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Confocal fluorescence microscopy for real-time breast cancer diagnosis: current advances and future perspectives

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Background: Confocal fluorescence microscopy (CFM) is a powerful optical biopsy technique which captures cellular resolution images of the tissue surface without the need for tissue fixation or sectioning. The evolution of CFM with miniaturization and fibre-based optics now allows rapid capture of wide field images with microscopic resolution. For *in-situ* diagnostics, there is growing evidence that CFM systems could rapidly and accurately identify breast cancer with clinically actionable results.

Review Focus: This comprehensive review discusses different technological advances in CFM systems and explores emerging trends in Artificial Intelligence (AI) and robotic integration in breast cancer imaging. The review further discusses the clinical implications of these technologies, including their potential to reduce re-excision rates following breast conserving surgery (BCS) and improve surgical workflow efficiency.

Methods: A comprehensive literature review using PubMed, Embase and Web of Science databases was conducted by three reviewers independently covering studies published from January 2013 to December 2024. We included studies that provided human tissue data (preclinical and clinical) relevant to breast cancer imaging, focusing on the technological features, intra-operative usability, and ease of use of different bench-top and fibre-based CFM systems. Research focusing on future trends and emerging challenges in standardizing imaging protocols for breast cancer CFM imaging and automating diagnostic workflows were also considered.

Results and conclusion: Of 1382 articles identified from database screening, 28 fulfilled the inclusion criteria. Only 10 clinical studies reported statistical differentiation among specimens. Bench-top CFM systems demonstrated high-resolution imaging with accuracy ranging 83%–99.6% making them effective for detailed tissue analysis. However, their size and operational complexity limit their use during live surgery. In contrast, fibre-based CFM systems offer miniaturized flexible micro-endoscopes that enable real-time, *in-situ* imaging with accuracy upto 94% demonstrating suitability for intra-operative diagnosis. Notably, fibre-bundle based Cellvizio® confocal laser endomicroscopy (CLE) system and line-scan CLE system can identify breast pathology but data is lacking on intra-operative diagnostic accuracy for margin assessment on wide local excision specimens. New developments like the commercial Histolog® Confocal Microscopy system (SamanTree Medical SA,

Lausanne, Switzerland) has potential to identify missed tumour margins in up to 75% of cases, enhancing the accuracy of margin assessments. While these technologies are promising, several obstacles must be overcome before CFM can be widely adopted in routine surgical practice. Additionally, AI-powered automation in CFM, although promising, requires large-scale validation to ensure accurate real-time tissue classification. Integrating robotics and AI-enhanced CFM could greatly improve real-time surgical decision-making, minimizing interpretation errors and enhancing workflow efficiency.

KEYWORDS

confocal laser microscopy, confocal endomicroscopy, breast cancer, breast conserving surgery, fluorescence, resection margins, margin assessment technology, intra-operative imaging

1 Introduction

Breast cancer is second most common cancer diagnosed globally in 2020 with more than 55,000 new diagnoses each year in the United Kingdom (UK) alone [Cancer Research UK \(2023\)](#), [Bray et al. \(2024\)](#). The lifetime risk of breast cancer for women in the UK and United States of America (USA) is estimated at one in eight with a global estimate of over 2.3 million new breast cancer diagnoses resulting in nearly 685,000 deaths from the disease in 2020 [Sung et al. \(2021\)](#). Surgery remains the primary line of treatment, with breast conserving surgery (BCS) performed in 60%–70% of early-stage cases [Macmillan et al. \(2022\)](#). The adoption of oncoplastic BCS techniques has increased significantly in the past decade, emphasizing intra-operative breast margin assessment to preserve normal tissue without compromising oncological safety [Fleming et al. \(2020\)](#).

1.1 Current challenges in intra-operative margin assessment

A key determinant in local and distant recurrence after BCS is the histopathological status of the resection margins of wide local excisions (WLE) [Moran et al. \(2014\)](#), [Chagpar et al. \(2018\)](#). Despite advancements in localization techniques, positive or close margins persist in 15%–30% of WLE cases, often mandating a second operation to excise further tissue to clear margins [Chagpar et al. \(2018\)](#), [Houssami et al. \(2014\)](#). Surgical guidelines vary globally, with the Association of Breast Surgery (ABS) recommending a 1 mm margin for invasive cancer and a 2 mm margin for ductal carcinoma *in-situ* (DCIS), while American Society of Clinical Oncology (ASCO) accepts no tumour on ink as sufficient in preventing local recurrence [Moran et al. \(2014\)](#), American Society of Clinical Oncology (ASCO) (2020). Positive margins more than double the risk of ipsilateral breast tumour recurrence, leading to revision surgery, causing anxiety, inferior cosmesis and healthcare costs [Lundgren et al. \(2019\)](#). Due to the burden placed on patients and hospitals, it is of great importance to reduce the requirement for further surgery through obtaining negative margins intra-operatively.

The gold standard for determining margin status is post-operative histopathology. Tissue sections (5–20 μm) are cut from different locations for fixing, staining and histopathological examination. While such post-operative pathology techniques

provide high diagnostic accuracy, it is labour-intensive, time consuming and poorly suited for intra-operative margin assessment (IMA). Current intra-operative techniques to assess breast cancer margins, include 2D or 3D radiographic assessment of the excised tissue and microscopic assessment with *in-vivo* imprint cytology or frozen section [Krishnamurthy et al. \(2009\)](#). Despite widespread use of specimen radiography, re-excision rates have persisted at around 20% for the last 15 years [Jeevan et al. \(2012\)](#), [Kaczmariski et al. \(2019\)](#). Frozen section and imprint cytology have been shown to have high sensitivity and specificity, although processing time, requirement of trained staff and high volume of breast tissue to assess have precluded the routine use of either technique [Krishnamurthy et al. \(2009\)](#), [St John et al. \(2017\)](#). A recent expert consensus on breast surgery highlighted the urgent need for novel intra-operative imaging techniques that can improve margin assessment accuracy and reduce re-excision rates [Chowdhry et al. \(2022\)](#). Commensurate with this, there has been tremendous progress in development and validation of real-time optical imaging for BCS.

1.2 Optical imaging for breast cancer margin assessment

Optical fluorescence microscopy is one such promising intra-operative tool that enables real-time, cellular-level imaging, allowing surgeons to differentiate normal and malignant breast tissues. Fluorescence microscopic imaging of fresh breast specimens has been predominantly demonstrated using multiphoton microscopy (MPM) [Wu et al. \(2015\)](#), [Yoshitake et al. \(2016\)](#), [Chen et al. \(2020\)](#). Although the diagnostic accuracy of MPM is comparable to a histological assessment, the high cost of femtosecond lasers used for these systems creates a significant challenge to their clinical utility. Single photon fluorescence microscopy systems, such as CFM are less expensive allowing real-time, high-resolution visualization of tissues at cellular level. The working principle is shown in [Figure 1](#).

By using laser light as a point-source and pin-hole aperture, the detector collects light only from the illuminated focused spot and rejects all the other out-of-focus light from the sample. This enables the CFM to image thin slices through a sample resulting in sharp focused cellular images without physically sectioning it. To create a 2D image, the laser spot is typically scanned point-by-point in raster-scan or spiral-scan patterns using a scan mirror. This

makes CFM a promising tool for improving accuracy during surgery and reducing the need for additional procedures.

There have been significant developments in the use of CFM technique for the assessment of breast cancer tissues and resection margins in BCS Sandor et al. (2022), Gareau et al. (2012), Ragazzi et al. (2014), Krishnamurthy et al. (2019). Early studies demonstrated the feasibility of bench-top confocal strip mosaicking microscopes for *ex-vivo* breast tissue imaging, enabling large-area evaluation with microscopic resolution Larson et al. (2013), Abeytunge et al. (2013a). Parallely, the introduction of fibre-based confocal laser endomicroscopy (CLE) has further expanded real-time intra-operative applications, with *ex-vivo* and *in-vivo* studies demonstrating its potential for rapid breast tissue diagnosis De Palma et al. (2015), Chang et al. (2015), Vyas et al. (2017). Both bench-top and fibre-based approaches offer diagnostic, cellular-level imaging in fresh tissues, supporting real-time intra-operative decision-making.

1.3 Recent advances and emerging frontiers

As the field of intra-operative imaging progresses, several new technologies have emerged that aim to address the limitations of conventional CFM approaches. Recently, Histolog[®] Confocal Microscopy and *ex-vivo* Fusion Confocal Microscopy (EVFCM) have demonstrated high levels of accuracy for IMA, offering an alternative to traditional histopathology Sandor et al. (2022), Togawa et al. (2023), Mathieu et al. (2024), Humaran et al. (2024). The Histolog[®] Scanner is a rapid confocal laser scanner for real-time imaging of excised tissue. Using a 488 nm laser and Histolog[®] Dip stain, it provides 2 μm resolution in under 45 s without sectioning Sandor et al. (2022), Togawa et al. (2023), Mathieu et al. (2024). With a 17 cm² FOV, Histolog[®] scans large tissue areas quickly, detecting missed tumor margins in upto 75% of cases, reducing re-excisions, and enhancing intraoperative decisions Mathieu et al. (2024). Additionally, EVFCM has emerged as a viable alternative to frozen section analysis, providing histology-like imaging in real time Humaran et al. (2024).

Beyond hardware advancements, the integration of artificial intelligence (AI) in confocal imaging is transforming the way intra-operative diagnostics are performed. AI-powered machine learning algorithms are being developed to enhance real-time tissue classification, reducing human interpretation errors and variability Gu et al. (2017), Gu et al. (2018), Gu et al. (2020). AI-driven fluorescence analysis is also helping to standardize imaging protocols and improve automated tumour detection in surgical workflows Sung et al. (2021).

Additionally, robot-assisted confocal imaging is emerging as a way to enhance intra-operative precision. The integration of fibre-based CFM with surgical robotic platforms has demonstrated the potential to perform large area tissue imaging, improve motion stabilization, and optimize tissue penetration depth Zuo et al. (2016), Giataganas et al. (2018), Abdelaziz et al. (2024). The CRUK-EPSRC “MAMMOBOT” project has recently been presented by developing a millimeter scale steering soft robot integrated with flexible endomicroscope for real-time virtual histology of the ductal system and breast micro-architecture Berthet-Rayne et al. (2021). Further, a high-speed line-scan

confocal laser endomicroscope (LS-CLE) system has been developed that can pass through the working channel of the MAMMOBOT platform well as soft polymer-based fiberbots and get real-time images of tissue micro-architecture at 120 fps, 10 times faster than conventional CLE systems Vyas et al. (2022), Abdelaziz et al. (2024). Feasibility studies have demonstrated effectiveness of LS-CLE to provide cellular resolution images of breast ducts, progressing from normal ducts to DCIS and invasive ductal carcinoma (IDC) in real-time by comparison to histopathology. AI-guided robotic-assisted CFM could play a pivotal role extending the use of fibre-based CFM systems in IMA and improving surgical outcomes.

1.4 Aims of this review

This review provides a comprehensive and critical update on novel CFM imaging techniques with potential for application in breast cancer surgery and with an emphasis on.

1. Comparing bench-top and fibre-based CFM systems for intra-operative diagnosis of breast cancer.
2. Assessing the effectiveness of CFM techniques in the detection of breast margin status against histopathology as the reference standard. Assessing imaging performance, diagnostic accuracy and clinical utility.
3. Identifying new frontiers such as integration with robotics and computer-aided diagnosis and discuss their role in improving the tumour margin assessment and clinical relevance.

2 Materials and methods

2.1 Study scope and definition of reference standard

The primary objective of this review is to evaluate the effectiveness of CFM techniques for intra-operative breast cancer margin assessment. Since this study did not involve direct patient data, ethics committee approval was not required. The reference standard for comparison was the routine hematoxylin and eosin (H&E) histopathological evaluation.

2.2 Literature search strategy

A comprehensive literature search was conducted following PRISMA (Preferred Reporting Items for Systematic Reviews and Meta-Analyses) guidelines. The databases searched were PubMed, Embase, and Web of Science databases by three independent reviewers assessing articles published from January 2013 to December 2024. The study was conducted in August 2023 with a final revision on December 2024. The following search terms were selected in the title and/or abstract.

1. Confocal microscopy OR con-focal microscopy OR confocal adj3 mosaicking OR fluorescen* adj3 microscop* OR confocal adj3 microscop* OR con focal adj3 microscop* AND

2. Breast/OR breast. mp AND
3. breast* adj3 margin* OR breast adj5 tissue* OR biops* OR needle adj3 biops* OR fresh adj3 tissue* OR frozen adj3 tissue*

The literature search was restricted to English-language publications, covering all studies from January 2013 to December 2024. COVidence software (Veritas Health Innovation, Melbourne, Australia) was used for literature management, and all retrieved records were screened by two independent reviewers (AE and NH), with a third expert adjudicator (KV) resolving any discrepancies.

Following screening of papers, all articles underwent full-text review by AE, NH and KV. The review included both technical and clinical studies. The focus was to identify studies evaluating fluorescence-based imaging in *in-vivo* and rapid *ex-vivo* imaging during breast cancer surgery. The following parameters were used to guide inclusion/exclusion of articles:

Inclusion criteria:

- Human studies (Pilot, cohort, or clinical trials)
- Peer-reviewed Journal publications
- Book chapters on CFM
- Studies evaluating intra-operative CFM imaging

Exclusion criteria:

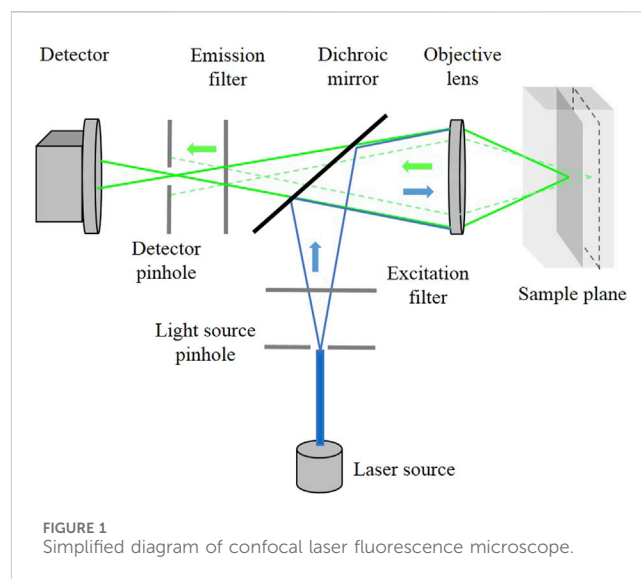
- Non-human studies (Animal, cell line, phantom models, tissue culture)
- Fluorescence spectroscopy-only studies
- Published in languages other than English
- Conference abstracts, editorials, or opinion pieces
- Review articles that did not present new experimental findings

Our focus on human breast tissue imaging reflects the transition of CFM from technical validation to clinical translation. We prioritized studies that offer insights into real-world tissue acquisition, staining, and interpretation protocols, which are critical for clinical deployment in BCS. Animal and phantom models, while relevant for early-stage system development, were excluded to maintain clinical applicability.

2.3 Data extraction

All eligible studies included in this review were analyzed using a structured extraction table, developed by AE, NH, KV, and senior author DRL. The extraction framework was created in Microsoft Excel and all discrepancies were discussed until 100% agreement was achieved. The following parameters were extracted.

- Study meta-data: Author, publication year, journal, article type (technical/clinical).
- Sample characteristics: Number of patients, number of tissue samples, cancer types and subtypes examined.
- Imaging parameters: Imaging modality, optical resolution, sectioning depth, frame rate, FOV, duration of imaging, fluorescent contrast agent (if applicable)



- Diagnostic utility: Sensitivity, specificity, accuracy, concordance with histology, intra-operative usability (*in-vivo/ex-vivo*).
- Clinical relevance: margin assessment potential, re-excision rate impact, tissue architecture analysis.

All selected studies involved human breast specimens, either preclinical or clinical in nature. No animal or phantom studies were included.

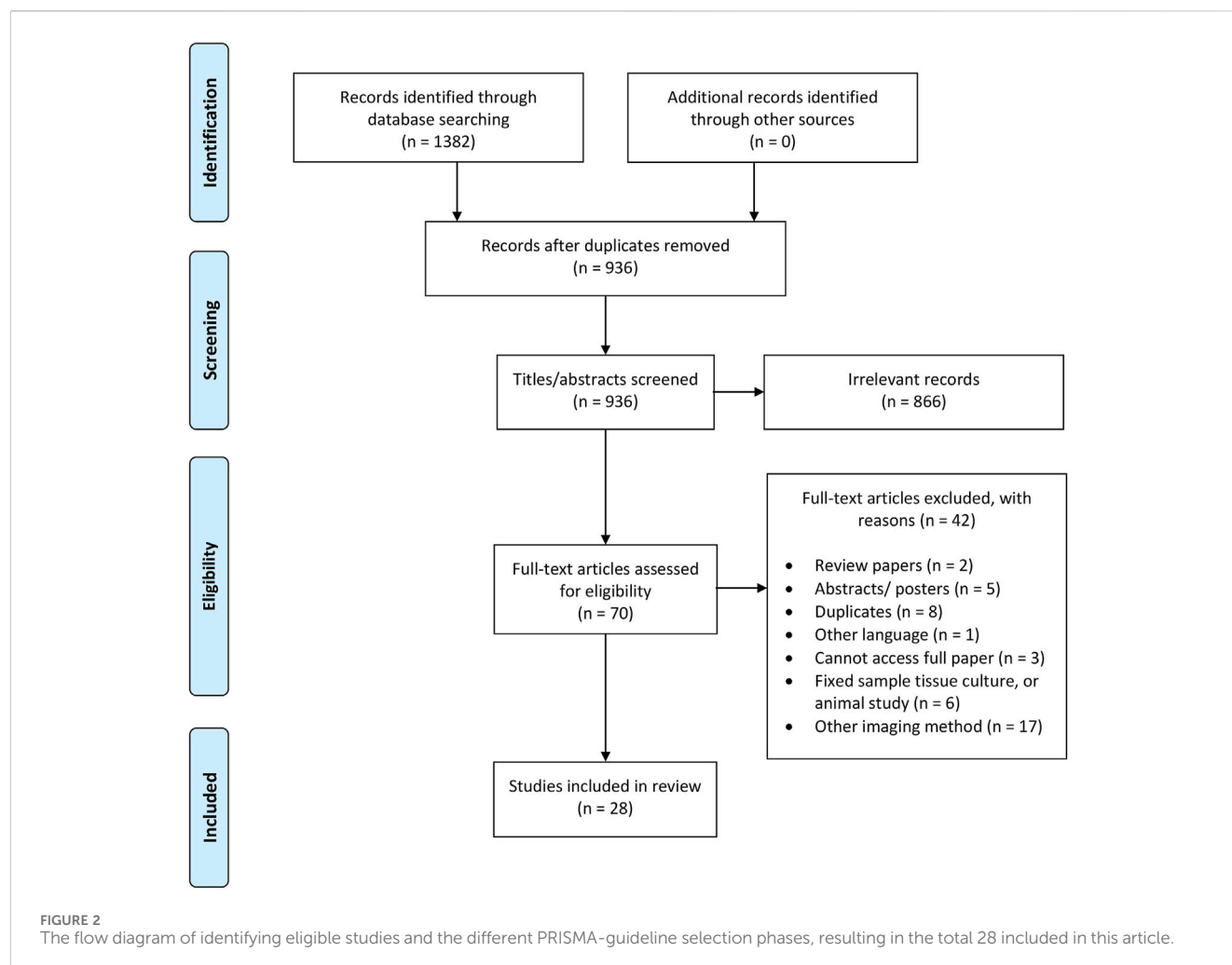
2.4 Statistical analysis

Due to the heterogeneity in study populations, imaging methodologies, and optical system specifications, a meta-analysis was not feasible. Instead, a qualitative synthesis of imaging performance was performed. Descriptive statistics were used to summarize diagnostic accuracy measures (sensitivity, specificity, accuracy) from individual studies.

3 Results

A total of 1382 references were imported to COVidence following preliminary database search. 445 duplicate studies were removed, and 936 articles were screened for title and abstract assessment. 68 were selected for full-text review, with 28 studies meeting the final inclusion criteria. A Preferred Reporting Items for Systematic reviews and Meta-analyses (PRISMA) flow diagram summarizing the study selection process is illustrated in Figure 2.

The articles included in this review consisted of experimental studies (clinical and technical) assessing the utility and diagnostic accuracy of novel CFM modalities in breast cancer assessment. Available studies were categorised into bench-top CFM systems (Table 1, n = 21) and hand-held fibre-based systems (Table 2, n = 7). To aid visual interpretation of the technical and workflow differences between these imaging modalities, Figure 3 presents representative examples of the gold-standard H&E



histopathology, alongside bench-top and fiber-based confocal fluorescence microscopy systems.

3.1 Bench-top CFM systems for breast tissue imaging

The majority of the literature on benchtop CFM for breast tissue imaging has used the commercial CFM systems, the Vivascope® 2000 and Vivascope® 2500 (Caliber Imaging and Diagnostics, Rochester, NY, USA). These systems are designed for *ex-vivo* tissue and both provide microscopic resolution ($\approx 1 \mu\text{m}$ lateral resolution) confocal images of un-fixed breast tissue at 5–10 fps for a FOV of 750 μmm .

In 2013, a confocal strip mosaicking microscope (CSMM) was developed as a modified version of Vivascope® 2000 to enable rapid scanning of large tissue areas while still maintaining microscopic resolution. With CSMM, a $10 \times 10 \text{ mm}$ area was imaged in 1 minute with sub-cellular resolution and optical sectioning of $3 \mu\text{m}$, prior to any fixation or slicing [Larson et al. \(2013\)](#), [Abeytunge et al. \(2013a\)](#). CSMM has previously been used to evaluate skin lesions, especially during Mohs surgery; however, the system was often operated only in reflectance mode. Larson et al. reported successful imaging of 25 WLE and mastectomy cases, demonstrating that fluorescence

imaging provided sufficient contrast to distinguish ducts and lobules from surrounding stromal tissue [Larson et al. \(2013\)](#). Further proof-of-concept studies demonstrated the capability CSMM for large-area *ex-vivo* imaging of invasive ductal carcinoma (IDC) and DCIS with an imaging area of $3.5 \times 3.5 \text{ cm}^2$, $0.8 \mu\text{m}$ lateral resolution, and an acquisition time of 13 min. A modified CSMM system featuring a customized tissue holder and tilt-levelling stage has recently been reported, to perform large-area imaging while maintaining microscopic resolution [Abeytunge et al. \(2017\)](#). In a pilot study of 34 freshly excised specimens from 18 patients, the authors reported an imaging area of 400 mm^2 could be scanned with $1 \mu\text{m}$ lateral resolution in 10 min. The system used 0.6 mM acridine orange as a nuclear contrast agent and have demonstrated applications towards rapid imaging of whole core needle biopsies. A recent study investigated the effectiveness of CSMM to tackle challenging tasks such as diagnosing DCIS and benign lesions in *ex-vivo* breast tissue cut-outs, reporting high diagnostic performance (95.5% sensitivity and 97.3% specificity in 40 patients) [Krishnamurthy et al. \(2019\)](#). CSMM may therefore be effective in future for margin assessment during BCS, with minimal tissue preparation, and warrants further investigations.

The commercial Vivascope®2500 has shown promise in breast cancer imaging, particularly for core needle biopsies and small surgical excisions [Dobbs et al. \(2013\)](#), [Dobbs J. et al. \(2015\)](#),

TABLE 1 Bench-top CFM (21 works). Legend to the table: FOV- Field of view, FPS- frames per second, DCIS- Ductal carcinoma *in-situ*, IDC- Invasive ductal carcinoma, ILC- Invasive lobular carcinoma, IC- Invasive carcinoma.

Study, year	Confocal system used	Resolution, FOV and frame-rate	Fluorophore used	Sample set	Pathologies examined	Main findings
Larson et al. (2013)	confocal strip mosaicking microscope (Larson et al., 2013)	1 μm ; 3 μm	Acridine orange (0.6 mM, topical administration)	25 patients 75 tissues	-	<i>ex-vivo</i> study demonstrating large-area imaging in BCS $3.5 \times 3.5 \text{ cm}^2$ of tissue is imaged in 13 min
Abeytunge et al. 2013a)	confocal strip mosaicking microscope (Abeytunge et al., 2013a)	-	Acridine orange (0.6 mM, topical administration)	1 patient 1 tissue	1 slide with IDC and DCIS	<i>ex-vivo</i> study for large-area imaging in breast surgery $10 \times 10 \text{ mm}^2$ of tissue is imaged in 75 s
Abeytunge et al. 2013b)	confocal strip mosaicking microscope (Abeytunge et al., 2013b)	1 μm ; 2.3 μm	Acridine orange (0.6 mM, topical administration)	1 patient 1 tissue	1 slide with tumour and surrounding fat and stroma	<i>ex-vivo</i> study for large-area imaging in breast surgery $2.5 \times 3.5 \text{ cm}^2$ of tissue is imaged in 13 min
Abeytunge et al. (2017)	confocal strip mosaicking microscope (Abeytunge et al., 2017)	1 μm ; 3 μm	Acridine orange (0.6 mM, topical administration)	18 patients 34 tissues	23 Benign 3 DCIS 8 IDC	<i>ex-vivo</i> study for large-area imaging in breast surgery
Dobbs et al. (2013)	VivaScope 2500 (Dobbs et al., 2013)	1 μm ; 5 μm 750 μm at 9 FPS	Proflavine (0.01%, topical administration)	31 patients 70 tissues	25 Normal or benign 11 DCIS 19 IDC and 13 ILC 2 mucinous carcinoma	Experienced reviewers demonstrate higher accuracy in detecting neoplasia than novices. Sensitivity 93% Specificity 93%
Dobbs et al. (2015a)	VivaScope 2500 (Dobbs et al., 2015a)	1 μm ; 5 μm 750 μm at 9 FPS	Proflavine (0.01%, topical administration)	34 patients 68 tissues 259 sites	179 Benign sites 80 Malignant sites	computer-aided classification to segment and measure nuclear and ductal parameters. Sensitivity 81% Specificity 93% Accuracy 90%
Dobbs et al. (2015b)	VivaScope 2500 (Dobbs et al., 2015b)	1 μm ; 5 μm 750 μm at 9 FPS	Proflavine (0.01%, topical administration)	23 patients 23 tissues, 3 inadequate	16 IDC 4 ILC	Moderate agreement by kappa coefficient $\kappa = 0.48 \pm 0.09$ ($p < 0.001$) for grayscale and histology images; poorer for false coloured images
Dobbs et al. (2016a)	VivaScope 2500 (Dobbs et al., 2016a)	1 μm ; 5 μm 750 μm at 9 FPS	Proflavine (0.01%, topical administration)	22 patients 44 tissues	2 collagen, DCIS and IDC 20 collagen and IDC	Adipocytes near neoplastic margins are significantly smaller than those adjacent to non-neoplastic stroma
Krishnamurthy et al. (2019)	VivaScope 2500 (Krishnamurthy et al., 2019)	1 μm ; 5 μm 750 μm at 9 FPS	Acridine orange (0.6 mM, topical administration)	38 patients 38 tissues	16 Normal 2 DCIS 18 IDC and 2 ILC	Sensitivity 100% specificity 100%
Krishnamurthy et al. (2018)	VivaScope 2500 (Krishnamurthy et al., 2018)	1 μm ; 5 μm 630 μm at 9 FPS	Acridine orange (0.6 mM, topical administration)	~16 tissues	8 Normal 1 DCIS 6 IDC and 1 ILC	Suitability of rapid <i>ex-vivo</i> is similar to H&E

(Continued on following page)

TABLE 1 (Continued) Bench-top CFM (21 works). Legend to the table: FOV- Field of view, FPS- frames per second, DCIS- Ductal carcinoma *in-situ*, IDC- Invasive ductal carcinoma, ILC- Invasive lobular carcinoma, IC- Invasive carcinoma.

Study, year	Confocal system used	Resolution, FOV and frame-rate	Fluorophore used	Sample set	Pathologies examined	Main findings
Krishnamurthy et al. (2019)	RS-G4 confocal strip mosaicking microscope (Krishnamurthy et al., 2019)	1 μm ; 5 μm 400 μm at 6 FPS	Acridine orange (0.6 mM, topical administration)	40 patients 40 tissues	25 Normal or benign 2 DCIS 9 IDC and 1 ILC	Sensitivity 95.5% Specificity 97.3%
Ragazzi et al. (2014)	VivaScope 2500 (Ragazzi et al., 2014)		Acridine orange (0.6 mM, topical administration)	12 patients 12 tissues	4 Normal or benign 1 DCIS, 1 LCIS 4 IDC and 2 ILC	-
Patel et al. (2012)	Custom confocal microscope (Patel et al., 2012)	0.9 μm ; 3–6 μm 350 μm at 9 FPS	Methylene blue	–17 tissues	1 Intracystic papillary carcinoma <i>in-situ</i> 11 IDC and ILC	IDC and ILC correlated well with both wide field microscopy and routine H&E
Yoshitake et al. (2016)	Zeiss LSM510 inverted confocal microscope (Yoshitake et al., 2016)	0.88 μm ; 3–6 μm 900 μm at 0.16 FPS	40 $\mu\text{g}/\text{mL}$ PI, 200 $\mu\text{g}/\text{mL}$ EY (topical)	-	-	
Elfgen et al. (2019)	Histolog [®] Scanner (Elfgen et al., 2019)		Acridine Orange (0.01%, topical administration)	24 Patients 24 tissues	2 DCIS 13 IDC and 4 ILC 3 Other IC	Rapid large-area scanning
Tanei et al. (2019)	KeyenceBZ-X710 with optical sectioning algorithm (Tanei et al., 2019)		click-to-sense probe 1 and Hoechst 33,342 + 33,258	30 Patients 65 tissues	30 Normal or benign 5 Ductal hyperplasia 10 DCIS 20 IDC and 4 ILC	Result difficult to assess
Sandor et al. (2022)	Histolog [®] Scanner (Sandor et al., 2022)		Acridine Orange (0.01%, topical administration)	40 Patients 40 tissues 240 images	10 IDC and 4 ILC 7 DCIS 17 IDC and DCIS 1 ILC and DCIS	6 sides of WLE specimen imaged in $\approx$ 13 s
Togawa et al. (2023)	Histolog [®] Scanner (Togawa et al., 2023)		Acridine Orange (0.01%, topical administration)	50 Patients 50 tissues	35 IDC and 4 ILC 7 DCIS 2 IDC and ILC 2 others	Surgeon: sensitivity 37.5%, specificity 75.2% and pathologist: sensitivity 37.5%, specificity 78.2%
Conversano et al. (2024)	Histolog [®] Scanner (Conversano et al., 2024)	-	Acridine Orange (0.01%, topical administration)	181 Patients 55 patients (training)	6 Normal 31 IDC and 11 ILC 7 DCIS	7 surgeons and 2 pathologist performed blinded assessment of 126 tissues: accuracy 83%–98%, and 99.6% respectively
Mathieu et al. (2024)	Ex-Vivo Fusion Confocal Microscopy (EVFCM) (Mathieu et al., 2024)	-	Autofluorescence (Label-Free)	258 Patients 109 assessed	18 Normal 74 IDC and 17 ILC 21 DCIS	Pathologist assessment showed 94.5% sensitivity, 98.2% specificity; significant agreement with histopathology
Humaran et al. (2024)	Ex-Vivo Fusion Confocal Microscopy (EVFCM) (Humaran et al., 2024)	-	Autofluorescence (Label-Free)	100 Patients 40 assessed	12 Normal 55 IDC and 14 ILC 19 DCIS	Demonstrated real-time intraoperative imaging capability with comparable accuracy to conventional histopathology

Krishnamurthy et al. (2018). Using 0.01% topical proflavine, an inexpensive topically applied dye, the researchers evaluated the ability of CFM to distinguish normal and neoplastic breast pathologies from gold standard histopathology. In this work, single $750 \times 750 \mu\text{m}^2$ regions of interest were obtained from acquired confocal images and correlated with corresponding histological images acquired at $\times 10$ magnification. A board-certified breast pathologist reviewed the H&E stained images,

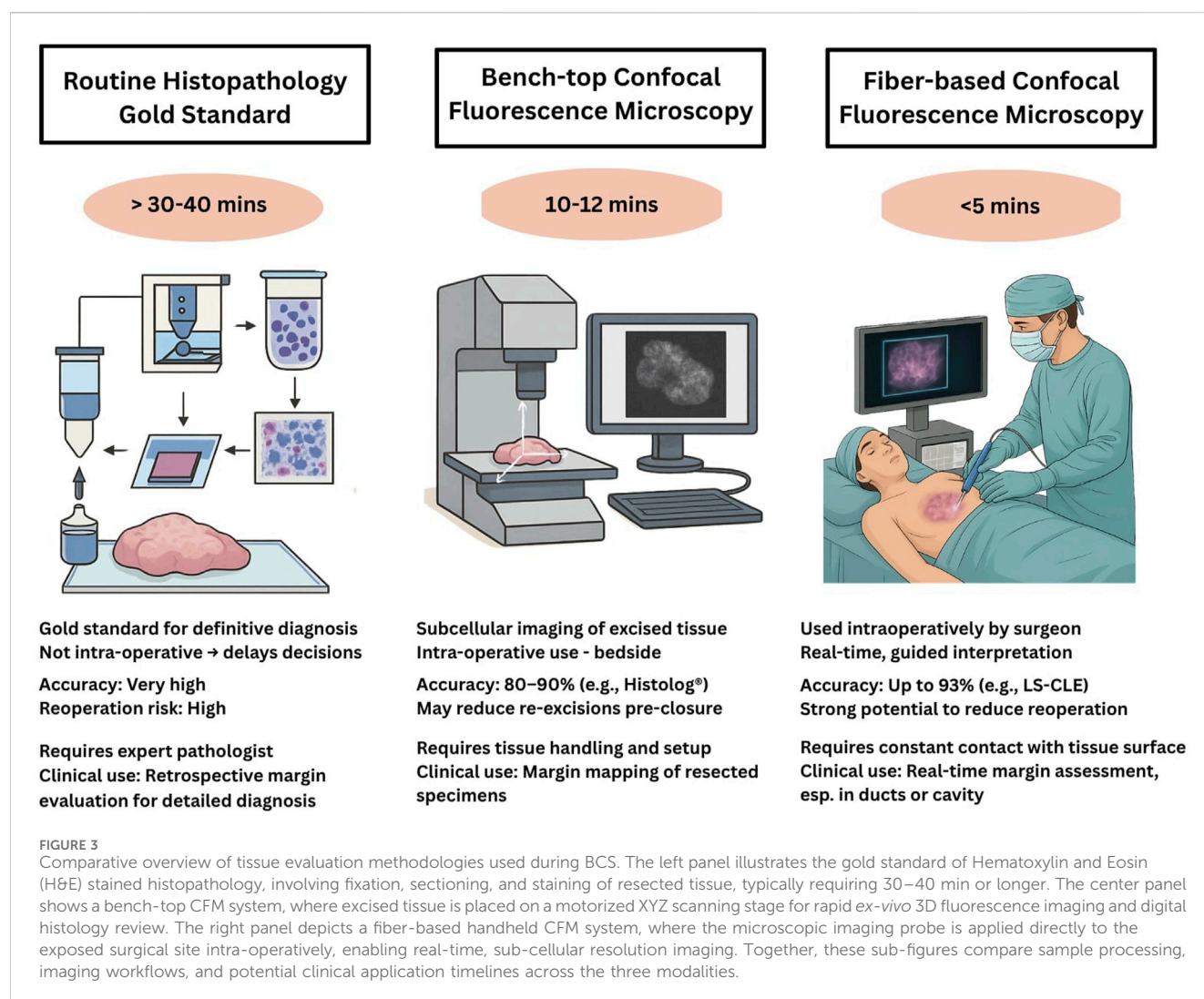
TABLE 2 Fibre based CLE (7 works). Legend to the table: p-CLE- Probe-based confocal laser endomicroscopy, LS-CLE- Line-scan confocal laser endomicroscopy, SI-HRME- Structured illumination based high resolution microendoscopy, FOV- Field of view, FPS- frames per second, DCIS- Ductal carcinoma *in-situ*, IDC- Invasive ductal carcinoma, ILC- Invasive lobular carcinoma.

Study, year	Confocal system used	Image resolution FOV and frame rate	Fluorophore used	Sample set	Pathologies examined	Main findings
Chang et al. (2015)	Cellvizio p-CLE (Chang et al., 2015)	1 μm ; 60 μm 240 μm at 12 FPS	Acriflavine (0.01%, topical administration)	50 patients 71 tissues	21 Normal 12 DCIS 38 IDCs	<i>ex-vivo</i> pilot study demonstrating the feasibility of p-CLE imaging in breast cancer surgery. Sensitivity 96% Specificity 92%, Accuracy 94%
Zuo et al. (2014)	Cellvizio p-CLE (Zuo et al., 2014)	1 μm ; 60 μm 240 μm at 12 FPS	Acriflavine (0.01%, topical administration)	-	-	First study integrating robotics with p-CLE for large area scanning and imaging inside the breast cavity
Vyas et al. (2017)	Line-scan CLE (Vyas et al., 2017)	2.2 μm ; 11 μm 240 μm at 120 FPS	Methylene blue (0.1%, topical administration)	5 patients 15 tissues	-	First study demonstrating use of high speed LS-CLE and methylene blue for breast cancer diagnosis
Zuo et al. (2015)	Custom p-CLE with leached fibre bundle (Zuo et al., 2015)	8 μm ; 15 μm 670 μm at 10 FPS	Acriflavine (0.01%, topical administration)	1 patient 2 tissues	1 Normal 1 IDC	Integrating robotics with very flexible fibre based p-CLE for large area scanning and imaging inside the breast cavity
Palma et al. (2015)	Cellvizio p-CLE (Palma et al., 2015)	1 μm ; 60 μm 240 μm at 12 FPS	(10%, intravenous Fluorescein, 5 min before excision)	13 patients 13 tissues	2 FAD 2 benign phyllodes 5 IDCs and 1 ILC 1 mucinous carcinoma	Rapid <i>ex-vivo</i> pilot study demonstrating the feasibility of p-CLE imaging in breast cancer surgery
Dobbs et al. (2016b)	Vivascope 2500 [®] and SI-HRME (Dobbs et al., 2016b)	1 μm ; 5 μm 750 μm at 9 FPS	Proflavine (0.01%, topical administration) and Lugol's Iodine (2%, topical application)	19 patients 70 tissues	21 Non-neoplastic 3 DCIS 20 IDC	<i>ex-vivo</i> pilot study demonstrating the feasibility of SI-HRME imaging with Lugol's Iodine and proflavine staining for breast cancer diagnosis
Vyas et al. (2022)	Line-scan CLE (Vyas et al., 2022)	2.2 μm ; 11 μm 240 μm at 120 FPS	Methylene blue (0.1%, topical administration)	5 patients 15 tissues	-	First study demonstrating use of high speed LS-CLE and methylene blue for breast cancer diagnosis

providing diagnoses. A smaller dataset of 49 matched ROIs was used in a validation study based on visual assessment by seven pathologists. The validation set included 18 images with histologically normal breast morphology, 10 images of benign change, and 21 images with neoplastic breast morphology, representative images presented in [Figures 4](#). A single-centre clinical trial further demonstrated a sensitivity and specificity of 93% for detecting *in-situ* and invasive carcinoma in 31 patients, reinforcing the potential of CFM as a real-time imaging tool for breast cancer detection [Dobbs et al. \(2013\)](#). Additional diagnostic accuracy studies have assessed the performance of CFM in core needle biopsies from 23 patients with suspected inflammatory breast carcinoma, reporting moderate agreement ($\kappa = 0.62$) between histologic and pseudo-coloured confocal imaging [Dobbs J. et al. \(2016\)](#). While variability in cellular estimation was noted, CFM's rapid imaging capabilities were highlighted as a benefit for preliminary diagnosis.

To expand CFM's clinical utility, researchers have explored techniques in computer-aided diagnosis (CAD) to automate tissue segmentation and improve diagnostic accuracy. One

approach focused on automatically segmenting adipocytes to evaluate changes in adipocytes in the tumour microenvironment associated with IDC and DCIS in 22 cases [Dobbs JL. et al. \(2016\)](#). Dobbs et al. analyzed 179 benign and 80 malignant tissue sites from 34 patients. The study reported a sensitivity of 81%, specificity of 93%, and an overall diagnostic accuracy of 90% in distinguishing malignant from benign tissue using CAD-assisted CFM imaging. CAD analysis revealed that adipocytes near tumour margins were significantly smaller than those adjacent to non-neoplastic collagenous stroma, suggesting that changes in adipocyte morphology could serve as an indicator of tumour progression. These findings highlight the potential of CFM in assessing changes in the tumour micro-environment and understanding progression in malignancy with high accuracy. Additionally, a feasibility study involving 12 breast surgical specimens from 12 patients explored the use of Vivascope[®] 2500 with acridine orange, a rapid nuclear-staining agent, for intra-operative tissue evaluation [Ragazzi et al. \(2014\)](#). The study demonstrated that CFM could differentiate various breast tissue structures with sufficient contrast, reinforcing its potential as an intra-operative imaging tool.



However, while these findings are promising, the study did not assess relevant clinical outcomes such as the accuracy of margin assessment, impact on re-excision rates, or long-term diagnostic reliability. Indeed whilst the studies highlighted above demonstrate the potential of CFM for breast cancer diagnostics, they have primarily been conducted on small tissue sections and biopsy samples, and further research is needed to assess its effectiveness in evaluating whole-margin status in larger WLE specimens.

The Histolog[®] Scanner (SamanTree Medical SA, Lausanne, Switzerland), is being explored as a tool for *ex-vivo* whole margin assessment in BCS. Sandor et al. conducted one of the earliest evaluations of Histolog[®] for WLE margin assessment [Sandor et al. \(2022\)](#). The authors analyzed 40 WLE specimens, comparing Histolog[®] imaging to intra-operative ultrasound and specimen radiography, rather than histopathology. Retrospective image review suggested that 30%–75% of patients requiring re-excision ($n = 12$) could have been identified earlier with confocal imaging. However, the wide range was attributed to tissue variability, differences in interpretation, and imaging method limitations. While the study demonstrated Histolog[®]'s potential for intra-operative use, it also highlighted the need for direct histological validation and real-time clinical trials. While the

study demonstrated Histolog[®]'s potential for intraoperative use, it underscored the need for direct histological validation and standardized interpretation protocols.

Togawa et al. further investigated Histolog[®]'s accuracy for breast WLE margin assessment using 50 tissue specimens [Togawa et al. \(2023\)](#). Surgeons had a sensitivity of 37.5% and specificity of 75.2%, whilst unsurprisingly pathologists achieved higher specificity of 78.2%. These results suggest that while Histolog[®] may support IMA, variability in interpretation indicates a need for additional standardization and training before it can be reliably implemented in routine surgical workflows. Conversano et al. as part of the HIBISCUSS project, evaluated 181 breast tissue samples, using 55 for training and the remainder for blinded tissue assessment [Conversano et al. \(2024\)](#). They found that pathologists demonstrated near-perfect diagnostic accuracy (99.6%), while surgeon interpretation ranged between 83% and 98% accuracy. These findings reinforce Histolog[®]'s potential for high-resolution, *ex-vivo* imaging, but also emphasize that specialized training is necessary to optimize interpretation accuracy. Beyond WLE margins, recently Mathieu et al. and Mazzucchelli et al. explored Histolog[®]'s applications in core needle biopsy assessment and patient-derived breast cancer organoid research, showing over

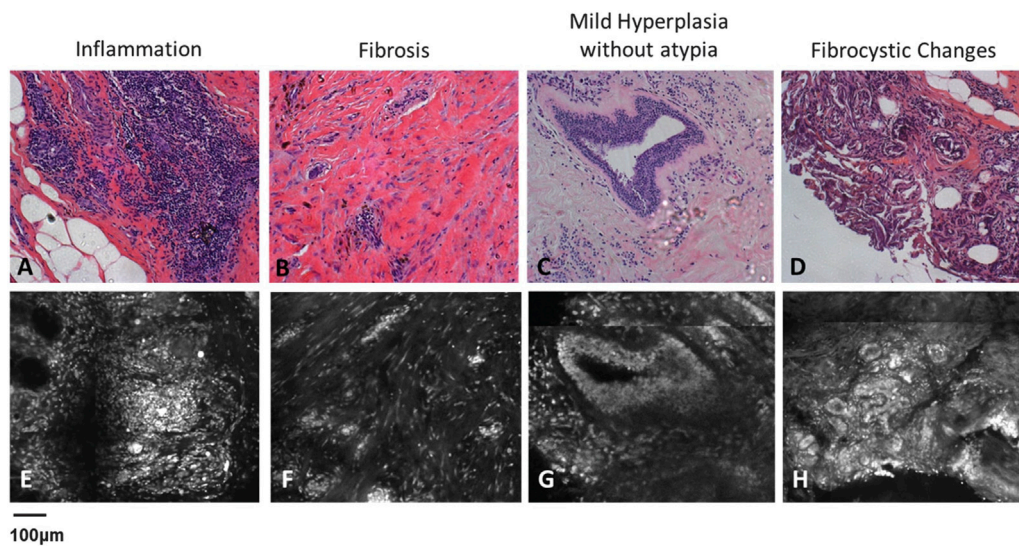


FIGURE 4
Demonstrates characteristic features of benign, non-neoplastic breast tissue. (A–D) represents H and E-stained images of inflammation, fibrosis, mild hyperplasia without atypia and fibrocystic changes while (E–H) represents features using CFM. Figure reproduced from [Dobbs et al. \(2013\)](#).

93% concordance with histopathology [Mathieu et al. \(2024\)](#), [Mazzucchelli et al. \(2024\)](#). Their findings suggest that Histolog[®] could be valuable in rapid breast tissue evaluation and personalized oncology research.

Collectively, these studies highlight the potential for wide-field confocal systems in intra-operative breast cancer diagnostics and precision BCS. However, despite the diagnostic accuracy shown in many studies conducted in academic research centres, up-front investment and the need for expert pathologists remain barriers to widespread adoption of existing bench-top CFM systems for breast cancer imaging. In addition, its low acquisition rate and small FOV can be prone to sampling errors, limiting its application for IMA of large specimens like whole breast margins in a timely manner.

3.2 Fibre-based CFM systems for breast tissue imaging

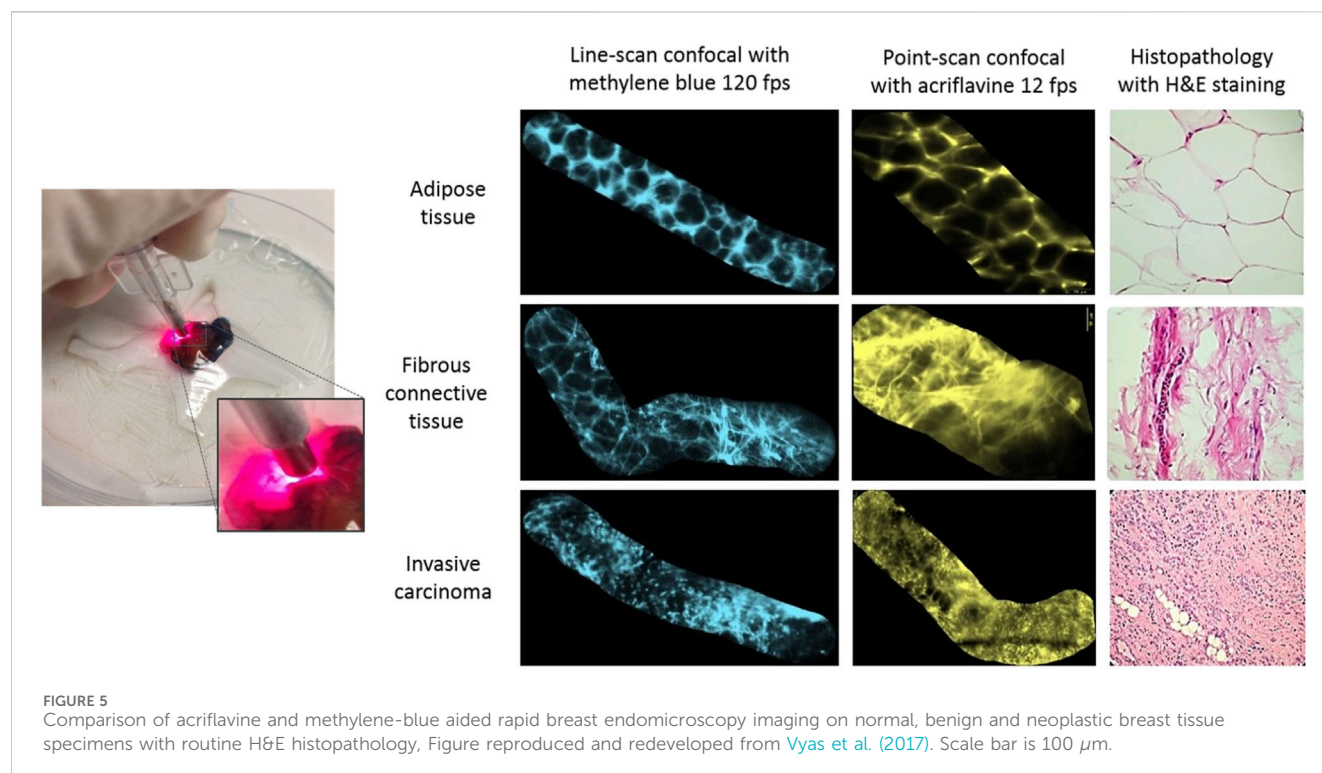
Fibre-based confocal laser endomicroscopy (CLE) is a popular optical biopsy technique that translates conventional CFM into a real-time *in-vivo* clinical modality [De Palma et al. \(2015\)](#), [Chang et al. \(2015\)](#). Flexible optical fibre bundles are used as small diameter imaging probes to acquire images at confined sites within the body by scanning the probe tip on the tissue surface. These probes can be inserted into the working channels of conventional endoscopes enabling rapid and non-invasive detection and classification of a tissue's histopathological status [Vyas et al. \(2022\)](#).

Point-scanning probe-based confocal laser endomicroscopy (p-CLE) system and a range of imaging probes with different diameters and specifications are commercially available by Cellvizio[®] (Mauna Kea Technologies, Paris, France). For such systems, lateral and axial resolutions down to 0.5 and 3 μm , respectively, FOV of typically $240 \times 240 \mu\text{m}$, have been achieved for frame rates of 12 fps. Prior studies demonstrate the capability of

p-CLE to detect breast tissue architecture using a commercial Cellvizio[®] p-CLE system [Chang et al. \(2015\)](#), [De Palma et al. \(2015\)](#). Chang et al. used topically applied acriflavine hydrochloride, excited at 488nm, which stains cell nuclei and enables high contrast imaging of tissue morphology. Cellular resolution confocal images with FOV of $240 \mu\text{m}$ were acquired at 12 fps. The study included fresh breast cancer samples with adjacent healthy tissue from 50 patients and overall a 93% accuracy was achieved by pathologists and breast surgeons. The study was investigational as acriflavine is currently not FDA approved or commercially available for *in-vivo* human use.

Another pilot study reported the application of optical fibre bundle based structured illumination endomicroscopy (SI-HRME) using topical application of 0.01% proflavine and 2% Lugol's iodine as contrast agents to detect breast cancer [Dobbs J. et al. \(2016\)](#). Fresh breast tissue specimens from 19 patients were stained with proflavine alone or Lugol's Iodine and proflavine. Images of tissue specimens were acquired using a confocal microscope and an HRME system with and without structured illumination. Dobbs et al. reported that structured illumination along with proflavine staining could potentially be used to increase contrast in HRME images of breast tissue for rapid image acquisition. Further, the addition of Lugol's Iodine did not increase mean contrast significantly for HRME or SI-HRME images. This study was also investigational as proflavine is currently not cleared to be routinely used for *in-vivo* human use.

For *in-vivo* imaging in routine clinical practice, it is desirable to use endomicroscopes with approved fluorescent dyes which are both safe for human use and provide sufficient contrast to distinguish between different tissue morphologies. Methylene Blue is one of the few FDA approved dyes for *in-vivo* human application and has been demonstrated previously for the treatment of methemoglobinemia and is an alternative to isosulphan blue for *in-vivo* localisation of non-palpable lesions and sentinel lymph node mapping during breast surgeries [Zhang et al. \(2019\)](#). In a 2017 study, a custom high-speed line-scan confocal laser endomicroscopy (LS-CLE) system



operating at 660 nm was used in combination with topical 0.1% methylene blue to successfully demonstrate the first rapid morphological assessment of freshly excised breast cancer tissues Vyas et al. (2017). Images and mosaics of normal, benign and neoplastic breast tissue, acquired with a lateral resolution of 2.2 μ m at 120 Hz, showed distinctive morphological features, which visually and qualitatively correlated well with histology. Representative images of tissue scanning and different breast pathologies imaged with p-CLE are shown in Figure 5. The high-speed of LS-CLE acquired long contiguous mosaics at frames rates up to 120 fps which was an order of magnitude improvement over the previous systems. Results were comparable to those obtained using the Cellvizio[®] p-CLE system with acriflavine staining, but with the advantage of a higher frame-rate and use of a safe and regulatory-approved (off-label) stain.

While all the previous studies have been performed using non-specific dyes for imaging morphology of different breast pathologies, a recent study by Gao et al. demonstrated the molecular imaging capabilities of confocal endomicroscopy Gao et al. (2017). A handheld near-infrared dual axis confocal endomicroscope was developed to detect ErbB2 positive cells in breast tissue by using a specific targeting peptide labelled with IRDye800CW maleimide (LiCor Biosciences) fluorophore. Images were acquired at five fps and *in-vivo* molecular imaging capabilities were demonstrated by assessing the uptake of specific peptide binding to human xenograft breast tumours expressing ErbB2.

3.3 New frontiers for p-CLE imaging

3.3.1 Computer-assisted image interpretation

Traditionally, histology images of biopsy samples are assessed by trained pathologists for disease diagnosis, classification, and

treatment planning. While CFM images closely resemble histology, their interpretation remains a challenge for clinicians and surgeons with limited histopathology training. The high variability in p-CLE images and the presence of atypical conditions further complicate accurate manual diagnosis. As a result, CAD systems have been developed to improve diagnostic accuracy and reduce dependency on expert pathologists, enhancing the clinical utility of p-CLE imaging.

A component-based segmentation approach that utilizes internuclear distance, nuclear shape, ductal lumen diameter, and fluorescence intensity has demonstrated the potential of CAD for CFM imaging Dobbs JL. et al. (2015). Using Vivascope[®] 2500 with 0.01% proflavine staining, researchers evaluated freshly acquired breast tissues from 34 patients, including 22 malignant cases of IDC and DCIS. A decision tree model, validated against expert pathologist-confirmed histopathology, demonstrated high diagnostic accuracy in classifying IDC (92%) and DCIS (96%) Dobbs J. et al. (2015). The overall model sensitivity, specificity, and accuracy were 75%, 93%, and 88%, respectively.

The mosaicking feature adapted by most CLE systems enable to create a larger FOV by stitching adjacent image frames together. An Unsupervised Multimodal Graph Mining (UMGM) approach is reported to learn the discriminative features for p-CLE mosaics of breast tissue on a database of 700 p-CLE mosaics Gu et al. (2017). The mosaicking capability of p-CLE systems enables the reconstruction of large fields of view by stitching adjacent image frames together, improving the spatial coverage of imaging.

Since p-CLE is a relatively new imaging technique, the size of clinical dataset is also limited, making CAD analysis even more challenging. To address this, a Transfer Recurrent Feature Learning (TRFL) framework has been introduced for p-CLE video classification Gu et al. (2018). This two-stage method first learns

discriminative features from individual p-CLE frames using generative adversarial networks (GANs) trained on both p-CLE and histology data. In the second stage, recurrent neural networks (RNNs) process frame-based features, handling variations in mosaic length and shape to achieve a classification accuracy of 84.1%.

3.3.2 Integration with robotics for large-area p-CLE imaging

One of the primary limitations of p-CLE is the restricted FOV, which is constrained by the fibre bundle probe size, typically ranging from 0.25 to 0.8 mm. This limitation poses significant challenges for surgeons in maintaining consistent probe-tissue contact, particularly when imaging large surgical areas such as breast WLE cavities. To address this, there has been increasing interest in robotic-assisted scanning systems to enhance p-CLE imaging capabilities, improve tissue coverage, and standardize image acquisition.

The first study to integrate robotics with p-CLE was conducted by Zuo et al., who demonstrated miniaturized and flexible robotic scanning systems designed for whole-breast cavity imaging [Zuo et al. \(2016\)](#). More recently, Giataganas et al. developed a portable robotic scanning probe capable of achieving micrometer-scale accuracy and generating mosaicked imaging fields of up to 14 mm². Their system allows for stable, high-precision tissue scanning, reducing manual variability and improving intra-operative imaging reliability [Giataganas et al. \(2018\)](#).

Expanding on these advances, Abdelaziz et al. recently introduced a polymer-based flexible robotic system demonstrating high-precision large area CLE imaging applications on *ex-vivo* breast tissue [Abdelaziz et al. \(2024\)](#). p-CLE fibre was passed through the working channel of this flexible robot, enabling automated scanning across large tissue surfaces. This approach is designed to improve p-CLE probe positioning, reduce motion artifacts, and optimize tissue-contact consistency, thereby enhancing imaging accuracy and diagnostic confidence in intra-operative breast cancer assessment. In another study, Berthet et al. demonstrated the design and operation of Mammobot: A mm-scale steerable soft growing robot for precise fibre based ductoscopy for early breast cancer detection [Berthet-Rayne et al. \(2021\)](#). MAMMOBOT aims to access the breast through the nipple and navigate the mammary ducts to detect precursors of invasive breast cancers. Addressing limitations of the state-of-the-art, MAMMOBOT maintains a hollow inner lumen throughout its soft body, enabling the passing of instruments such as miniature endoscopes, biopsy needles, and optical probes for *in-situ* histopathology. Vyas et al. demonstrated the feasibility of LS-CLE as a flexible micro-ductoscope that could pass through the hollow lumen of MAMMOBOT and aid in identifying discernible features corresponding to normal ducts, DCIS and distinguishing them from IDC at sub-cellular scale. Such a miniaturized tool could be very useful as the next-generation mammary ductoscope for full duct outline mapping by detecting cancer or pre-cancerous lesions [Vyas et al. \(2022\)](#). Such robotic tools could be used hand-held or with an articulated or passive arm for large-area scanning applications. This could allow p-CLE systems to cover large areas of breast specimens or even for scanning breast resection

cavities *in-situ* in a timely manner, increasing their clinical utility.

4 Discussion

4.1 Overview of findings and clinical implications

This review evaluated the role of confocal fluorescence microscopy (CFM) in breast cancer imaging, focusing on its technical features, clinical applications, and potential for IMA. Our analysis demonstrated that both bench-top and fibre-based CFM systems provide high diagnostic accuracy in distinguishing malignant from normal breast tissue, with reported sensitivities ranging from 81% to 96% and specificities from 85% to 99.6%.

Although all the reviewed studies focused on *ex-vivo* breast tissue imaging, the results highlight CFM's potential for intra-operative integration in BCS. Accurate IMA remains a major challenge in breast cancer surgery, with re-operation rates ranging from 20% to 30% due to postoperative identification of positive margins. By enabling real-time, high-resolution imaging, CFM could improve intra-operative decision-making by enhancing intra-operative margin evaluation. However, further trials are required to demonstrate that CFM can reduce re-excision rates, improving oncological and cosmetic outcomes for patients undergoing BCS.

4.2 Diagnostic utility of CFM in breast cancer imaging

Our review found that CFM reliably provides sub-cellular resolution, enabling identification of nuclear and stromal features characteristic of malignant and benign breast tissue. Studies utilizing bench-top CFM systems (e.g., Histolog[®] Scanner, Vivascope[®] 2500) have demonstrated high diagnostic concordance with histopathology, with pathologists achieving accuracy rates as high as 99.6% in differentiating malignant from normal breast tissues. However, surgeon interpretation of confocal images was less consistent, with accuracy ranging from 83% to 98% [Conversano et al. \(2024\)](#).

Similarly, fibre-based p-CLE systems (e.g., Cellvizio[®], LS-CLE) have been investigated for intra-operative applications, offering potential for real-time, *in-vivo* tissue imaging. Chang et al. demonstrated that topical acriflavine staining allowed p-CLE to distinguish neoplastic from normal breast tissue within 3 minutes, making it a potentially faster alternative to frozen section analysis. Palma et al. similarly showed that p-CLE with fluorescein was capable of accurately identifying IDC in breast quadrantectomy patients, reinforcing the potential of fibre-based CFM systems for rapid IMA during breast surgery.

Despite these promising results, CFM-based margin evaluation has been limited to retrospective and *ex-vivo* studies on small tissue cut-outs, with no current evidence supporting its real-time use in assessing breast cavity margins intra-operatively. This gap in research underscores the need for clinical trials evaluating CFM's ability to guide surgical decision-making in real-time.

4.3 Margin assessment and implications for Re-operation reduction

One of the most critical applications of CFM in breast cancer surgery is its potential for IMA, allowing surgeons to determine tumour involvement at resection margins in near real-time. Several studies investigated CFM imaging for margin evaluation, particularly using the Histolog[®] Scanner for *ex-vivo* assessment of WLE specimens. Sandor et al. analyzed 40 WLE specimens, demonstrating that CFM-based retrospective review could have identified tumour-positive margins in up to 75% of cases [Sandor et al. \(2022\)](#). However, substantial variation in diagnostic performance was observed between surgeons (30% accuracy) and pathologists (58% accuracy), underscoring the need for improved standardization of image interpretation and AI-assisted classification tools. Further supporting these findings, the HIBISCUSS project demonstrated pathologist-led CFM image interpretation achieved near-perfect accuracy (99.6%), while surgeon interpretation ranged from 83% to 98% [Conversano et al. \(2024\)](#). While these results highlight the diagnostic capabilities of CFM, they also indicate that its optimal role may be as an adjunct to pathology workflows rather than a direct intra-operative imaging tool for surgical decision-making.

A major limitation in current CFM studies is the absence of data on its real-time application for *in-vivo* margin assessment. While CFM has demonstrated high diagnostic accuracy for localized areas of excised tissue, its feasibility for evaluating entire WLE specimens intra-operatively remains untested. Tissue heterogeneity, and variability in optical plane depth present additional challenges in obtaining high resolution imaging across large surgical specimens, necessitating further validation studies focusing on whole-margin assessment. Additionally, cancer-induced tissue stiffness variations may further hinder uniform imaging, and the high-resolution nature of CFM generates vast image datasets that could complicate real-time interpretation and clinical workflow integration. Combined with the issue of tissue stiffness, a single duct containing DCIS would need to be in the optical plane and detected correctly by the reader, which would pose further challenges to widespread use. Moreover, the shallow imaging depth (100 μm), may be insufficient for accurate margin assessment given that surgical guidelines recommend 1–2 mm clear margins for breast cancer excision. While lipid-clearing agents and near-infrared imaging have extended penetration depth to 900 μm , further technological advancements are needed to improve deep tissue visualization with CFM. Technical innovations to current CFM systems are needed to enable disease to be detected at depths that parallel current guidelines on resection margins will need to be developed. Given these constraints, specimen imaging may be more reliable than cavity imaging, as the latter lacks spatial information on margin proximity.

4.4 Fluorescent contrast agents in confocal imaging of breast tissue

CFM relies on exogenous dyes to generate high-contrast images that reveal critical cellular features—particularly nuclear and epithelial structures—which are essential for identifying

malignancy. This is particularly relevant in BCS, where rapid assessment of surgical margins can influence real-time decision-making.

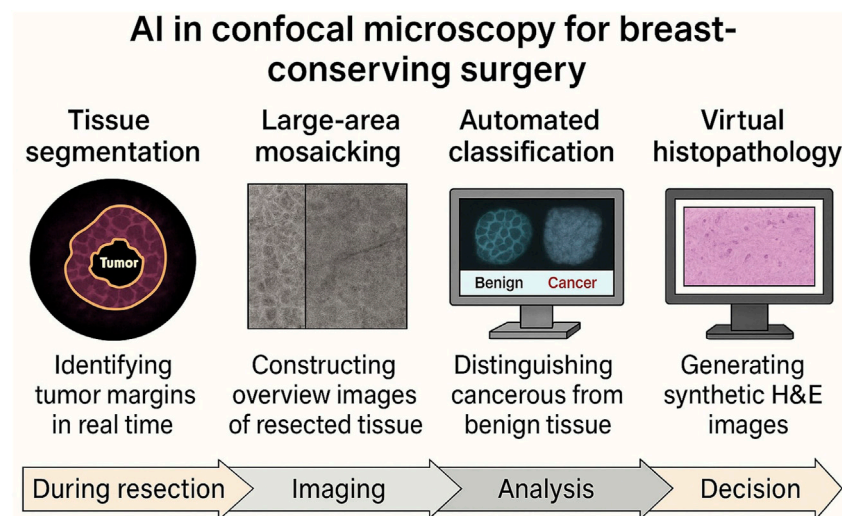
Since most breast cancers originate from the epithelial lining of ducts and lobules, accurate visualization of this surface layer becomes central to intra-operative imaging. Fluorescent contrast agents enable such visualization and are typically delivered via topical application or intravenous (IV) administration. Topical application allows for fast penetration (30–120 s), is safer for real-time surgery, and does not interfere with downstream histopathology. IV dyes circulate systemically and accumulate in tumor regions with leaky vasculature but require longer uptake times and raise safety/regulatory considerations. Several contrast agents have been evaluated in pre-clinical and early clinical studies.

- Acriflavine and proflavine are nuclear-binding agents that provide excellent contrast and have been widely used in *ex-vivo* CFM studies for breast tissue and shown to delineate malignant ductal structures with high sensitivity. However, due to their mutagenic potential, they are not FDA-approved for human use and their use remains restricted to investigational settings.
- Methylene Blue, an FDA-approved dye commonly used for sentinel lymph node mapping, has demonstrated promising results when applied topically in confocal imaging workflows. It offers reasonable nuclear contrast and has been successfully employed in studies using line-scan confocal microscopy (LS-CLE) for assessing ductal carcinoma and margin status.
- Fluorescein Sodium, another FDA-approved dye, has been trialed in breast imaging via IV administration. Although it accumulates in regions of increased vascular permeability, its lack of nuclear specificity limits its utility for detailed margin evaluation. Additionally, its systemic nature and pharmacokinetics make it less practical for real-time intra-operative use.
- The Histolog[®] Dip Stain, a proprietary agent used with the Histolog[®] Scanner, allows for rapid, contrast-rich imaging within 1–2 min of topical application. However, it is not yet FDA-cleared and remains available only for research use.

While several fluorescent dyes show strong potential for margin assessment in BCS, most remain investigational or used off-label for intra-operative use. Clinical translation requires dyes that are both biocompatible and FDA-cleared, with rapid uptake and minimal impact on histology. Given the epithelial origin of most breast tumors, topical staining remains the most practical route for margin imaging during BCS.

4.5 Artificial intelligence – a critical frontier for clinical translation of confocal imaging

AI has emerged as a powerful enabler for the clinical translation of CFM, particularly in the context of BCS where real-time, high-resolution imaging of tumor margins is crucial. While the imaging capabilities of CFM are well established, its adoption in routine surgical workflows remains constrained by several key challenges—limited field of view, grayscale contrast

**FIGURE 6**

Integration of AI in the intraoperative confocal fluorescence microscopy (CFM) workflow for BCS. The schematic outlines the role of artificial intelligence across four key modules in the surgical imaging pipeline: 1) Tissue segmentation, assisting real-time tumor margin delineation at the point of resection; 2) Large-area mosaicking, enabling reconstruction of wide surgical margins from high-resolution confocal tiles; 3) Automated classification, supporting diagnostic interpretation of benign vs. malignant tissue patterns; and 4) Virtual histopathology, translating grayscale confocal images into H&E-like visual formats for enhanced interpretability. Each application aligns with a specific phase in the surgical workflow and addresses key limitations of conventional confocal imaging—positioning AI as a driver of real-time, scalable clinical adoption in BCS.

interpretation, lack of standardization, and dependency on expert users. AI offers a compelling pathway to overcome these limitations and enhance the reliability, scalability, and usability of intraoperative confocal imaging platforms.

We identify the following four key application domains where AI can propel the clinical translation of CFM, enabling scalable, reliable, and surgeon-friendly intraoperative imaging platforms.

1. **Automated Tissue Segmentation and Margin Identification.** Deep learning models—particularly convolutional neural networks (CNNs) and recurrent neural networks (RNNs)—have shown success in segmenting epithelial structures, stromal boundaries, and tumor margins in grayscale confocal images with high precision. Accurate margin delineation is pivotal in BCS to reduce positive margins and avoid re-excision. AI-assisted segmentation reduces inter-observer variability and can offer real-time guidance during intraoperative imaging workflows [Dobbs J. et al. \(2015\)](#).
2. **Large-Area Mosaicking and Margin Mapping.** Due to the inherently small field-of-view of high-resolution CFM, real-time evaluation of surgical margins necessitates large-area mosaicking. AI-driven algorithms have demonstrated the capability to stitch thousands of confocal tiles into seamless wide-area maps (e.g., $> 1 \text{ cm}^2$) within minutes. These mosaics can approximate the diagnostic breadth of traditional histology while preserving cellular detail—supporting comprehensive, digital margin assessment.
3. **Tissue Classification and Decision Support.** Supervised AI classifiers trained on annotated confocal datasets have achieved promising results in distinguishing between benign, malignant, and normal tissue types with diagnostic sensitivities exceeding 90% [Gu et al. \(2017\)](#), [Gu et al. \(2018\)](#),

[Gu et al. \(2020\)](#). These tools hold significant potential to support intraoperative interpretation—especially in settings lacking on-site pathology—by offering rapid, standardized decision support to surgeons or radiologists.

4. **Virtual Histopathology and Enhanced Visualization.** To bridge the familiarity gap between grayscale fluorescence imaging and routine histopathology, researchers have applied generative adversarial networks (GANs) to generate virtual H&E-like images from confocal data. Such AI-driven translation tools can improve interpretability for clinicians accustomed to color-stained histological images, easing the transition to digital and real-time modalities.

These innovations, while encouraging, are still at a developmental stage. Most AI models have been trained on small, single-center datasets, with limited validation across diverse histological subtypes or imaging devices. Interpretability remains a concern, particularly for high-stakes surgical decision-making. Regulatory frameworks are also evolving, and ethical concerns—such as bias, liability, and data privacy—must be addressed before clinical adoption can scale.

Nonetheless, the integration of AI into CFM represents a promising frontier in image-guided surgery. By enabling robust segmentation, classification, visualization, and surgeon-AI collaboration, these tools have the potential to transform intraoperative decision-making and reduce reoperation rates. As multi-center datasets become available and explainable AI methods mature, we anticipate these technologies to serve not only as interpretive aids, but as essential components of future digitally guided cancer surgery. A schematic of this evolving workflow is illustrated in [Figures 6](#).

4.6 Challenges and future directions

Despite its promising applications, several challenges must be addressed before CFM can be routinely integrated into intra-operative breast cancer surgery.

- **Field-of-View Constraints**—fibre-based CFM systems necessitate multiple targeted scans for complete margin assessment, increasing operative time.
- **Operator Dependency**—Variability in surgeon-led CFM image interpretation underscores the need for standardized AI-assisted diagnostic algorithms.
- **Contrast Agent Limitations**—Certain nuclear contrast agents, such as acriflavine, remain restricted for clinical use, necessitating the development of safer, tumour-specific alternatives.
- **Pathological Coverage Gaps**—Current CFM studies predominantly focus on IDC, the most common breast cancer subtype, while benign lesions (e.g., fibrosis, fibroadenosis), atypical conditions (e.g., flat epithelial atypia), and non-invasive diseases (e.g., DCIS) remain under-represented. Expanding CFM image classification to encompass the full spectrum of breast pathology is essential for its broader clinical applicability.

Given that lobular carcinoma (6.6%) often presents with diffuse infiltration and DCIS (2.5%) lesions can be multi-focal, it is essential to examine and validate whether CFM exhibits the same level of diagnostic accuracy across different histological subtypes. Additionally, rarer breast pathologies, including mucinous carcinoma, fibroadenoma, phyllodes tumour, and lobular carcinoma *in-situ* collectively accounted for only 1.7% of analyzed specimens, highlighting a significant gap in confocal imaging validation.

For CFM to be effectively integrated into intra-operative surgical workflows, future research must go beyond validation in small tissue fragments and focus on real-time, *in-vivo* imaging of resection cavities and whole WLE specimens. This will provide direct evidence of its ability to assess margins intra-operatively and determine its clinical impact on reducing re-excision rates and optimizing surgical decision-making.

4.7 Limitations and barriers to clinical translation

While CFM offers promising capabilities for real-time margin assessment in breast conserving surgery, several barriers hinder its widespread clinical adoption. First, cost remains a significant constraint. Bench-top systems such as VivaScope or Histolog® can exceed USD 200,000, and even fiber-based systems—though relatively more affordable—require integration with surgical monitors, staff training, and dedicated imaging workflows. Unless clearly demonstrated to reduce re-excision rates or accelerate intra-operative decisions, their cost-effectiveness may be difficult to justify in resource-limited settings.

Second, training and usability pose practical challenges. Most confocal systems produce grayscale fluorescence images that differ significantly from conventional H&E slides.

Interpretation requires familiarity with the imaging modality, and current systems rely heavily on expert visual assessment. This raises the need for structured training pathways, decision-support tools, and ideally, AI-assisted pattern recognition to aid non-pathologist users such as surgeons or radiologists. Some attempts to circumvent these challenges include depicting confocal images using post-processing techniques that provide a pink/purple contrast which is more akin to H&E (e.g., Histolog®).

Third, regulatory hurdles continue to limit routine use. Many of the fluorescent dyes used for nuclear contrast—such as acriflavine and proflavine—are not FDA-approved for clinical application. Devices like the Histolog® Scanner remain cleared only for research use in many jurisdictions, slowing adoption despite promising early results. Broader clinical integration will require not only regulatory approval of hardware, but also validation of contrast agents for safe and effective intra-operative use.

Finally, histological diversity presents interpretive challenges. While many studies have focused on invasive ductal carcinoma, breast cancer includes a spectrum of subtypes—such as lobular carcinoma, papillary lesions, and benign mimickers like sclerosing adenosis—that can appear deceptively similar on fluorescence imaging. Additionally, it is critical that work be done to better characterise cellular atypia that falls short of DCIS. These variations require further validation in large, histologically diverse datasets to ensure safe clinical deployment across tumor types.

5 Conclusion

Confocal fluorescence microscopy (CFM) holds significant potential as an intra-operative imaging tool for real-time tumor margin assessment in breast conserving surgery. By providing high-resolution, subcellular imaging, CFM addresses a key challenge in breast cancer surgery—reducing positive margin rates and re-operation rates (20%–30%). Both bench-top and fibre-based CFM systems offer high diagnostic accuracy, with bench-top systems like Histolog® excelling in resolution and wide-area imaging, while fiber-based systems like Cellvizio® p-CLE and LS-CLE allowing real-time intraoperative scanning.

The capacity to visualize cellular architecture within minutes offers a potential alternative to conventional frozen section analysis and may reduce re-excision rates. Yet, despite encouraging technical validation, clinical adoption remains nascent. Key barriers include the cost and complexity of implementation, training requirements for non-pathologist users, lack of standardized workflows, and the limited regulatory approval of contrast agents suited for intra-operative imaging.

Nonetheless, we view this as a moment of inflection and opportunity. CFM lies at the intersection of emerging technological trends: AI-driven diagnostic support, robotic-assisted imaging, and virtual histopathology. These developments are no longer distant ambitions—they are gradually entering clinical research pipelines. AI-enabled platforms that can segment, mosaic, and classify confocal images in real time are increasingly feasible. Robotic delivery systems offer the potential for precise probe control in anatomically constrained spaces, including intraductal regions.

Together, these tools could shift intraoperative care from a reactive to a guided paradigm of real-time.

For the field to mature, the next steps must prioritize.

- The development and approval of clinically compatible contrast agents
- The assembly of diverse, annotated confocal image datasets for AI model training
- The design of interoperable systems that integrate with existing surgical workflows
- And the execution of multi-institutional trials to validate diagnostic performance across tumor subtypes and clinical environments

As engineers and clinicians work more closely at this frontier, we believe CFM offers more than just technical novelty - it signals a paradigm shift toward real-time digital pathology. With continued interdisciplinary effort, this technology may not only enhance the precision of cancer surgery but also redefine how we visualize and act on disease, in a timely manner.

Author contributions

KV: Investigation, Writing – review and editing, Formal Analysis, Writing – original draft, Methodology, Data curation, Validation, Visualization, Resources, Conceptualization. AE: Formal Analysis, Writing – original draft, Data curation, Conceptualization, Validation, Investigation, Writing – review and editing, Methodology, Software. NH: Writing – review and editing, Visualization, Investigation, Validation, Methodology. RR: Supervision, Writing – review and editing, Validation, Conceptualization. DL: Project administration, Supervision, Conceptualization, Writing – review and editing, Funding acquisition, Resources.

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