

Staining in Gastrointestinal Endoscopy: The Basics

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Introduction

Chromoendoscopy or tissue staining is an “old” endoscopic technique that has been used for decades. It involves the topical application of stains or pigments to improve localization, characterization, or diagnosis [1]. It is a useful adjunct to endoscopy; the contrast between normally stained and abnormally stained epithelium enables the endoscopist to formulate a diagnosis and/or to direct biopsies based on a specific reaction or enhancement of surface morphology. In recent years, there has been a resurgence of interest in chromoendoscopy as an endoscopic technique that can improve endoscopic diagnosis. This may have occurred because it is a simple, safe, quick, widely available, and inexpensive diagnostic tool. Also, new applications of staining techniques to the diagnosis of conditions such as Barrett’s esophagus, celiac sprue, and the presence of *Helicobacter pylori* underscore the potential for routine clinical use by the gastroenterologist. Vital staining and tattooing have become even more relevant in clinical gastrointestinal practice because of evolving endoscopic therapies (such as endoscopic mucosal resection, photodynamic therapy, multipolar electrocoagulation, etc.) which require careful selection of patients suitable for treatment.

Stain Equipment and General Technique for Chromoendoscopy

The various agents used for chromoendoscopy are categorized into absorptive or vital stains, reactive stains, contrast stains, and agents for tattooing (Table 1). Tissue staining generally refers to the application of stains or pigments by spraying through a catheter. Endoscopic tattooing indicates injection of dye (such as India ink) through a needle to mark a site for future identification.

Chromoendoscopy requires minimal equipment and reagents are generally available. A special spray catheter (such as the Olympus PW-5L, Olympus America, Melville, New York, USA) is essential for delivery of stain in a fine mist to the mucosa. The reusable catheters can last several years, even with routine use in a busy endoscopy unit. A new biopsy channel cap is useful for minimizing the amount of stain that leaks out. If staining is being performed for cancer surveillance in a patient with biopsy-proven Barrett’s esophagus, a large-channel/therapeutic upper endoscope is required to accommodate the “jumbo” biopsy forceps.

The technique for staining is simple and easy to learn. The steps and reagents for the most common staining agents are summarized in Table 2. When spraying in the esophagus or colon, the endoscopist needs to direct the endoscope and catheter tip towards the esophageal mucosa and use a combination of rotational clockwise-counterclockwise movements with simultaneous withdrawal of the endoscope tip. This will maximize the amount of reagent applied to the epithelium. Atropine or glucagon can be administered immediately before staining to minimize contractility and decrease loss of reagent. The interpretation of staining patterns may need to be learned and requires an understanding of what type of tissue is stained and what is not stained (Table 1).

Vital Stains

Lugol’s Solution

Lugol’s solution (named after a French physician Jean Guillaume Auguste Lugol) [1] is a readily available reagent containing potassium iodine and iodine, which has an affinity for glycogen in nonkeratinized squamous epithelium. Lugol’s staining involves dilution of stock solution to 1–4% (usually 2–3% strength works best) followed by spraying of 20–50 ml through a spray catheter. Normal squamous epithelium will stain black, dark brown, or green-brown after a few minutes (Figure 1a). Similar to the Schiller’s test for the cervix, an abnormal staining pat-

Table 1 Tissue stains

| Stain type | What is stained | Mechanism of staining | Positive staining | Clinical uses in gastrointestinal endoscopy |
|--|--|--|---------------------------------|---|
| Vital stains | | | | |
| Lugol's solution (iodine + potassium iodide) | Normal glycogen-containing squamous cells | Binds iodine in nonkeratinized cells | dark brown | 1. Squamous cell esophageal cancer (nonstaining) 2. Columnar epithelium in the esophagus, including residual Barrett's esophagus following mucosal ablation (nonstaining) 3. Reflux esophagitis (nonstaining) |
| Methylene blue (methylthionine chloride) | Small or large intestinal cells or intestinal metaplasia | Active absorption into cells | blue | 1. Specialized epithelium (intestinal metaplasia) in Barrett's esophagus* 2. Intestinal metaplasia in the stomach 3. Early gastric cancer* 4. Gastric metaplasia in the duodenum (nonstaining) 5. Celiac and tropical sprue |
| Toluidine blue (tolonium chloride or dimethylamino-toluphenazothionchloride) | Nuclei of columnar (gastric and intestinal-type) cells | Diffuses into cell | blue | 1. Squamous cell carcinoma of the esophagus 2. Gastric or intestinal metaplasia in Barrett's esophagus |
| Reactive stains | | | | |
| Congo red (Biphenylene naphthadene sulfonic acid) | Acid-containing gastric cells | Acid pH < 3.0 results in color change | Turns red to dark blue or black | 1. Acid-secreting gastric mucosa (including ectopic locations) 2. Gastric cancer (nonstaining); may be combined with methylene blue to outline intestinal metaplasia |
| Phenol red (Phenolsulfon phthalein) | <i>Helicobacter pylori</i> -infected gastric cells | Alkaline pH (from hydrolysis of urea to NH ₃ and CO ₂ by urease) results in color change | Turns yellow to red | Diagnose <i>H. pylori</i> infection (positive color change) and map its distribution in the stomach |
| Contrast stain | | | | |
| Indigo carmine [†] | Cells are not stained | Pools in crevices and valleys between mucosal projections | blue (indigo) | 1. Colon, gastric, duodenal, esophageal lesions 2. Barrett's esophagus |
| Agents for tattooing | | | | |
| India ink | Injection site | Mark location of a lesion (permanently) | black | 1. Location of colon polyps or cancers for subsequent endoscopic or intraoperative identification 2. Location of the original squamocolumnar junction prior to mucosal ablation of Barrett's esophagus |
| Indocyanine green | Injection site | Mark location of lesion | green | Same as India ink |
| Methylene blue | Injection site | Mark location of lesion (temporarily) | blue | Same as India ink |

* Methylene blue does not stain nonspecialized or gastric metaplasia; specialized columnar epithelium stains blue but highly dysplastic and malignant specialized columnar epithelia in Barrett's esophagus generally take up little to no dye; low grade dysplasia in Barrett's esophagus may or may not take up stain.

[†] With or without Congo red.

[‡] Also used in combination with high resolution or high magnification endoscopy; may be used with or without cresyl violet (for early colorectal cancers).

tern (absence of dye uptake) is associated with conditions that result in depletion of glycogen in squamous cells, such as inflammatory change (reflux esophagitis), dysplasia, or early malignancy.

Lugol's staining has been shown to be helpful in detecting high grade dysplasia and early squamous cell cancers of the esophagus. Unsuspected early esophageal cancers can be diagnosed in high risk patients such as those with head

and neck cancers [2,3], or alcoholics [4], who undergo screening since these lesions fail to show the characteristic color.

Nonsquamous mucosa, such as columnar cells at the gastroesophageal junction (Figure 1b) or metaplastic Barrett's epithelium within the esophagus should also not pick up Lugol's stain. The extent of Lugol's staining has also been used to improve the detection of Barrett's esophagus [5].

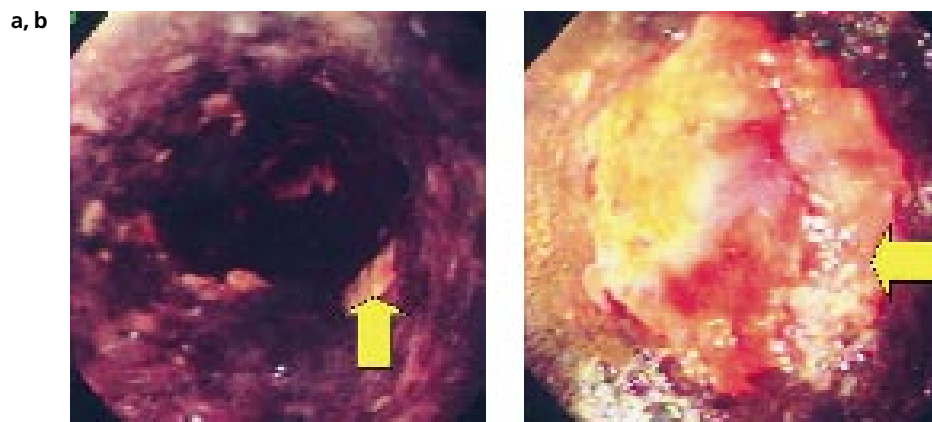


Figure 1 Stained images of the proximal esophagus (a) and normal gastroesophageal junction (b) showing dark brown-stained squamous epithelium. The yellow arrows show unstained columnar epithelium (b) and areas of nonspecific inflammatory squamous mucosal change (a)

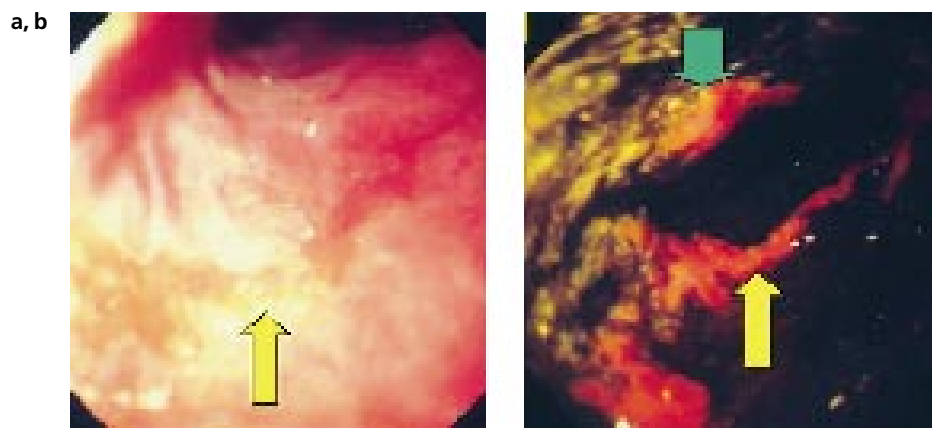


Figure 2 Unstained (a) and stained (b) images of areas of residual Barrett's esophagus following photodynamic therapy. The yellow arrows indicate an unstained columnar mucosa on the posterior esophageal wall that is clearly demarcated by the dark brown Lugol-stained normal squamous mucosa. The green arrow shows a small island of columnar mucosa that was not appreciated prior to Lugol's staining

By increasing the demarcation between squamous mucosa (which will stain dark brown) from unstained, pink metaplastic columnar epithelium, Lugol's staining can increase the sensitivity, specificity, and accuracy of endoscopic diagnosis of Barrett's esophagus to 89%, 93%, and 91%, respectively [5]. Although this has not been formally studied, Lugol's staining is also particularly useful in differentiating regenerating squamous epithelium from small areas of residual Barrett's mucosa in patients who have undergone mucosal ablation (e.g. after photodynamic therapy or multipolar electrocoagulation) (Figure 2).

Toluidine Blue

Toluidine blue (also called toloum chloride) is a basic dye that stains cellular nuclei. It has been used to identify malignant tissues, which have an increased DNA synthesis and a high nuclear-to-cytoplasmic ratio [6]. It will stain abnormal tissues blue. It has been used to help screen for early squamous esophageal cancers in alcohol and tobacco abusers [7] and in patients with head and neck cancers [8, 9]. It can also selectively stain gastric cancers and can help to discriminate between benign and malignant ulcers [10]. It can stain esophageal columnar epithelium, such as Barrett's esophagus with a sensitivity of 98% and specificity of 80% [11]. Toluidine blue can stain columnar-type mucosa in Barrett's esophagus but it cannot discriminate between gastric and intestinal metaplasia.

Toluidine blue staining is accomplished by spraying 1% acetic acid (which acts as a mucolytic) before and after a 1% aqueous solution of toluidine blue. The second application of acetic acid serves to wash off excess dye. No adverse effects have been reported.

Methylene Blue

Methylene blue is a vital stain taken up by actively absorbing tissues such as small intestinal and colonic epithelium. It does not stain nonabsorptive epithelia such as squamous or gastric mucosa. It has recently been used to highlight subtle mucosal changes in the small intestine (e.g. celiac disease [12]) and colon (flat adenomas and carcinomas). It has been used to positively stain metaplastic absorptive epithelium, such as intestinal-type metaplasia in the stomach [13]. It will not stain nonabsorptive epithelium, such as ectopic gastric metaplasia in a background of positive staining duodenal mucosa.

The technique of methylene blue staining was originally described by Japanese investigators for improving the diagnosis of early gastric cancer, either alone [14] or in combination with Congo red dye [15]. Methylene blue staining has been also used in the esophagus to aid in the detection of Barrett's esophagus [16]. Barrett's esophagus is the metaplastic replacement of squamous epithelium in the esophagus by columnar epithelium, which resembles cells

in the gastric fundus, gastric cardia, or intestine. It is the intestinal-type metaplasia or specialized columnar epithelium (SCE), with characteristic crypts and villi lined by mucus-secreting columnar cells and goblet cells, which is considered pathognomonic of Barrett's esophagus. The similarity between SCE in Barrett's esophagus and the incomplete intestinal metaplasia in the stomach resulted in the use of methylene blue for selective staining of SCE. In a controlled pilot study, Canto et al. showed that methylene blue can selectively and reproducibly stain SCE in Barrett's esophagus with high accuracy, even in patients with very short segments [16] (Figure 3).

Methylene blue staining involves application of a mucolytic, followed by dye, followed by washing off excess dye. Surface mucus must be removed to increase the uptake of dye into epithelial cells. This can be accomplished by spraying a mucolytic agent. The technique for methylene blue staining reported by Japanese endoscopists involves ingestion of a proteolytic enzyme solution (proteinase or Pronase) followed by methylene blue in a capsule [14, 17]. This technique was adapted by Fennerty et al. for use in the United States [13] by substituting 10% solution N-acetylcysteine for pronase and a 0.5% solution of methylene blue for the capsule. Both reagents are sprayed on the mucosa with a washing catheter in sequence. Excess dye is then washed off with water. The endpoint of staining is somewhat subjective and is the most difficult part to learn. Positive staining is defined as the presence of blue-stained mucosa that persists despite vigorous water irrigation.

In Barrett's esophagus, methylene blue staining may be either focal (Figure 3) or diffuse (> 75% of Barrett's mucosa stains blue; Figure 4) [16]. The majority of patients with long-segment (> 6 cm) Barrett's esophagus have diffuse staining because SCE comprises most of the columnar mucosa [16]. The pattern of methylene blue staining is important because dysplastic and malignant Barrett's esophagus appears to behave differently from nondysplastic epithelium [18]. An increasing grade of dysplasia is significantly associated with focal areas of decreased stain intensity and/



Figure 3 Methylene blue-stained image of short segment of Barrett's esophagus with focal staining. The endoscopic biopsies directed towards stained areas just distal to the squamocolumnar junction showed specialized columnar epithelium with goblet cells and no dysplasia (yellow arrows). Biopsies from unstained areas showed cardiac-type columnar epithelium

or increased stain heterogeneity (i.e., an increasing proportion of light blue or pink unstained mucosa compared with dark blue mucosa). High grade dysplasia and endoscopically inapparent adenocarcinomas in Barrett's esophagus can be diagnosed by directed biopsy to the heterogeneously stained or light blue/unstained epithelium (Figure 5).

Cresyl Violet

Cresyl violet has been used for staining uterine cervical lesions and to enhance endoscopic diagnosis of early malignancies. Cresyl violet dye spraying has also been combined with fiberoptic magnification endoscopy to diagnose a characteristic staining pattern of early gastric carcinomas [19]. A 0.2% solution of cresyl violet can be sprayed after indigo carmine to enhance the diagnosis of characteristic pit patterns of depressed early colorectal carcinomas.

a, b

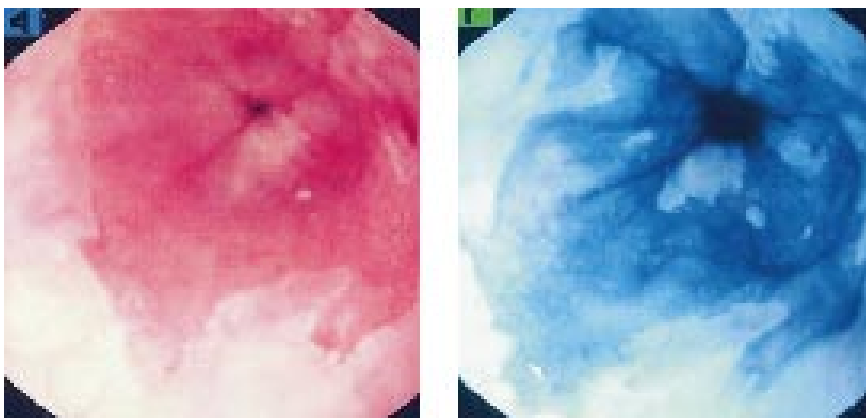


Figure 4 Unstained (a) and stained (b) images of limited-segment Barrett's esophagus. The methylene blue staining in b is diffuse, and biopsies from stained areas showed specialized columnar epithelium with goblet cells and no dysplasia

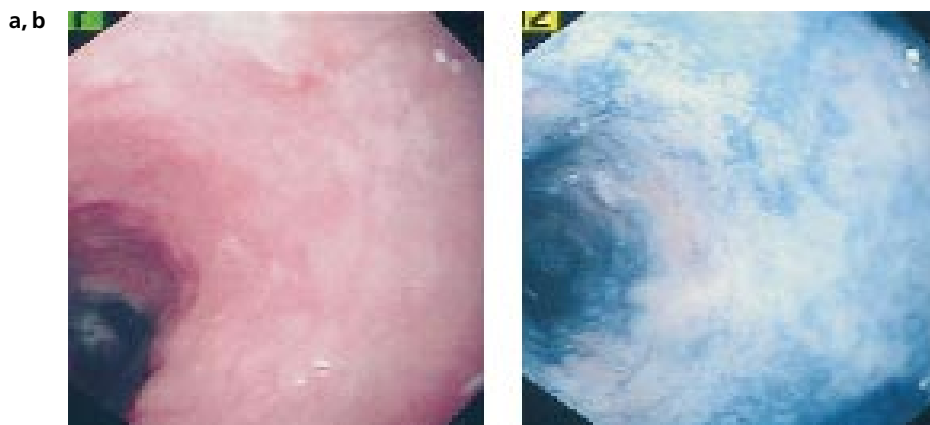


Figure 5 Unstained (a) and stained (b) images of long-segment Barrett's esophagus. The unstained (pink) and light blue, heterogenous area shown in (b) corresponds to the specialized columnar epithelium with high grade dysplasia not endoscopically apparent in (a)

Contrast Stains

Indigo Carmine

Indigo carmine is derived from a blue plant dye (indigo) and a red coloring agent (carmine) [1]. Unlike vital stains, this deep blue stain is not absorbed by gastrointestinal epithelium. It pools in crevices between epithelial cells and highlights small or flat lesions and defines irregularities in mucosal architecture, particularly when used with high magnification or high resolution endoscopy.

Indigo carmine has been used in combination with high magnification endoscopy to diagnose the villiform appearance of Barrett's esophagus [20]. In the stomach, indigo carmine can be used to diagnose small gastric cancers [14], and in the duodenum, it has been used to evaluate villous atrophy in patients suspected of having malabsorption from celiac disease or tropical sprue [21]. In the colon, it has been used to study the surface appearance of colonic crypts and to discriminate between hyperplastic polyps which have a typical "pit" pattern and adenomatous polyps, which have a "groove" or "sulci" pattern [22]. It can also aid in the diagnosis of minute, flat or depressed colorectal tumors [23,24].

Several techniques are described for indigo carmine staining. The oral route involves ingestion of a capsule or colonic electrolyte lavage solution with dye. The second route involves direct spraying of 0.1–0.5% indigo carmine onto the mucosa. Indigo carmine has also been injected into the celiac artery (intra-arterial dye method) to permit more precise endoscopic delineation of the size and extent of gastric cancers [25].

Reactive Stains

Congo Red

Congo red is a pH indicator that changes from red to dark blue or black in acidic conditions. It has been used to map acid-producing epithelium in the stomach or in ectopic sites. It can help with the evaluation of post-vagotomy patients

[26]. It has been used primarily for screening for early gastric cancers and for detecting synchronous lesions, in combination with methylene blue, which stains gastric intestinal metaplasia [15]. The early gastric cancer will usually be identified as a "bleached" area of mucosa that did not stain with either Congo red or methylene blue [1]. Synchronous gastric cancers are found to be present in up to 9% of patients when this combination staining technique is used. Congo red can also aid in the detection of intestinal metaplasia of the stomach, which is accompanied by gastric atrophy and decreased or absent acid production.

The technique for staining with Congo red involves stimulation of acid production with 250 µg of pentagastrin given orally. Then, during endoscopy, 0.5% sodium bicarbonate solution is sprayed prior to a 0.3–0.5% Congo red solution. A positive reaction (black color change) results within minutes that delineates acid-secreting areas (blue-black) from non-acid-secreting areas (red).

Phenol Red

Like Congo red, phenol red is a pH indicator. It detects alkaline pH by a color change from yellow to red. A promising useful clinical application of phenol red is the detection of *H. pylori* infection in the stomach. The urease produced by the bacterium catalyses hydrolysis of urea to NH₃ and CO₂, which results in increased pH. Hence, *H. pylori* can be observed in red-stained mucosa. Investigators have used the endoscopic phenol red test to improve the diagnosis of *H. pylori* and map its distribution in the stomach [27]. The reported sensitivity and specificity in 108 patients studied were 100% and 84.6%, respectively. This technique has also been recently used to clarify the role of the organism in gastric carcinogenesis [28,29]. A prospective study by Japanese investigators of 79 patients with (n = 65) and without (n = 14) gastric adenocarcinoma [29] showed good correlation of red-color change with actual *H. pylori* infection. Indeed, 50 of 65 patients with gastric cancer were found to be infected with *H. pylori* using this endoscopic test, particularly those with differentiated tumors. Hence, the endoscopic phenol red test may potential-

Table 2 Summary of commonly used stains and staining techniques

| Stain technique | Stain concentration and volume | Mucolytic needed | Washing step | Procedure |
|-----------------------------|--|------------------|--------------|--|
| Vital stains | | | | |
| Lugol's | 20–50 ml of 1–4 % Lugol's solution | No | No | Spray dye directly on esophageal mucosa; positive results are seen within a few minutes |
| Toluidine blue | 30 ml of 1 % acetic acid before and after (total of 60 ml of acetic acid) 10 ml of 1 % toluidine blue | Yes | Yes | Wait 20 seconds after first acetic acid rinse before applying toluidine blue. Then, after 30 seconds, rinse with acetic acid |
| Methylene blue | Volumes required vary depending on the site and lesion under evaluation; for Barrett's esophagus, 20 ml of 10 % acetylcysteine or other mucolytic followed by 20 ml of 0.5 % methylene blue followed by 120 ml of tap water for each 5 mm length | Yes | Yes | Wait 2 min after mucolytic and 2 min after methylene blue stain application. Wash vigorously with tap water until persistent blue staining remains |
| Cresyl violet | 0.2 % cresyl violet | No | No | Spray directly on colonic mucosa (usually after indigo carmine) |
| Reactive stains | | | | |
| Congo red | 0.5 % bicarbonate followed by 0.3–0.5 % Congo red | No | No | Stimulate acid production first with 250 µg pentagastrin. Then, spray bicarbonate before Congo red and wait for the color change |
| Phenol red | 0.1 % phenol red and 5 % urea | Yes | No | Dissolve mucus with dimethylpolysiloxane or other mucolytic. Spray reagents evenly on gastric mucosa and wait for the color change |
| Contrast stain | | | | |
| Indigo carmine | 0.1–0.5 % indigo carmine | No | No | Spray directly on epithelium |
| Endoscopic tattooing | | | | |
| India ink | 50 % (1 : 1 dilution with sterile water) to 70 % (5 parts ink to 2 parts water) India ink | No | No | Inject 0.2–0.5 ml with sclerotherapy needle submucosally to make a bleb |

ly help researchers to investigate the association of *H. pylori* with other disease conditions.

The technique of the endoscopic phenol red test involves treatment with a potent acid-suppressive agent in all patients and application of a mucolytic agent, dimethylpolysiloxane, and an anticholinergic drug just before endoscopy [29]. Then, during endoscopy, a solution of 0.1 % phenol red and 5 % urea is sprayed evenly over the gastric mucosal surface. A positive test consists of a color change from yellow to red, which indicates the presence of *H. pylori* [29]. Areas of gastric intestinal metaplasia will not change color to red [29].

Endoscopic Tattooing

India Ink

India ink consists of suspended inert carbon particles in aqueous or nonaqueous stabilizers and diluents [1]. Injection of India ink with a sclerotherapy needle is used to permanently mark the location of lesions. This is possible because the ink remains in the gastrointestinal wall for long

periods of time, perhaps for life. This allows for easy intraoperative localization of colonic lesions [30] or endoscopic surveillance of colonic tumors [31], such as malignant polyps or large sessile adenomas removed by piecemeal polypectomy. It has also been used to mark the proximal and distal extent of Barrett's esophagus [32]. The latter indication is a relatively new and potentially useful one in the research setting. For example, india ink could mark the squamocolumnar junction for longitudinal follow-up of the length of Barrett's esophagus and for surveillance after mucosal ablation. The black tattoo reportedly persists for at least 36 months [32].

The dilution and technique of India ink tattooing is highly variable. India ink is frequently used unsterilized [33]; however, the preferred methods for tattooing involve sterilization by autoclaving or gas sterilization. One described technique involves dilution of commercial grade India ink with an equal volume of water, followed by sterilization by standard autoclave processing [30]. Alternatively, the ink can be diluted with sterile bacteriostatic water (ratio 5 parts ink to 2 parts water) and passed through a Travenol 5 µm pore size filter into sterile 5 ml vials (to remove large par-

ticulate material) before autoclaving for 40 minutes [31]. The opened India ink vials are sterile for 2 hours while unopened ones can be used up to 6 months after preparation.

Prior to injection, the vial should be shaken well and the ink draw up into a 3- or 5 ml syringe attached to a sclerotherapy needle. Injections of 0.1 to 0.5-ml should be made to create intramucosal or submucosal blebs just proximal and distal to the colonic lesion and on the other quadrants of the colonic wall nearby. Multiple injections are recommended.

Although India ink tattooing is generally considered safe over the long term [31], several reports have raised concern about adverse effects associated with India ink tattooing. In a comprehensive review of colonic tattooing with India ink, Nizam et al. [33] found at least 447 cases reported in the literature, with only five reports of complications (risk of 0.22%). These include allergic reactions [34], fat necrosis and inflammatory pseudotumors [35], and colonic abscess and focal peritonitis [36].

Indocyanine Green

Indocyanine green makes a persistent tattoo and does not cause secondary tissue inflammatory change, unlike India ink [1]. This dye has not been extensively studied for endoscopic tattooing but deserves further evaluation.

Methylene Blue

Methylene blue has been used to tattoo the colon wall for localization of lesions during surgery. However, it appears to cause a significant tissue reaction and fibrinoid necrosis of vessel walls and does not persist as long as India ink. In a study evaluating the tissue injury associated with India ink and methylene blue as tattoo agents, the latter was not grossly visible 7 days after injection, unlike the former which lasted at least 7 weeks [37]. Hence, methylene blue is a poor tattoo agent compared to India ink.

Conclusion

Compared to other evolving diagnostic modalities such as fluorescence spectroscopy, fluorescence endoscopy, and optical coherence tomography, chromoendoscopy is an effective technique, that is already available to the endoscopist, to improve visualization and diagnosis. It is simple, quick, widely available, inexpensive, and free of adverse effects. It is useful in both clinical practice and endoscopic research. In the future, new applications of "old" stains or the development of new staining agents may expand the role of staining in gastrointestinal endoscopy.

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