation of early neoplastic lesions in BE.

### **Clinical impact of autofluorescence imaging**

The results of these two randomized crossover trials were disappointing, given the anticipated benefit of AFI as a “red-flag technique”, as reported in earlier feasibility studies. However, these studies only investigated the diagnostic yield of AFI and did not assess the implications of primary AFI detected lesions on subsequent therapeutic management and clinical decision making.

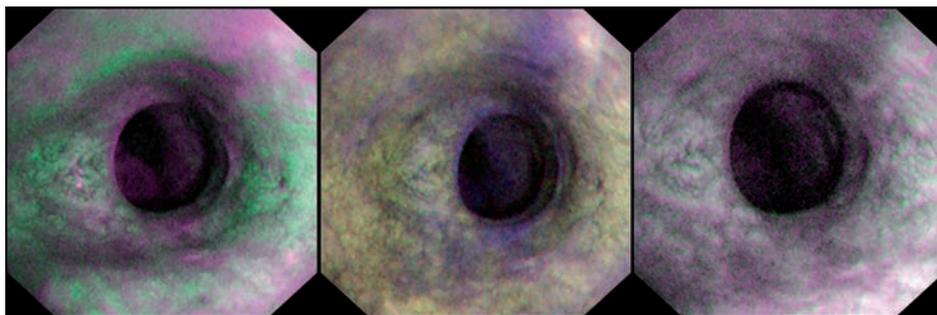
The cornerstone of endoscopic treatment in early Barrett’s neoplasia is endoscopic resection of all visible abnormalities (Paris classification 0-IIa, 0-IIc or combination<sup>18</sup>)<sup>19</sup>. Flat lesions (Paris class 0-IIb) or focal neoplasia detected on random biopsies can be adequately eradicated using radiofrequency ablation<sup>20</sup>. AFI may detect additional lesions that otherwise would have been missed with WLE and/or random biopsies (diagnostic value) leading to a clinically relevant change in treatment strategy (therapeutic value).

In a pooled analysis including follow-up data on (endoscopic) treatment, we evaluated 5 prospective trials on AFI, to investigate the clinical relevance of AFI for the diagnosis of neoplasia and the clinical impact of AFI on therapeutic decision making in 371 patients<sup>21</sup>. In those patients where AFI was used for surveillance of non-dysplastic BO, only 2% of cases that were diagnosed with HGIN/EAC were detected with AFI alone and missed by WLE and/or random biopsies.

In those patients where AFI was used in the work-up of early neoplasia, 57 patients had one or more HGIN/EAC lesions detected with AFI, that were not primarily seen with WLE. In 26 patients, the AFI lesions were ablated with RFA. All patients achieved complete remission of neoplasia and no recurrent neoplasia was seen during follow-up (median follow-up: 45 months). In 31 patients AFI prompted endoscopic resection (ER) of the AFI detected lesion. Histological assessment of the resection specimens did not reveal submucosal invasion, poorly/non-differentiated cancer or lymphovascular invasion. These data suggest that, after adequate inspection with WLE, lesions identified with AFI rarely contain more advanced stages of neoplasia that have an impact on the clinical decision making.

### **Third generation autofluorescence imaging**

The aforementioned disappointing reports triggered renewed attention to the AFI algorithm, and recently a new ETMI system was developed. This third generation AFI system is based on a dual wavelength excitation algorithm using three components to create the pseudocolor images: autofluorescence after 380-390nm excitation, autofluorescence after 400-420nm excitation and green reflectance (540-560nm). By narrowing the two specific bands of excitation light, it was hypothesized that biochemical changes in malignant cells would be specifically targeted, rather than focussing on mucosal architectural changes and submucosal fluorescence (*figure 2*)<sup>22</sup>. A recent feasibility study was performed, comparing the second and the third generation AFI in a mixed population of Barrett’s patients. Second and third generation AFI increased the targeted detection of neoplasia from 47% to 79% and from 47%



**Figure 2.** Third generation AFI image of a neoplastic area, showing 3 variations of the image composition. The last modus (right) creates the image by combining 3 channels of pure autofluorescence, while the first two (left, mid) are composed of dual bandwidth autofluorescence and a reflectance channel to increase image contrast.

to 89% respectively. Yet, both systems had a false positive rate of 86%. These results do not suggest that third generation AFI performs significantly better than second generation AFI<sup>23</sup>.

## FUTURE PERSPECTIVES

### Biomarkers and autofluorescence imaging

There is a need for better risk stratification of Barrett's patients. Hopes are set that a biomarker or a panel of biomarkers may replace histology and allow better and more objective identification of patients who are at low or high risk of progression to cancer<sup>24,25</sup>. The low-risk group could potentially be discharged from endoscopic surveillance, while high-risk group may undergo intensified follow-up, or even prophylactic ablation therapy.

Despite recent efforts, biomarkers have not yet reached implementation into clinical practice. One of the drawbacks of molecular biomarkers in terms of clinical applicability is related to the presumed molecular heterogeneity of BE, with adjacent crypts demonstrating hallmarks of separate clonal origin<sup>26 (27,28)</sup>. In the presence of a heterogeneous disease, the reliability of biomarkers assessed on random biopsies might fall significantly short, since random biopsies are subject to sampling error during standard endoscopy<sup>7,29</sup>. AFI false positivity may relate to early cellular changes that alter the fluorescence characteristics, based on the accumulation of molecular abnormalities prior to the morphological development of dysplasia. We hypothesized that AFI may therefore allow targeted detection of areas with a higher rate of molecular abnormalities. The apparent disadvantage of AFI – a high false positive rate – may thus actually serve as a detection tool of very early markers for progression and potentially provide an endoscopic risk-stratification tool. Recent studies have shown that, irrespective of the presence of dysplasia in the actual sample, AFI positivity was associated with an increased content of biomarkers and dysplasia. The use of a biomarker panel on a limited number of AFI targeted biopsies may thus effectively classify Barrett's patients according to their dysplasia status, avoiding laborious random sampling<sup>30,31</sup>.

## Probe based autofluorescence spectroscopy

All studies on wide field autofluorescence imaging are based on prior experiences with probe based autofluorescence spectroscopy. A small probe is passed through the working channel of the endoscope to deliver excitation light and capture the emitted fluorescent light. Despite promising sensitivity and specificity, these point measurements appeared to be unsuitable for the detection of lesions, due to its small area of imaging and the resulting risk of sampling error. Moreover, these studies had practical limitations due to difficulties in correlating measured tissue spectra with histology; the spectroscopy probe has to be removed from the working channel of the endoscope and the biopsy forceps introduced to sample the exact same location that has been scanned<sup>32,33</sup>.

Recently, an optical biopsy forceps was developed, which triggered new attention to probe based spectroscopy. This biopsy forceps consists of an integrated optical fiber probe in a regular biopsy forceps enabling optimal correlation between the optical signal and the physical biopsy (*figure 3*). Spectroscopy can thus be used to construct a highly specific, operator independent, optical algorithm for stratification between suspicious and non-suspicious sites. Such an optical algorithm can be integrated in an optical biopsy system, that consists of the optical biopsy forceps and an operating console. By quickly scanning multiple areas throughout the Barrett's segment with the opened biopsy forceps, and only physically sampling areas considered suspicious by the optical biopsy system, the yield of random biopsies might be significantly increased. The optical biopsy forceps will take away many of the practical limitations associated with prior spectroscopy studies, while allowing future clinical applications.

The WavSTAT optical biopsy system (OBS) is such a promising technique that combines the optical biopsy forceps with a 405 nm laser to induce and capture tissue autofluorescence. A recent study on the OBS for flat type neoplasia are in accordance with previous reports on fluorescence spectroscopy and demonstrated a decent sensitivity of 80% for the OBS alone, with a specificity of 58%. As an adjunctive tool to the endoscopists assessment, sensitivity increased to 91%, with a specificity of 50%. This resulted in a 50% reduction of the number of random biopsies required,



**Figure 3.** Optical biopsy forceps: when the jaws of the biopsy forceps are opened, the optical probe protrudes and enables spot-on correlation between the scanned area and the corresponding histology. Image courtesy of SpectraScience Inc, San Diego, CA, USA.



yet at the cost of misclassifying 9% of neoplastic areas as unsuspecting. In its current form, the clinical applicability of the autofluorescence based OBS therefore seems limited<sup>34</sup>.

Prevalent neoplasia can cause remote genetic alterations and genomic instability in histologically normal tissue surrounding the site of neoplasia – a so called field defect of carcinogenesis<sup>35</sup>. A carcinogenic field defect has been demonstrated for various cancers, including Barrett's associated adenocarcinoma. Recently, the optical detection of field carcinogenesis was reported. Data on spectroscopic scanning techniques at histologically inconspicuous areas showed high accuracy in predicting the presence of distant colonic adenomas<sup>36–38</sup> and squamous neoplasia elsewhere in the aerodigestive tract<sup>39–41</sup>.

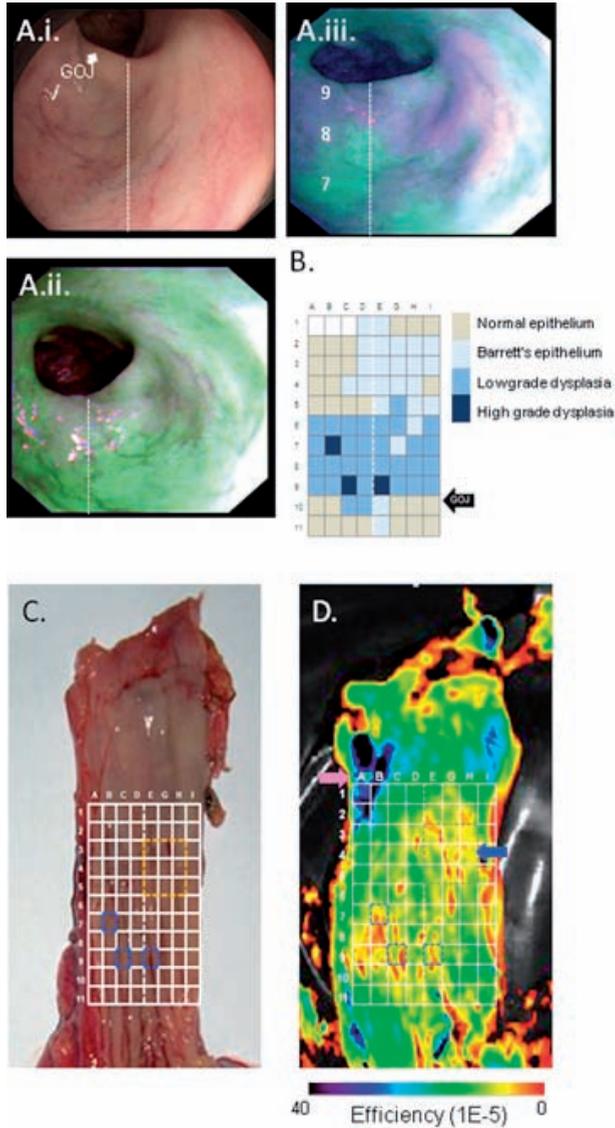
Therefore, a second opportunity for fluorescence spectroscopy next to the detection of neoplasia, is the identification of an optical marker for a carcinogenic field defect, much alike risk-stratification using molecular markers. Scanning distant, easy-to-reach areas with a spectroscopy probe may aid the endoscopist during surveillance endoscopy, and may even serve as a minimal invasive screening and risk stratification tool.

In order to pursue spectroscopy for endoscopic differentiation and optical identification of field carcinogenesis, research should focus on large, prospective cohort studies. This will involve scanning not only possible neoplastic areas, but also more distant locations, such as the proximal Barrett's segment, squamous mucosa and even oral mucosa. The optical biopsy forceps offers an excellent tool for integrating (multi wavelength) fluorescence spectroscopy with other modalities (e.g. scattering spectroscopy). Synchronous genetic biomarker analysis on biopsy material, combined with optical scanning and long term follow up may yield a multimodal risk-stratification model that can individualize care for Barrett's patients.

### Fluorescent contrast agents

The concept of administering fluorescently labeled exogenous substances that bind to specific molecular targets for malignant progression has recently emerged. Peptides as a molecular probe have shown tissue specific binding in ex-vivo studies using screening experiments and fluorescence microscopy<sup>42</sup>. Peptides are similar to antibodies in terms of binding affinity, but can be produced relatively cheaply and in large quantities. Another promising approach are fluorescently labelled lectins, that have a low toxicity and high stability and are inexpensive to produce. Lectins bind with high affinity to glycan targets in intestinal metaplasia, compared with low binding to HGIN/EAC. In an extensive ex-vivo study early neoplasia demonstrated reduced fluorescence intensity compared to non-neoplastic Barrett's, which despite negative contrast showed good sensitivity<sup>43</sup>. These two approaches have different strengths and weaknesses. The peptide probes are more specific but require a narrow field of view and may be costly to produce. Lectin probes are very cheap and for agents such as WGA (wheat germ agglutinin) safety is not likely to be an issue since it is a foodstuff. However, lectins lack specificity and it would be preferable to image out of the auto-fluorescent range in order to improve contrast. In-vivo studies are now in process for these techniques which will enable the clinical accuracy to be determined (*figure 4*).

Despite the developments in advanced imaging for the detection of early neoplasia in Barrett's esophagus, white light endoscopy with protocolized random biopsies remains the



**Figure 4.** (a) Images taken with an endoscope. White-light image (left), imaging fluorescence at 490–560 nm before the application of wheat germ agglutinin (WGA; middle) and imaging fluorescence at 490–560 nm after application of WGA and Alexa Fluor 488 (right). The areas of low WGA binding appear in purple. The dashed white line is placed longitudinally along the posterior wall of the esophagus to facilitate orientation between the different images, and the numbers 7, 8 and 9 refer to the y coordinates on the reference grid in b. (b) Grid showing the pathological diagnostic of each block made from the resection specimen. This same grid can be compared with the endoscopic and fluorescence images in a, on the right, and d. The dashed line represents the longitudinal axis along the posterior wall of the esophagus. (c) The same specimen after being opened longitudinally along the anterior border of the esophagus with the overlying grid from b. (d) The WGA fluorescence signal from the esophageal specimen taken with the IVIS200 camera. The pink arrow marks an area of artifact from the exposed submucosal tissue, and the blue arrow indicates the site of a previous endoscopic mucosal resection (outlined with a dashed gray box). Image courtesy of Bird-Lieberman et al.



gold standard. Relevant abnormalities are generally visible with HD-WLE, yet often remain undetected by endoscopists. A substantial number of patients referred with early neoplasia in random biopsies actually present with clear visible abnormalities. A training program, which is currently being developed by the international workgroup for the classification of oesophagitis (IWGCO<sup>44,45</sup>), may increase the WLE detection of relevant lesions in community hospitals. However, in the absence of visible abnormalities – which represents the majority of Barrett's patients – an improved risk-stratification model, rather than random biopsies and standard histological assessment still is a dire necessity.

In summary, fluorescence based light-tissue interaction has been successfully designed in point-probe differentiating spectroscopy systems and integrated into wide-field endoscopic systems such as AFI. However, the clinical value of current AFI systems is limited, since the additional detection of neoplastic lesions is compensated by random sampling and most AFI-detected lesions have no major implications for the endoscopic treatment of these patients. In the future, fluorescence imaging may play an important role in the development of an individualized risk-stratification model. Either by identifying areas with an enriched content of molecular abnormalities, or by finding an optical carcinogenic field effect that can identify BE patients at risk for malignant progression.

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