REVIEW

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Utility of confocal laser endomicroscopy in pulmonology and lung cancer

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Abstract

Primary lung cancer is an increasing health issue worldwide, with an ever-growing incidence due to various risk factors dispersed in all settings of modern society. Late discovery and poor survival rates for patients that do not qualify for surgical treatments greatly decrease overall mortality. Imaging methods remain powerful tools for early detection; however, molecular profiling is currently required for better understanding treatment options and for developing novel agents. Most procedures that are associated with tissue collection are invasive and, if performed in suboptimal conditions, may lead to morbidity and mortality. The need for better optical biopsy tools has thus arisen, directing further tissue collection and minimizing the chance of misdiagnosis. A series of methods have been proposed, including optical coherence tomography, narrow band imaging or autofluorescence. Lately, a novel *in vivo* tool for rapid and non-invasive microscopy gained traction – probe based confocal laser endomicroscopy became available in an increased number of referral centers worldwide. Miniaturization and the use of optical fibers allowed for the development of a dedicated device for pulmonary applications; lung cancer diagnosis and characterization are key issues targeted by this novel technology. We present here recent advancements in the field of optical biopsy of lung tissue, with a focus on emerging technologies and their involvement in cancer diagnostics and future therapeutic options.

Keywords: optical biopsy, probe-based confocal laser endomicroscopy, lung cancer, diagnosis, therapy.

Introduction

Primary lung cancer is one of the most frequent cancers today, also displaying a very high mortality rate. Approximately 55% of all cases are registered in developing countries, with the real incidence hard to determine due to incomplete screening and gathered data [1]. In all settings and irrelevant to economic conditions, men are more predisposed compared to women – approximately 1.1 million cases, 16.5% of the total neoplasia. Higher rates are reported in Central and Southern Europe; slightly lowest rates are found in Northern America and Eastern Asia, while lowest being reported in Western and Middle East Africa [1]. A lower incidence is recorded in women; however, globally, it still occupies fourth place (516 000 cases, 8.5% of the total incidence) and the second place as cancer-related death, following breast cancer [1].

As over 50% of cases are discovered in late, inoperable cases, when the five-year survival rate is only 15%, the prognosis of lung cancer is extremely severe [2, 3]. It is also one of the most invasive malignancies, with numerous opportunities for blood and lymphatic dissemination from the lungs. This accounts for early metastasis, as cancerous cells tend to migrate in incipient stages of aggressive forms of disease [3]. High-yield imaging methods can improve detection rates and accurately stage discovered cases; computed tomography, magnetic resonance imaging and positron-emission tomography may provide sufficient preliminary data to confirm an initial suspicion of a space-occupying tumor.

However, the complete and final diagnosis requires histological confirmation; this also constitutes a solid base for oncological treatment and plays a key role in modern chemotherapy strategies. Bronchoscopy provides good results, with over 70% successful confirmation rates, following either cytology or histology from biopsy fragments. Other invasive maneuvers, such as thoracoscopy or mediastinoscopy may provide necessary data, along with the possibility of curative maneuvers, at the cost of higher maneuver-related morbidity and mortality [4].

Therefore, accurate, fast and minimally invasive methods for accurate tissue analysis are required for positive identification and may provide insights on staging and further therapeutic methods.

Aim

The aim of our review is to present recent advancements in the field of optical lung tissue sampling, with

an accent of a novel technology – probe-based confocal laser endomicroscopy and its applications in lung cancer diagnosis.

Principles of optical biopsy

In vivo imaging of tissue histology has been a long-time goal for practitioners and basic scientist alike. Improvements upon classic endoscopy techniques have been made in order to allow better tissue visualization and rapid characterization of lesions [5]. The need to differentiate early lesions and pre-malignant conditions from normal mucosa fueled the change from classical white-light optical methods to special techniques performed at different wavelengths.

Autofluorescence (AF) techniques using blue-light were associated to conventional endoscopic techniques, successfully detecting precancerous lesions of the mucosa such as dysplasia or higher-grade precancerous lesions [6]. The functioning principle of AF is based on the difference in fluorescence between altered and normal tissue, detectable at certain wavelengths. However, this technique is unable to properly differentiate inflammatory changes from pre-cancerous lesions, therefore limiting its specificity [7].

The use of narrow band imaging (NBI) and optical coherence tomography (OCT) further improved the specificity of special endoscopic techniques. NBI uses a filter that limits the wavelengths received by the optical sensor, thus selecting specific features visible in conventional red-green-blue color schemes [8]. It can be successfully used to visualize blood vessels beneath the upper mucosal layers, thus identifying abnormal vascular patterns [9].

On the other hand, OCT uses a principle similar to ultrasound (US) imaging, using near infrared light in a manner similar to sound waves: delay between the time of emission and receiving the signal back after bouncing on structures behind tissues is measured, thus creating an image of everything in-between [10]. Since light does not need liquid to stop from dissipating, as ultrasound does, using this technique is particularly effective on air-filled organs such the lungs [11]. Low coherence interferometry is the technique used to measure light delay, because travel time of light is much superior to that of ultrasound; the resolution of OCT is far superior to US, bringing it closer to optical biopsy [12].

Recent advances in laser technology have facilitated the development of microendoscopic devices that use the principle of confocal fluorescent microscopy to provide *in vivo* imaging at a cellular level. The high degree of miniaturization is accomplished by the use of an optical fiber bundle instead of a classical objective. This is attached to a flexible miniprobe with a diameter of only 1.4 mm – fit to be used through the working channel of a bronchoscope. A laser source scans the thousands fiber cores by using two rapidly moving mirrors that channel the laser beam to and from the tissue, forming an image. Different wavelengths are used – 488 nm is used for AF microimaging and 660 nm in conjunction with the topical application of exogenous fluorophores. The lateral resolution achieved by this system is 3.5 μm, with a field of view of 600×500 μm and a depth of focus of up to 50 μm, producing 9 to 12 frames per second. Given its multiple advantages, probe-based confocal laser endomicroscopy (pCLE) soon rose as an interesting alternative to conventional *in situ* cellular characterization during normal bronchoscopy procedures.

Clinical applications of pCLE

A relatively low number of studies present significant findings after usage of pCLE in lung disease; the majority of these being only descriptive. Low patient numbers and diverse pathology investigated in the same report prevent any conclusion on the diagnostic accuracy of this investigation when compared with gold standard techniques. A major issue with pCLE is data consistency. One study published by Yserbyt *et al.* in 2013 [13] included 26 patients who underwent two consecutive pCLE alveoloscopies within a median time of 90 days in order to analyze the test-retest reliability. The authors reported moderate intra-class correlation coefficients of 0.56 (thickness of elastic fibers), 0.62 (alveolar duct diameter), poor coefficients of 0.74 for cellularity and 0.78 for alveolar cell autofluorescence and good coefficients for alveolar cell diameter – 0.29. Their study concluded that the technique shows good test-retest reliability for cellularity and autofluorescence quantification and only moderate for morphometry of elastic fibers and alveolar ducts. The heterogeneity of cells found within the alveolar spaces account for poor consistency between the two investigations; dynamic inflation of the lung may account for differences encountered when evaluating elastic fibers and alveolar ducts. Another drawback of all confocal endoscopy studies is the need to review numerous images in order to produce a viable classification of the observed lesions. Another study [14] showed high inter-observer agreement when assessing pCLE imaging of central airways and alveoli autofluorescence and only fair for elastic fibers; alveolar cellularity was found to be good by the authors. However, when comparing pCLE findings with pathology, the correlation was not observed.

Some studies used fluorescein as the main contrasting agent; however, this substance cannot penetrate into epithelial cells of the airways, therefore limiting its use for successful diagnosis of lung malignancies. A study involving 15 consecutive patients exhibiting various lung conditions and four healthy volunteers showed that only non-cellular structures could be identified [15]. This limited the technique, as no central airwave cellularity could be observed. Only fibrillar components could differentiate between pathological and normal areas. The study showed that no additional information was gained by using fluorescein over native imaging except for lesion areas, where changes in the alveolar spaces and interstitium were enhanced by the contrasting agent. In contrast, numerous studies performed on gastrointestinal mucosa showed great benefits from using fluorescein to enhance visibility of epithelial cells [16–18].

One pilot study including 32 consecutive patients diagnosed with nodular lesions of the central airwaves demonstrated the usefulness of acriflavine in increasing the accuracy of pCLE investigations [19]. Its main conclusion was that acriflavine-aided pCLE may be suited for detecting cancerous lesions during bronchoscopy examination of the airways, despite some limitations. Methylene blue

showed similar diagnostic power to acriflavine in a recent study, without having genotoxic effects in the tested conditions [20].

Lung cancer diagnosis

Lung cancer imaging is a challenging pathology, with the recent rise in incidence, primarily due to solitary lung nodules (SLNs) identified in almost all adult age groups [1, 2]. Few studies have explored the usefulness of pCLE in characterizing lung malignancies. One report of three cases diagnosed with SLNs described interesting findings by use of 488 nm and 660 nm (in conjunction with topical methylene blue fluorescence) [21]. Different mini-probes (Cholangioflex and Alveoflex) were introduced through the guide sheath once nodules were found by endobrochoscopy ultrasound (EBUS), virtually navigated by magnetic tracking. Biopsies were later obtained through the same guide sheath during endoscopy and molecular analysis was performed to confirm diagnosis and establish treatment when nodules proved malignant. Biopsy report in the third case confirmed the suspected diagnosis of lipid pneumonitis, as observed by pCLE. The report shows potential utility of pCLE – through the probe dedicated to lung applications – and contrasting agents in characterizing the structure of lung nodules. Targeting the lesions remains challenging and it involved using a fusion technique [EBUS with computed tomography (CT) guidance] when identifying either infracentimetric or distal nodules that were otherwise difficult to reach. One interesting observation was that, while normal biopsy may result in substandard samples due to crushing or small quantity of material, pCLE may facilitate the characterization of such lesions. However, standard pathology examination was still required in all three cases. It would be possible that pCLE can optimize the site of targeted biopsies and vastly improve diagnosis by reducing false negative results or inconsistent tissue harvesting.

The study performed by Fuchs *et al.* [19] on 32 patients with suspected lung cancer compared data from 75 522 images collected by pCLE from 56 different locations with pathology data obtained from biopsy specimens. The authors identified different patterns by pCLE, specific for neoplasms: no overlapping of cells and their chaotic distribution, with distinct changes in nuclei – heterogeneous size and variable distances between them. In addition, acriflavine was not homogeneously absorbed by malignant tissue, leaving dark areas with no uptake. Based on this pattern classification system, the study claims high accuracy for pCLE (91%), as neoplastic changes were detected with 96% sensitivity and 87.1% specificity – as it allowed subsurface imaging and differentiation of cellular and subcellular structures. Malignant features were thus clearly differentiated from both inflammatory infiltrates and normal mucosa.

Another study [22] compared pCLE findings with pathology features obtained from the same areas; the study included 25 patients with proven primary lung malignancies recruited between 2012 and 2013. Three pathologists and two pulmonologists, who knew the final diagnosis, reviewed both microscopic and pCLE images in a side-by-side comparison. As the number of patients that were included was small, and since physicians were not blind to the diagnosis, the results were considered preliminary by the authors, who refrained from trying to quantify the data; also, imaging and sampling was not performed simultaneously. However, the authors describe similar features found both by *in vivo* pCLE as well as later pathology examination. Disorganized and fragmented tissue, with multiple areas of low fluorescent uptake seem to correlate well with malignancy.

A comparative study between pCLE and light microscopy was performed on 18 post-lobectomy specimens for lung cancer nodules from 18 different patients [23]. They found alterations of stromal components, which allowed fluorescein leaks and an appearance of dark areas in cancerous areas. Other features were different, depending on the type of lung tumor examined. Their conclusion was that at least a subset of features visible in normal microscopy can be successfully identified by pCLE.

To date, pioneering work has established its usefulness in characterization of airways epithelium and alveoli, as well as several important lung diseases, mainly precancerous and malignant lesions. Distal *in vivo* lung imaging using pCLE represents an exciting field; it is known that up to 50% of connective fibers of the lung tissue contains elastin [24]; furthermore, elastin fibers are fundamental for acinary structures and compose the external sheats of microvessels at this level [25]. Alveolar proteinosis can be identified through pCLE, as not only elastic fibers can produce autofluorescence after laser excitation [26]. A recent study on six patients with pulmonary alveolar proteinosis prospectively recruited over a three-years period revealed that amorphous fluorescent material, characteristic for the disease, can be identified by pCLE [27]. Other pathognomonic aspects can be identified and a positive diagnosis can be rapidly achieved after investigating multiple areas by pCLE during normal bronchoscopy, before whole-lung lavage and cytology (which is a mandatory part of the current standard diagnostic procedure). Also, the authors compared pCLE with high resolution computed tomography (HRCT) and revealed better diagnostic abilities of pCLE in a patient 1.5 years after treatment, by discovering specific changes not only in the segments identified by the HRCT scan. Similar results – fluorescent complexes in areas that were not identified by HRCT alone – were observed in two other patients at three and six months after treatment, respectively. The authors obtained good interobserver agreement.

A cross-sectional study performed on 16 patients (eight with controlled asthma and eight controls) revealed that *in vivo* pCLE is able to show the pattern of elastic fibers of the human airways, without discerning between controls and asthmatic patients. However, comparison between pCLE and histology findings suggested a structure–function relationship between the extracellular matrix of the airway walls and the lung function [28].

Another possible use for pCLE could be in the qualitative assessment of lung tissue following surgery. A group of authors describes real-time imaging of the pulmonary acini following lung transplantation, with the purpose of identifying acute cellular rejection [29]. They evaluated a series of 105 bronchoscopies in patients subjected to liver transplant and used pCLE to evaluate biopsy pieces; autofluorescent cells were present in 73%

of recorded frames from acute rejection patients, compared to only 42% in controls. Moreover, applying three pCLE criteria in the evaluation resulted in 93% sensitivity and 83% specificity for detection of acute cellular regression, thus suggesting specific characteristics that can be evaluated by pCLE.

In a series of five cases published by a research group in 2013 [30], imaging was performed by inserting the confocal probe into the ventral, lateral and dorsal segments of each upper lobes. Authors evaluated alveolar duct diameter, the thickness of elastic fibers, counted the alveolar cells per microscopic field and their dimension and autofluorescence intensity. Their assessment was that pCLE could be used to determine a set of characteristics for specific to different diseases, thus providing sufficient information for targeted biopsy and successful diagnosis.

From our own experience with *ex vivo* analysis of lung cancer tumor tissue and surrounding parenchyma, we could also observe a basic set of characteristics that could lead to enhanced diagnostic capabilities at minimal costs for the patient. Following already described procedures of acriflavine dying [19], we subjected resected surgery pieces to pCLE analysis. Normal lung parenchyma displayed normal, regular fibrinous structures and homogeneous tissue distribution, without acriflavine leakage or dark areas (Figure 1, A and C). We could observe cellular characteristics and identified normal nuclei, with regular shapes and distribution, suggesting almost invariable cellular sizes. On the other hand, when analyzing tumor tissue we could observe large dark spots and leaks of acriflavine due to the irregular distribution of fibers, which varied in shape and sizes (Figure 1, B and D). In addition, when we encountered cellular elements, we could identify various nuclear sizes and shapes, suggesting nuclear atypia. Distances between them varied greatly, thus implying cellular variability and heterogeneity. Furthermore, we could also observe what appeared as inflammatory infiltrate near necrosis areas, with dense small-bodied cellular populations (Figure 2). We could also observe the aspect of inflammatory cells in other areas adjacent to tumor tissue, within lung parenchyma and under pleural structures (Figure 3, false coloring).

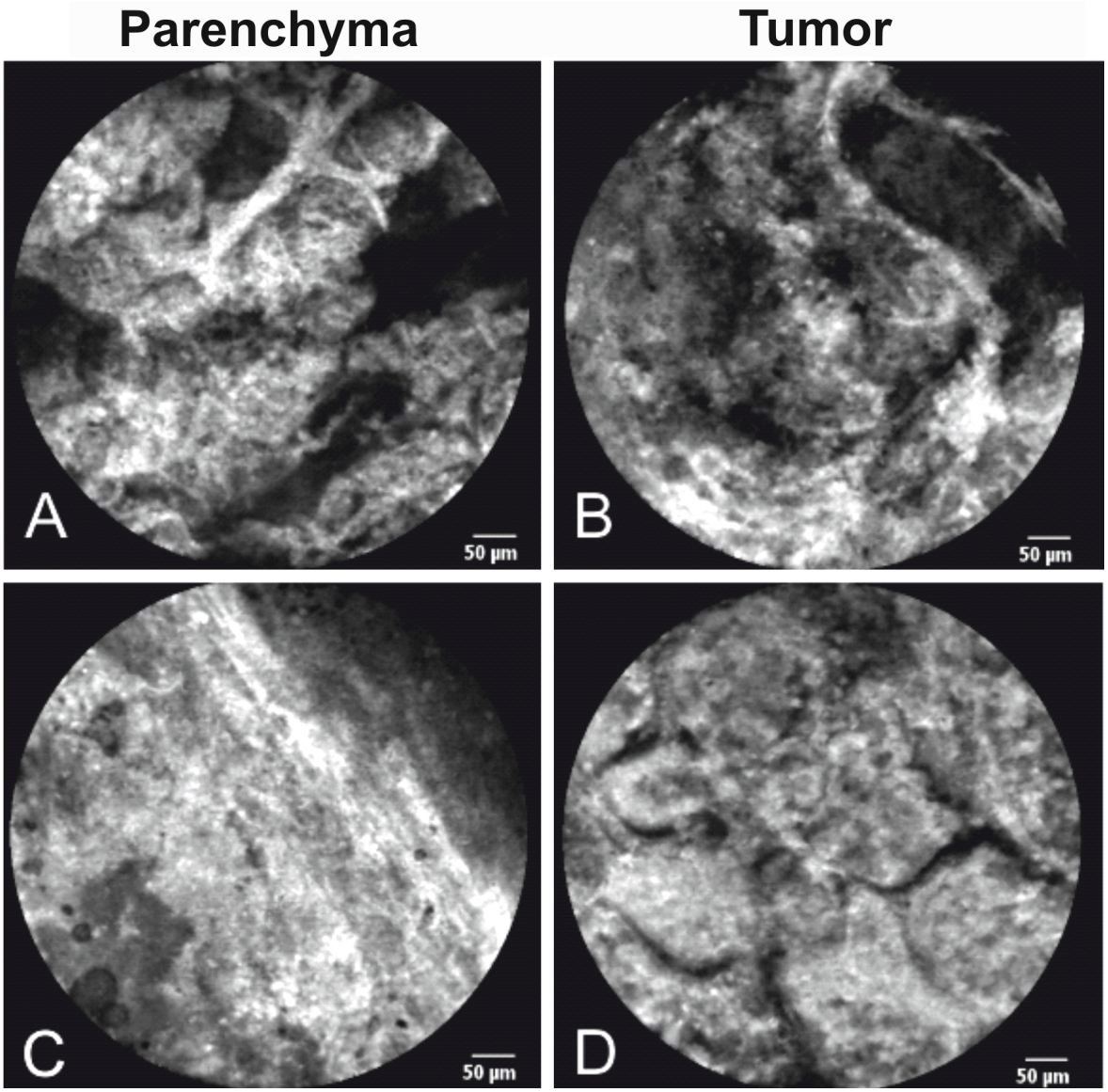

Figure 1 – ***Lung parenchyma versus tumor tissue. We could observe regular fibers and cellular structures within normal lung (A and C), while tumors had a chaotic arrangement of structures, with deep degradation of the initial architecture (B and D).***

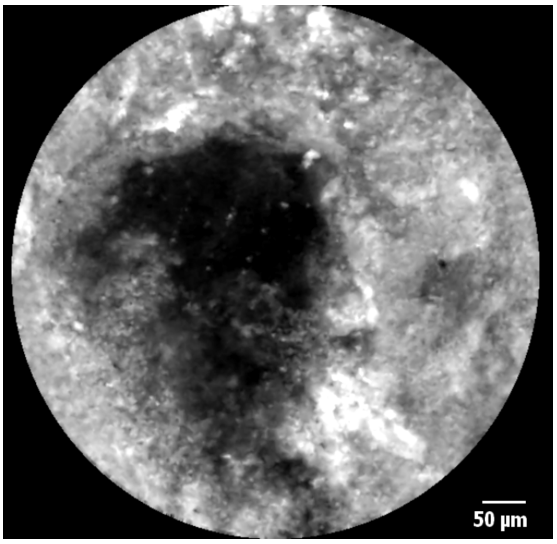

Figure 2 – ***Possible inflammatory cells near an area of necrosis, at the periphery of tumoral tissue.***

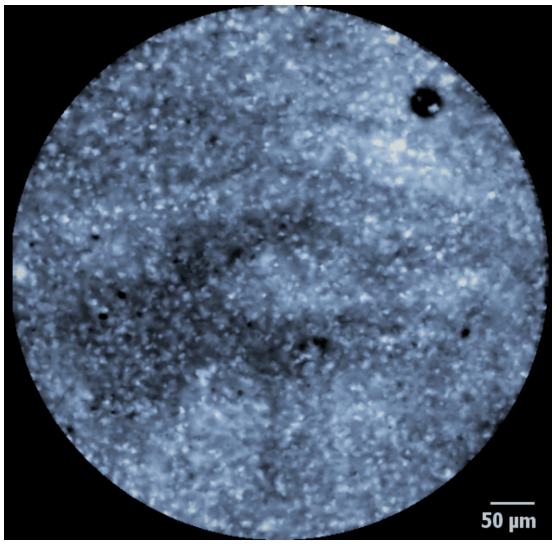

Figure 3 – ***Apparent inflammatory infiltrate adjacent to tumoral tissue. False coloring to highlight cellular nuclei.***

Future directions

Molecular or targeted CLE is defined by the usage of fluorescent molecular probes consisting of antibodies or peptides that bound to certain tissue structures, after topical or systemic administration, followed by CLE visualization [31]. The principle is that a targeted molecule linked with a fluorescent compound is connected directly at the level of desired tissue structures. Beside enhanced imaging and diagnosis, the method has important implications for the follow-up of anti-inflammatory and anti-angiogenic therapy, which can be tailored according to the presence or absence of certain receptors. Personalized therapy through targeted molecular approaches directed at activated oncogenes currently represents the shared vision of the future [32].

Treatment of non-squamous cell lung cancer (NSCLC) patients witnessed a rapid evolution of drugs targeting specific molecular pathways, including epidermal growth factor receptor (EGFR) or anaplastic lymphoma kinase (ALK) [33]. Angiogenesis is also involved through vascular endothelial growth factor (VEGF), with important consequences in proliferation, migration and metastasis. Dual inhibition of these two pathways (both EGFR and VEGF) might be beneficial in terms of progression free survival, although it does not seem to benefit the overall survival [34]. These receptors have been precisely characterized at a cellular level through CLE, although only in a few studies *in vivo*, with most of the research in animal models or *in vitro*.

Fluorescent-labeled EGFR antibodies have been used *in vitro* [human colorectal cancer (CRC) lines], but also *in vivo* in xenograft tumors and human tissue samples, to analyze EGFR expression patterns, for both CRC diagnosis, but also as a predictor of treatment response [35]. A similar approach allowed calculation of EGFR-specific fluorescence, being used *in vivo* in human patients with colorectal cancer and adenomas, as compared with normal mucosa [36]. Another approach is to use therapeutic antibodies like cetuximab, an anti-EGFR antibody, for both detection but also early prediction of response to targeted therapy [37, 38].

In vivo visualization of VEGF has been shown to be possible with CLE, in mice tumors, but also in surgical specimens of patients with colorectal cancer [39]. Other markers used for the evaluation of angiogenesis and microvascular density through CLE included fluorescently labeled anti-CD31 used as a pan-endothelial marker in cancer patients [40, 41]. In contrast to pan-endothelial markers, another approach consists of usage of CD105, which is expressed mostly in newly formed vessels (in activated endothelial cells) [42]. Due to specific visualization through CLE, quantification of microvascular density is feasible both *ex vivo*, but also *in vivo*, while the results are comparable to conventional immunohistochemistry.

To date, limited data on the feasibility of pCLE in observing pulmonary capillary morphology *in vivo* exist. One recent animal study [43] shows that pCLE placed through the trachea can observe blood flow within different regions of the lungs and assess vasculature after contrast intravenous administration. Executed on six porcine animal models, the study concluded that pCLE might be used as a standard diagnostic tool to visualize capillary structures and potentially quantify performance. Some of the main concerns are linked to the difficulty with which the probe has to be placed and maintained focused on an area of interest in order to provide sufficient image quality during respiratory movements.

Conclusions

In conclusion, *in vivo* applications of probe-based CLE will certainly open a plethora of applications, especially in the field of targeted therapy, with the aim of personalizing treatments in individual patients. This could have important consequences in terms of ensuing morbidity and mortality of lung cancer patients, but also in terms of an enhanced cost-efficiency of current chemotherapy and antiangiogenic treatment regimens.

Conflict of interests

The authors declare that they have no conflict of interests.

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Author contribution

Costin Teodor Streba and Ana Maria Gîltan equally contributed to the manuscript and share main authorship.

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