# Multiphoton Imaging and Optical Biopsy of Gastrointestinal Tumors Postprint

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## Abstract

The selection of surgical procedures for gastrointestinal malignant tumors and the determination of surgical resection margins urgently require an in-situ, real-time diagnostic technique to evaluate tumor infiltration depth, metastasis status, and the presence of residual cancer at surgical margins. Utilizing multiphoton imaging technology, multiphoton microscopy can provide real-time information on gastrointestinal tissue architecture and cellular morphology. Multiphoton imaging technology possesses characteristics such as label-free tissue imaging, high sensitivity to collagen, minimal photodamage to tissues, and deep penetration depth, making it potentially applicable for optical biopsy of gastrointestinal tumors. This review, from the perspectives of related research on tumor infiltration depth, metastasis status, and residual cancer at surgical margins, aims to comprehensively summarize the feasibility of employing multiphoton imaging technology for evaluating optical biopsy of gastrointestinal tumors and to explore its promising development prospects.

## Full Text

### Multiphoton Imaging and Optical Biopsy of Gastrointestinal Tumors

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#### Abstract

The selection of surgical procedures and determination of resection extent for gastrointestinal malignant tumors urgently require an in situ, real-time diagnostic technology to evaluate tumor invasion depth, metastasis, and residual cancer at surgical margins. Multiphoton microscopy, based on multiphoton

imaging technology, can provide real-time information on gastrointestinal tissue architecture and cellular morphology. This technology offers several distinctive advantages: it requires no exogenous labeling, exhibits extreme sensitivity to collagen, causes minimal photodamage to tissues, and provides deep penetration depth. These characteristics enable its potential application as a novel optical biopsy method for gastrointestinal tumors. This review comprehensively summarizes the feasibility of multiphoton imaging technology for evaluating optical biopsy of gastrointestinal tumors and explores its considerable development prospects, focusing on relevant research perspectives including tumor invasion depth, metastasis, and residual cancer at surgical margins.

**Keywords:** gastrointestinal tumor; multiphoton imaging; optical biopsy

## Introduction

Radical surgical resection represents a common and effective treatment for gastrointestinal tumors. However, selecting the appropriate surgical approach and defining the resection range require a real-time, in situ diagnostic technique to determine tumor characteristics, including benign or malignant status, invasion depth, metastasis, and residual cancer at margins. Preoperative endoscopic biopsy provides important histological evidence for diagnosing gastrointestinal tumors, and surgical approaches for gastric cancer—such as total gastrectomy, subtotal gastrectomy, partial gastrectomy, and endoscopic mucosal or submucosal resection—are selected based on tumor size, location, and invasion depth. Nevertheless, endoscopic biopsy has inherent limitations, including bleeding from the intestinal wall or tumor, artificial traction or compression, repeated biopsies due to endoscope passage failure causing time delays, and emergency hemostasis requirements in cases of severe hemorrhage.

Current imaging modalities such as CT and MRI cannot accurately determine invasion depth and lymph node metastasis in early gastrointestinal tumors. Endoscopic ultrasound (EUS) for T-staging of gastrointestinal tumors has reported accuracy rates of only 44.7%–78%[1-3], which is insufficient as a reliable diagnostic standard. EUS also performs poorly in preoperative evaluation for local resection, cannot precisely delineate gastrointestinal mucosal layers, and demonstrates poor N-staging capability[4]. Therefore, developing a new in situ, real-time diagnostic technology represents a major objective[5]. Such technology must simultaneously assess tumor invasion depth, metastasis, and margin status to provide robust guidance for surgical decision-making in gastrointestinal malignancies.

In 1989, Denk, Strickler, and Webb at Cornell University invented the multiphoton microscope (MPM)[6], employing multiphoton microscopic imaging based on nonlinear optics and femtosecond lasers. This technology utilizes intrinsic autofluorescence from cells and second harmonic generation from collagen to rapidly obtain real-time tissue architecture and cellular morphology[7]. As early as 1986, second harmonic generation was applied in skin and coronary

artery imaging studies, confirming its feasibility for biological tissue observation[8-9]. MPM has also emerged as an important tool for cancer research[10-11]. Cellular autofluorescence originates from nicotinamide adenine dinucleotide (NADH) and flavin adenine dinucleotide (FAD), with NADH emitting at 460 nm and collagen second harmonic generation at 370–390 nm. Consequently, multiphoton microscopes operating in the 740–780 nm range are typically used for tumor tissue observation. MPM imaging is not only comparable to standard histopathology but also provides additional information on tumor progression, such as metabolic levels reflected by the NADH/FAD ratio[12].

With advances in interdisciplinary research, renowned institutions including Cornell and Yale Universities are conducting translational medical research on multiphoton microscopy[13-16], aiming for clinical application in real-time "optical biopsy." Alongside developments in laser scanning and image processing technology, multiphoton microscopy has become increasingly popular as a minimally invasive bioimaging technique[17-21]. The ability to image tissues at cellular and subcellular levels without fluorescent staining is particularly advantageous. Multiphoton imaging causes minimal photodamage and offers deep tissue penetration, though potential thermal damage from infrared and near-infrared sources cannot be completely excluded. The focused beam produces a conical laser distribution, confining multiphoton excitation to a minimal region near the focal point, which significantly reduces photodamage to non-observed fluorescent dyes and enables "point imaging" with inherent three-dimensional imaging capability and high spatial resolution[22]. The femtosecond laser is critical for generating multiphoton absorption. When observing cells using high-intensity laser-induced nonlinear optical effects, both thermal and photonic energy must be controlled within levels that do not cause cellular damage. From a cell safety perspective, titanium-sapphire femtosecond lasers can achieve average output power of 10 mW with peak power of 1 kW, generating heat insufficient to damage cells while facilitating two-photon absorption[23].

In addition to exciting autofluorescent substances, femtosecond lasers play a crucial role in generating second harmonic generation through nonlinear polarization effects interacting with non-centrosymmetric biological molecules such as collagen. During this process, second harmonic oscillation (SHG) occurs without incident light absorption or energy loss, with photons produced at exactly half the wavelength of the incident light. Because SHG wavelengths are shorter than multiphoton-excited fluorescence wavelengths, SHG signals can be separated from autofluorescence signals to create contrast in tissue imaging[12,24-25]. While SHG imaging primarily focuses on collagen structural or content changes, multiphoton imaging covers larger areas when observing and quantifying collagen fibers across different tissue stages, including liver fibrosis staging[26-29]. For unstained tissue samples, TPEF/SHG microscopy can scan tissues with minimal invasiveness, making it an excellent replacement for conventional histological imaging[30]. Notably, Gailhouste et al.[29] proposed SHG imaging as a novel tool for evaluating fibrosis in lung, kidney, and heart diseases.

## 1. Clinical Multiphoton Imaging Principles and Characteristics

Clinical multiphoton imaging systems for gastrointestinal tumors primarily consist of three components: a laser source, scanning microscopy system, and detection system. The laser output wavelength is tunable from 700–980 nm. The laser scanning system employs META detectors composed of high-quality reflection gratings and 32-channel photomultiplier tube arrays. The detection system uses a 63× oil immersion objective with a numerical aperture of 1.4, which collects reflected optical signals and focuses dispersed TPEF/SHG signals from the specimen. The META detector has 32 independent channels that can be randomly selected to detect signals across 377–716 nm, enabling imaging.

Two distinct signal channels capture high-contrast frequencies from autofluorescent substances and collagen structures: the 397–419 nm channel acquires SHG signals to display collagen microarchitecture, while the 430–716 nm channel acquires TPEF signals to reveal morphological changes in fluorescent substances[5].

### 2.1 Gastrointestinal Tumor Invasion Depth

The deepest site of cancer cell invasion reflects tumor invasion depth. Given its deep penetration characteristics, multiphoton microscopy is feasible for assessing tumor invasion depth in gastrointestinal tissues.

Xu et al.[31] performed multiphoton microscopic imaging of esophageal tumors at early invasion stages, using MPM to image microstructures of normal human esophagus, carcinoma in situ, and early invasive carcinoma to investigate morphological changes during early tumor progression. Diagnosing early-stage tumors relies on identifying these specific morphological alterations as diagnostic criteria, such as using cancer cell presence to determine invasion depth and distinguishing cancer from normal tissue based on basement membrane integrity. For carcinoma in situ diagnosis, although the basement membrane remains intact, detecting cancer cells within this layer introduces diagnostic controversy. This demonstrates that MPM has made real-time, in situ diagnosis of early tumors at the cellular level possible. While preclinical studies on in vivo real-time diagnosis of early gastrointestinal tumors using multiphoton imaging have not yet been reported, the esophagus and gastrointestinal tract share the same digestive system, suggesting MPM could soon be applied to early gastrointestinal tumor imaging for invasion depth assessment. Studies have confirmed the feasibility of MPM for diagnosing early colorectal cancer[32]. Additionally, multiphoton imaging can differentiate advanced tumors through comparison with pathological sections.

Yan et al.[33] conducted real-time multiphoton imaging of gastric cancer with and without serosal invasion, comparing MPM-based T4 staging with endoscopic T4 staging through statistical analysis, demonstrating the specificity, accuracy, precision, and feasibility of MPM for real-time diagnosis of serosal invasion in gastric cancer. Multiphoton images of invasive gastric cancer showed

highly irregular collagen structures, significant collagen reduction, cancer cell infiltration, nuclear pleomorphism, and irregular glandular architectures.

### 2.2 Residual Cancer at Surgical Margins

Real-time evaluation of surgical margins is essential for preliminary assessment of R0 resection. Margin status directly correlates with surgical efficacy and patient prognosis. Negative margins generally indicate better outcomes, warranting heightened attention for tumors prone to margin involvement. Residual cancer in gastric cancer is defined as cancer infiltration or lymphovascular invasion within 0.5 cm of the resection margin. Upper gastric cancer and advanced gastric cancer (tumor diameter  5 cm, poorly differentiated or undifferentiated type, and serosal involvement) are prone to margin positivity, with the fundamental preventive measure being a minimum 5 cm tumor-free margin[34]. Due to anatomical constraints in low rectal cancer, achieving a 5 cm distal margin is impossible during sphincter-preserving surgery, yet a cancer-free distal margin is mandatory for sphincter preservation.

Postoperative MPM can real-time examine margins for residual cancer. Intraoperative multiphoton imaging of tissue within 0.5 cm or more from the resection margin showing tumor cells or irregular structures should raise suspicion for positive margins, providing significant guidance for sphincter-preserving decisions in low rectal cancer. Yan et al.[35] performed MPM imaging on fresh, unfixed, unstained full-thickness low rectal cancer resection margins, comparing MPM images with intraoperative frozen sections and routine pathology, thereby confirming the feasibility of real-time optical biopsy using MPM for low rectal cancer margins. MPM images showed high concordance with H&E staining. In negative margins, MPM revealed normal tissue architecture and cellular morphology, including typical central foveolae, circular crypt openings, and regularly arranged glands formed by epithelial and goblet cells. Conversely, positive margins displayed irregular tubular structures, reduced stroma, and cellular/nuclear pleomorphism. SHG signals could detect periglandular tissue, with signal intensity decreased in negative margins compared to positive margins containing cancer cells. Overall, multiphoton imaging can evaluate surgical margins in low rectal cancer [Figure 1: see original paper] and provide significant guidance for assessing tissue at intestinal anastomosis sites before bowel reconstruction. After intestinal resection, multiphoton endomicroscopy can perform in vivo real-time imaging of mucosa at proximal and distal ends. Positive imaging findings may prompt extended resection, while negative findings can ensure surgical success and provide prognostic value by reducing recurrence and metastasis risk. Zhuo et al.[36] used SHG imaging on unstained colonic basement membrane tissue to effectively differentiate normal, precancerous, and cancerous tissues, demonstrating that complete internal basement membrane dynamics could reflect different colorectal cancer stages.

### 2.3 Gastrointestinal Tumor Metastasis

Metastatic potential directly influences treatment approaches. Gastrointestinal malignancies can metastasize to liver, peritoneum, ovary, and lung. Mucinous adenocarcinoma exhibits high malignancy. In multiphoton imaging of mucinous adenocarcinoma, the mucosal layer shows distorted or elongated glands with blurred lumens and reduced reticular collagen fibers, while the submucosa reveals disorganized, elongated, sparse collagen fibers with destroyed or nearly absent elastic fibers. Some cancer cells appear as mucin-filled cavities surrounded by fibrin, occupying the entire submucosa[37].

Studies applying MPM to diagnose lung and liver cancer in mouse models[37-39] have addressed the design and development of miniaturized multiphoton microscopes. In colorectal cancer ovarian metastasis, some patients undergo oophorectomy only to receive final pathology reports negative for ovarian metastasis, causing significant impact particularly for young women desiring fertility. An in situ, real-time diagnostic method is urgently needed to accurately determine ovarian metastasis before performing oophorectomy, enabling precise surgical planning and better achieving lesion clearance, organ preservation, and damage control[40]. Multiphoton imaging technology can meet this need for real-time evaluation of gastrointestinal tumor metastasis.

## 3. Comparison of Multiphoton Imaging with Other Diagnostic Techniques

Imaging examinations primarily operate at the organ and tissue level with relatively macroscopic results. CT and MRI are routinely used to assess tumor size, location, progression, and metastasis. Literature reports CT has high sensitivity for detecting mesenteric lymph node metastasis, while MRI shows higher sensitivity than CT for detecting colorectal liver metastases[41]. However, both require specialized radiological expertise and frequently underestimate tumor progression compared with postoperative pathological staging. Multiphoton microscopy offers greater application potential than CT or MRI due to its non-destructive, real-time examination capability, faster image acquisition, and superior temporal and spatial resolution.

Compared with imaging examinations, single-photon confocal endomicroscopy better reveals microstructures and provides cellular morphological information, making it a favored non-invasive imaging tool in gastrointestinal oncology[42]. However, compared with MPM, confocal endomicroscopy provides limited extracellular tissue information, has shallow imaging depth, requires exogenous contrast agents, and offers poor "optical biopsy" capability. Confocal microscopy excites dye molecules throughout the entire laser illumination region, causing premature bleaching in non-focal areas. In contrast, multiphoton microscopy combines autofluorescence excitation with SHG, eliminating the need for exogenous labeling while providing histological, cellular, extracellular matrix, and even chemical information. MPM imaging quality in fresh intestinal mucosa

exceeds that of confocal microscopy[16]. While confocal microscopy has limited subcellular resolution, multiphoton imaging clearly visualizes cellular and subcellular structures. Multiphoton photobleaching and phototoxicity are minimized, with deeper tissue penetration. Overall, multiphoton imaging' s greatest advantages are its strong penetration depth, clear visualization of cellular and subcellular structures, and elimination of exogenous dyes, enabling "optical biopsy" and "optical tomography" [43].

## 4. Discussion

Preoperative biopsy has drawbacks including bleeding, needle tract seeding, metastasis risk, and time consumption. Postoperative histopathological diagnosis requires tissue processing, fixation, embedding, sectioning, and staining. MPM can rapidly capture tumor histological features, reflect cellular metabolic levels, and detect collagen signal changes in surrounding stroma without these cumbersome steps, offering real-time, non-destructive, high-penetration, and high-resolution advantages. For example, in real-time evaluation of rectal cancer surgical margins, intraoperative frozen pathology is currently used. If positive margins are found, surgeons must re-excise the already anastomosed bowel —an awkward, costly, and damaging scenario, with stapler costs exceeding ten thousand yuan and significant patient impact. Multiphoton microscopy enables rapid, non-invasive "optical biopsy" without traditional histological processing.

Although MPM has proven effective for cellular-level tissue morphology imaging comparable to gold-standard H&E pathology, clinical practice relying solely on multiphoton imaging without histological confirmation requires extensive validation. Detecting cellular redox ratios (NADH/FAD) can reveal tumor metabolic levels[37] and potentially quantify differentiation grades (well, moderately, poorly, undifferentiated) and histological types (papillary adenocarcinoma, tubular adenocarcinoma, mucinous adenocarcinoma, signet ring cell carcinoma, squamous carcinoma). However, surgeons prefer direct visualization of pathological morphology. Therefore, accurate display of tissue architecture and cellular structure is critical. Multiphoton imaging' s limitations include its restriction to fluorescent excitation and high equipment costs due to sophisticated laser system requirements.

Nevertheless, multiphoton imaging of gastrointestinal tissues holds promise as an emerging optical biopsy method that may eventually parallel or replace traditional histological biopsy. It can provide compelling evidence for evaluating tumor progression and observing collagen structural changes in clinical practice. Based on nonlinear optics and femtosecond lasers, multiphoton microscopy utilizes intrinsic cellular autofluorescence and collagen SHG to rapidly obtain tissue architecture and morphology in real time. It can differentiate normal from tumor tissue, enable early tumor prediction, and assess margin status. The technology shows excellent application prospects in gastrointestinal tumor optical biopsy, with miniaturization trends being unstoppable. Developing multiphoton endoscopes, probes, and laparoscopes will promote translational medicine

and open new horizons.

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