Emerging applications of intra-vital smart micro-imaging: from bench-to-bedside

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Confocal laser endomicroscopy (CLE) combined with fluorescent probes (FP) targeting of biomarkers is expected to evolve into a new paradigm in the field of “standard- and point-of-care” bedside diagnostics and theranostics. FP-CLE is expected to provide physicians with accurate and real-time data on organ state and cellular/molecular mechanisms underlying pathology progression and resolution. A CLE commercial device (Cellvizio®) has been recently approved by the FDA for human biopsy support. FP-CLE will tremendously realize CLE potential, and preclinical validation of FP-CLE methods is mandatory stepping stone towards clinical trials in humans.

This chapter discusses the validation and usefulness of several FP in pre-clinical models of systemic and organ inflammation/remodeling through three examples:

i) Lung inflammation is a key-concept in pneumonitis because it can jeopardize lung repair after injury when unresolved. Myeloperoxidase (MPO) activity is linked to reactive oxygen species (ROS) production, and a representative and useful biomarker for inflammation. MPO can be monitored and semi-quantitatively measured in vivo using FP-CLE. This was done in acute lung injury models (i.e. lipopolysaccharide (LPS) and bleomycin challenged lungs) using an activatable and specific FP Identifying sustained inflammation can implement physicians to modify their decision-making strategies.

ii) Stem cell therapy application has been hindered by the lack of understanding regarding the fate of inoculated cells, and the mechanisms by which they heal targeted tissues. Mesenchymal stem cells (MSCs) are proposed for their immune-modulatory properties and their potential ability to mitigate radiation-induced lung damage (RILD), a side effect of lung cancer radiotherapy. FP-CLE tracking and quantification of DID-labeled MSCs in vivo in a rat model of RILD is shown to be achievable. This method can be complemented using other cell labeling methods or by combining MSCs monitoring with FP-CLE imaging of the pathology to assess the potential of stem cells in treating damaged organs.

iii) Vital organ functional assessment through microcirculation perfusion measurement has recently gathered interest because evidences suggest that macro-perfusion threshold parameters fail to properly predict major organ dysfunction. Acute kidney injury assessment is of particularly interest as it is associated with high treatment costs. Using optical blood staining eventually combined with circulating cell labeling, FP-CLE can be performed in vivo to measure renal cortical flow and micro-vessel density/recruitment assessments in shock models (i.e. LPS challenge and cecal-ligation puncture models). This procedure, once properly translated to the clinic, will allow a fine-tuned modulation of physicians’ interventions in addition to current vital sign parameters. These potential applications clearly illustrate how FP-CLE approaches can provide key- and added value data during patient monitoring, at a low-cost and non-invasively. Integrating microscopy and molecular information should be part of the decision-making strategies of upcoming clinical management.

Keywords: micro-imaging in vivo, fluorescent probes, intra-vital molecular assessment

Confocal laser endomicroscopy (CLE) combined with fluorescent imaging probes (FP-CLE) targeting of biomarkers is expected to evolve into a new paradigm in the field of “standard- and point-of-care” bedside diagnostics and theranostics. FP-CLE is expected to provide physicians with accurate and real-time data on organ state and cellular/molecular mechanisms underlying pathology progression and resolution. A CLE commercial device (Cellvizio®) has been recently approved by the FDA for human biopsy support. FP-CLE will tremendously expand CLE potential, and preclinical validation of FP-CLE methods are a mandatory stepping stone toward human trials.

1. Introduction

Fluorescence imaging probe (FP) assisted confocal laser endomicroscopy (CLE) is a new promising imaging method that allows non- or minimally- invasive image acquisition in vivo and in real time with sub-cellular spatial resolution,
CLE is used to detect fluorophores in vivo in real time. In “probe”-based CLE (pCLE), an imaging conduit made of fiber bundle is coupled to a scanning laser unit. There are several synonyms in the literature for this concept, including fibered confocal fluorescence microscopy, endoscopic confocal fluorescence microscopy, fiberoptic confocal system, and high resolution microendoscopy. In this concept, a fiber bundle is used to guide excitation photons and collect emitted fluorescence in the field of view (FOV). The FOV, spatial resolution and focal depth are defined by the diameter of the bundle, the diameter of individual fibers, and associated optical components. If the region of interest (ROI) is larger than the FOV, dynamic sequences can be registered using mosaicking algorithms to yield a complete map of the ROI. The cost of sending a distal image through a fiber bundle is that the raw image will be convoluted with the shape of fibers, and will need to be corrected accordingly. For any CLE device, the number of signal channels and the wavelengths of the fluorescence they can detect will define which fluorophores will be visible simultaneously. Fluorophores can be native to the host (endogenous) or can be instilled, injected or engineered into the host to provide a desired contrast (exogenous).

1.1 Confocal laser endomicroscopy

CLE can be employed to monitor fluorescent cells or organisms in vivo. We have used this strategy to detect neutrophils recruited in the lung using PKH2. In a recent study, mesenchymal stem cells (MSC) where engineered to co-express tumor necrosis factor (TNF)-related apoptosis-inducing ligand (TRAIL) and green fluorescent protein (GFP) to monitor the fate of MSC behaviour. Inoculation of egfp carrying Escherichia coli has been done to the validation of intra-mucosal bacterial invasion in bowel diseases. In this chapter, we discuss how an unspecific membrane label can be selected to monitor stem cells therapy. It would also possible to detect protein-protein interactions using Förster resonance energy transfer (FRET) experiment as is currently done in other fluorescence microscopy approaches.
Table 1. List of FPs used in CLE applications (non-exhaustive).

<table>
<thead>
<tr>
<th>Probe</th>
<th>Target</th>
<th>Context</th>
<th>Ref.</th>
</tr>
</thead>
<tbody>
<tr>
<td>FITC-Peptide (ASYNYDA)</td>
<td>H460 adenovarcinoma cells (exact target unknown)</td>
<td>Barrett’s neoplasia</td>
<td>27</td>
</tr>
<tr>
<td>Neurotrace®</td>
<td>Nissl bodies</td>
<td>Enteric nervous system</td>
<td>28</td>
</tr>
<tr>
<td>FITC-adalimumab (TNFα-targeted antibody)</td>
<td>TNFα receptors</td>
<td>Crohn’s disease</td>
<td>29-31</td>
</tr>
<tr>
<td>FITC-Peptide c(CGGRGPFC)-NH2</td>
<td>Aspergillus fumigatus</td>
<td>Pulmonary aspergillosis</td>
<td>32</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>No specific target</td>
<td>Inflammation and cancer – urinary tract</td>
<td>7,33,34</td>
</tr>
<tr>
<td>Proflavine</td>
<td>DNA</td>
<td>Cervical neoplasia</td>
<td>4</td>
</tr>
<tr>
<td>Acriflavine hydrochloride</td>
<td>DNA</td>
<td>Lung cancer</td>
<td>35</td>
</tr>
<tr>
<td>DSAPF/SNAPF Multi-branched peptides (near-infrared &amp; fluorescein derivatives)</td>
<td>HOCl (Myeloperoxidase), Neutrophil Elastase</td>
<td>Lung inflammation</td>
<td>17,26,36</td>
</tr>
<tr>
<td>Alexa Fluor 488-dextrans-</td>
<td>No specific target</td>
<td>Congenital heart disease</td>
<td>16</td>
</tr>
</tbody>
</table>

iii) Genetic expression underlying in vivo physiological events can be detected using reporter systems transfected in animal models. A fluorescent-protein yielding gene, such as gfp, is co-expressed with that of a protein of interest, for example the endothelial NO synthase 37, melanocortin-4 receptor 38 or the mouse insulin I receptor 39. eGFP expression in rabbit tissues allowed visualization of anatomical structures and cell morphology with CLE 2. Transgenic fluorescent animals have the advantage to allow direct visualization with a sustained fluorescence, which is appropriate in longitudinal studies 40. Further investigations of transgenic fluorescent animal combined with CLE are needed, and will be promising in the study of biological processes without using exogenous FP. In fact, utilization of FP must be characterized to ensure validity within animal model.

1.3 Advantages and limitations of FP-CLE

FP-CLE houses a unique niche among imaging modalities used in the clinic. The lateral resolution of FP-CLE can vary (2.5-5 μM) depending of the fiber bundle properties 6. This is superior to what is achievable with magnetic resonance imaging (MRI) 41, whole body computed tomography (CT) 42, ultrasound or positron emission tomography (PET) 43,44, but inferior to confocal or two-photon microscopy (< 1 μm). The sensitivity of FP-CLE is high (10^{-9}-10^{-11} M) compared to MRI (10^{-3}-10^{-5} M) and close to that of PET (10^{-11}-10^{-12} M) 45,46. These properties position FP-CLE in a unique niche between macro- and microscopic methodologies. While MRI and PET require expensive infrastructure and specialized staff, FP-CLE can be performed by physicians during a typical fiberoptic session using the working channel of any endoscope. In vivo confocal and two-photon imaging require that the tissue of interest be accessible from the surface of the subject 47,48 – which limits applications in humans. FP-CLE does not suffer from this limitation. However, the size of the bundle will be the limiting factor to access tissues using natural cavities, especially in small animals. For example, in the lung, deep air spaces (alveoli) are reached through 200 μm wide conduct – which do not allow for non-invasive exploration with current fiber bundles. For now, there are few workaround for this limitation. We found that it is possible to punch through lung tissues to access deep airspace and alveoli-like structures, but this procedure is challenging and hazardous in the rat. In larger animals, fiber-guidance is possible, and needle-based CLE (nCLE) can be used to pierce and enter tissues distally 28,49. In the future, two photon CLE, or smaller fiber bundles or unique fiber CLE with diameter could mitigate this limitation 50.
Another key aspect of CLE devices is the number of data channels available, which will limit what and how many fluorophores will be visible. The Cellvizio® instrument has two lasers (488 and 660 nm), and two channels (filters) to detect fluorophores in two separate bandwidths (505 - 650 and 680 - 900 nm). The acquisition rate in FP-CLE is limited by the laser scanning unit. For the Cellvizio® instrument, this frame rate is 12 Hz, which is sufficient to detect rolling or adhering cells. This is much faster than typical microscopic, MRI or PET acquisitions. Results presented in the remainder of the chapter were acquired with the Cellvizio® instrument.

1.4 FP-CLE, from bench-to-bedside

FP-CLE methodologies can provide insight into patho-physiological processes by shedding light on underlying cellular and molecular mechanisms. In drug development, the ability to describe and quantify these mechanisms could significantly contribute to the development of more efficient therapeutics – by providing real-time information on drug delivery (e.g., delivery of antibody based therapy, or stem cell therapy), and in vivo efficacy (e.g., to evaluate anti-inflammatory treatment targeting the lung, as we will discuss below). Such tool would help physicians monitor the progression of diseases and/or adjust their therapeutic strategies based on real-time efficiency assessment. Clinical trials are already ongoing to explore the feasibility and added-value of using fluorescein combined to CLE to diagnose parenchymal lung diseases or gastric intestinal metaplasia. However, validation in small animals must be performed. Here, we present three applications of FP-CLE in the rat and challenges expected in the validation of FP-CLE methodologies.

2. Lung inflammation monitoring by CLE imaging of HOCl

2.1 Acute lung injury and unresolved inflammation

Acute Respiratory Distress Syndrome (ARDS) is the most severe form of Acute Lung Injury (ALI), and can result from several challenges, including bacterial infections, aggressors and pulmonary aspiration of gastric content. ALI/ARDS results into hypoxemia, acute inflammation and can even lead to sepsis. If the patient’s condition does not improve after one week of pharmacological treatments or mechanical ventilation support, physicians will consider open lung biopsy (OLB) to investigate the exact nature of the underlying cause, but OLB is accompanied by unacceptable risks and complications (up to 25%) for patients mechanically ventilated and cannot be repeated multiple times in the same patient for longitudinal assessment. Hence, OLB is seldom used in critically ill patients despite the fact that it would have contributed in patient management by guiding the selection of more specific/appropriate therapy in 60% of cases, or interrupting unnecessary therapy in 37% of tested cases. Optical imaging “virtual biopsy” at bedside would be highly valuable as a surrogate to assess lung condition.

Lung inflammation usually occurs in two phases: an inflammatory/exsudative phase followed by a proliferative/repair phase. When inflammation fails to resolve, the lung undergoes fibrosis, which affects compliance and can be more lethal than the cause of inflammation. Unresolved inflammation is characterized by increased capillary permeability and pleural infiltration of protein rich liquid. This triggers immune cell recruitment, including neutrophils and macrophages, which will release pro-inflammatory mediators. In inflammatory conditions, these cells express myeloperoxidase (MPO), and enzyme that converts hydrogen peroxide to hypochloric acid (OCl/HOCl) – a reactive oxygen species (ROS). MPO is concentrated in phagosomes or secreted by exocytosis in neutrophils extracellular traps (NETs).
2.2 Quantifying myeloperoxidase product HOCl in vivo as a marker of inflammation

Several fluorescent probes have been published to detect or quantify MPO activity. These molecules have low intrinsic fluorescence, but can react with OCl/HOCl molecules to yield fluorescent products. Here, we will discuss pCLE with 4-[6-(4-aminophenoxy)-3-oxo-3H-xanthen-9-yl]benzene-1-3-disulfonic acid (DSAPF), which was derived from a comparable probe synthesized by Shepherd et al. in order to obtain a probe that can be detected using the green channel of the Cellvizio®. Here, DSAPF was validated in a hyperoxia model of lung injury in Sprague-Dawley rats.

2.3 Material and Methods

Hyperoxia model preparation. Pathogen-free Sprague-Dawley adult rats (250-350 g) were exposed to 85% O2 for ~48 h in a poly(methyl methacrylate) chamber. This leads to ALI/ARDS-like inflammation (epithelium injury and type II proliferation) with neutrophil infiltration – more specifically, the pathophysiology of this model is reminiscent of the exudative and proliferative phases described above, and allows to study lung repair after injury.

Probe instillation and pCLE. Rats were anesthetized (1 ml/kg ketamine/xylazine), tracheotomised with 14G catheter, and positioned upright. DSAPF (150 μM x 250 μL) was nebulized using a manual micro-nebulizer (Penn-Century). Note that manual nebulization minimizes the quantity of probe required for imaging, but yields less homogeneous droplet distributions compared with jet or piezo nebulizers – with only part of the product reaching the lower respiratory tract. However, nebulization through tracheotomy is known to allow for better dispersion in distal airways compared with insufflator. Three hours post instillation, the ProFlex S-1500 miniprobe connected to the Cellvizio® was introduced through the intra-tracheal (IT) catheter, and the lungs of animal were imaged over 15 minutes – producing 2 min. long videos, between which the optical probe was removed and cleaned of debris and mucus.

2.4 Results and Discussion

Representative pCLE results are shown in Fig. 1A and B. Objects of intense signal, presumably cells, can be detected along with low intensity – and less circular objects. These can be dying cell material, NETs or other structures which can retain and accumulated activated probe molecules. The distribution of the DSAPF probe was validated using immuno-histochemistry (Fig. 1 D-F). These results show that there is a good correlation between neutrophils/macrophages and DSAPF, as expected. DSAPF was also observed outside cells, which is expected as MPO can be released, and neutrophils/macrophages are expected to eventually enter autophagy/apoptosis. There are three main limitations to the current setup. First, the tracheotomised animal must remain anesthetized over the DSAPF incubation, and this is an invasive procedure which limits the amount of longitudinal monitoring that is possible. While the trachea can be sutured and the animal awaken, in our hands this procedure is challenging and was found to have a...
variable rate of success. Then, the manual nebulization was found to have a variable delivery efficiency. An alternative method would be to use an electric nebulizer which produces smaller droplets (≈5 μm) which can more easily reach distal airspace. Finally, there is little guidance possible with this setup – we can guide the micro-probe left or right, and control how deep we lower the S1500 probe into the airways, but without endoscopic guidance, we cannot bring the probe to a specific ROI. This methodology was refined in the next section of this chapter.

3. Confocal Laser Endomicroscopy for Stem Cell Therapy Imaging: Application in Radiation-Induced Lung Damage

3.1 Stem cell therapy imaging

Stem cell therapy (SCT) aims to repair or regenerate damaged organs by one or several mechanisms. This includes differentiating into functional cells and replacing damaged ones, secreting growth factors in a paracrine fashion to stimulate resident stem cells to repopulate the damaged region, or by reducing further damage such as chronic inflammation through immune-modulation. Despite great progress in SCT research, its clinical use remains limited. Stem cells fate and interactions with host cells remain areas of intense research. Molecular imaging and cell tracking methodologies can shed light on these aspects, and can also contribute to identifying roadblocks in the way of successful and safe SCT or to compare and optimize different methods to achieve the desired goals, such as engraftment optimization or survival.

A stem cell labelling method must be selected based on the hypothesis or objective, which guides criteria such as cell detection sensitivity, resolution, monitoring period and cost-effectiveness. Labelling can be achieved in a direct or indirect way: with a fluorescent dye, for example, that passively enters the cell or with a reporter gene system where the signal is turned on when the gene is activated, respectively. The former is simpler to use and readily available but is unspecific. Direct labelling does not differentiate between labelled cells and released labelling agents (e.g., after the death of labelled stem cells) that are internalized by other cells. The latter is more challenging to implement but has the potential to answer more complex questions, such as the status of cell viability. Cells can be labelled directly with superparamagnetic iron oxide nanoparticles (SPIONs) and be followed with magnetic resonance imaging (MRI) or with a positron emitting radionuclide and followed by positron emission tomography (PET). PET can also be used with reporter gene systems (indirect labelling) to detect, track and quantify stem cell transplantation. MRI-compatible reporter genes can be used as well to monitor and track cells with high resolution.

In FP-CLE, labelling can be achieved using a fluorescent dye that passively enters/labels the cell or with a reporter gene system using a fluorescent protein. Intra-vital micro-imaging allows to visualize single cells in vivo at cellular resolution. Several applications have already been reported in the literature, including studying stem cells niches, homing and engraftment, and determining the safety of administering stem cells at varying concentrations. CLE was used to follow transplanted stem cells as a potential treatment for irritable bowel syndrome. In the following section, we discuss the monitoring of mesenchymal stem cells (MSCs) in a SCT rat model of radiation-induced lung damage.

3.2 Radiation-induced lung damage mitigation by mesenchymal stem cells (MSCs) therapy

In the scope of lung cancer treatment by radiation therapy (RT), radiation-induced lung damage (RILD) to healthy surrounding tissues is a limiting factor for dose escalation, and occurs in 30% of patients. RILD limits RT by reducing the total dose that can be delivered – which can be too low to destroy all cancer cells (small/no therapeutic window). RILD involves two main phases: an early inflammatory phase (pneumonitis) and a late fibrotic phase (fibrosis) that can be life threatening.

MSCs are an alternative treatment option which is currently explored. MSCs are adult stem cells derived from the bone marrow. They can differentiate to give rise to bone, cartilage and muscle tissue. They have been shown to promote organ repair in a variety of organs including the lung. Their mechanism of action is still being investigated but it was reported that their main role in repair relies on paracrine immunomodulatory effects rather than engrafting and differentiating in functional tissue.

3.3 FP-CLE imaging of MSCs therapy in RILD

To assess the potential of MSCs in mitigating RILD, we established a rat model of the disease following lung irradiation in a clinically relevant fashion, involving CT simulation, organ contouring, treatment planning, and radiation delivery with a clinical linear accelerator.
### 3.4 Material and methods

**Rat RILD model preparation and SCT** Sprague-Dawley female rats were anesthetized (isoflurane) and imaged in a CT simulation scanner (Philips Brilliance Big Bore, 120 kVp, 175 mA). Using this acquisition, RT was planned to mitigate surrounding organ dose while delivering a 18 Gy dose to the right lung with a 6 MV photon beam. Two or three weeks later, MSC were injected in the animals, and pCLE imaging was performed through a tracheotomy as described before – but in this experiment, animals underwent a CT scan prior to pCLE, and the latter procedure was performed under fluoroscopic guidance (Philips Pulsera).

**MSC culture, labelling and injection.** MSCs were harvested from the femur bone marrow and cultured as previously described. DiD (Vybrant R DiD Cell-Labeling Solution, Thermo Fisher Scientific) was added to MSC at a concentration of 10 μM for 30 minutes in normal culture conditions, and then washed twice with sterile DPBS, and fresh culture media was then added. The next day, MSC labelling was confirmed with fluorescence microscopy. MSC were then harvested, suspended in 1 mL PBS, and injected i.v. (1 M cells).

**pCLE procedure and collagen imaging.** The animals were prepared for pCLE as described above, and a PE50 catheter was inserted in the right jugular vein. To highlight collagen structures in the fibrotic lung, a collagen-binding probe derived from the structure of conjugate EP-3533P tagged with a single fluorescein moiety was i.v injected in the jugular vein 15 min before imaging at 10 μmole/kg in 1 ml of saline.

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### 3.5 Results and discussion

The pre-scan CT confirmed RILD in the irradiated right lung (blue outline) as shown in Fig. 2A. Increased CT signal in this lung was attributable to inflammation, edema and fibrosis. Using fluoroscopy, the distal tip of the S1500 probe and its airway positioning within the affected region is shown in Fig. 2B. Fluorescent-enhanced collagen microfibrils can be detected in selected areas (ROI) using pCLE in Fig. 2C, and the presence of MSCs in a similar area is displayed (Fig. 2D). More detailed data have been recently published.

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### 4. Microcirculation status as a biomarker of renal function in acute renal failure

**4.1 The role of microcirculation in organ failure**

Microcirculation (μc) vessels, the smallest blood vessels in the body, are responsible for and regulate exchanges between tissues and blood. Terminal arterioles are the flow resistant portion of the μc and are < 100-200 μm in size. Capillaries, usually below 10 μm, are permeable and allow nutrient and O2 diffusion from the blood to tissues. Post-capillary venules are responsible for the capacitance of the μc. Any significant and sustained decline of μc function, such as capillary permeability or density, or reduction in perfusion homogeneity (low or interrupted flow) can result in tissue injury and organ dysfunction.

In the clinic, the μc is mainly ignored in favor of macro-hemodynamics. This is problematic, because tissue & vital organs μc perfusion can be disturbed despite macro-hemodynamics preservation or restoration, including in critical illness such as sepsis – leaving physicians with no way of properly monitoring organ failure and adapt their therapeutic strategy. Thus, μc disturbance would be a better prognosis factor than macro-hemodynamics thresholds, which are currently monitored in the clinic – but this view remains a matter of debate due to the ongoing research on the relationship between systemic macro-hemodynamics and μc function. A key element for the discrepancy observed between both systems is that each is regulated differently: macro-hemodynamics (arteries, veins, heart) are primarily regulated by the sympathetic system, and the μc is mainly auto-regulated by autacoids oxygen sensors, such as prostacyclin; nitric oxide and the endothelium-dependent hyperpolarizing factor.
Evidence is mounting to support the role of $\mu c$ in organ failure. Multiple organ failure/dysfunction is frequently associated with life-threatening conditions in critical illness such as sepsis. Impaired $\mu c$ is one of the suspected culprits, although, as mentioned above, this is still a matter of debate. The kidney is a very sensitive system and becomes dysfunctional in more than 50% of sepsis, with an acute renal failure (ARF) often characterized by a decreased glomerular filtration rate and diuresis. These reductions can occur despite the fact that renal vascular resistance is depressed and blood flow is normal. The kidney is rich in small blood vessels topographically dedicated to distinct functions and regulation mechanisms, not only in glomeruli, but within the cortical-to-medulla peri-tubular gradient as well and dysregulation of this finely-tuned vascular network can have devastating effects. It is essential to develop diagnostic methods capable of tracking $\mu c$ disturbance in the context of ARF, both in animal models and in critically ill patients.

4.2 Imaging and characterizing renal microcirculation

In the last decade, diverse methods have been refined to observe and analyze $\mu c$ function. “Extra-vital” techniques have considerably progressed, including nuclear scintigraphy and PET, as well as contrast-enhanced MRI & ultrasound, and can potentially measure or estimate kidney perfusion and sometimes micro-perfusion. While those have wide FOV, lack of resolution and sensitivity are still limiting. “Intra-vital” techniques offer higher resolution, and targeted fields can be assessed, providing information on $\mu c$ perfusion, flow heterogeneity and density. Near-infrared spectroscopy is non-invasive and allows tissue oxygenation/perfusion quantification, but no direct imaging of microvessels structures and flow is obtained. More recent and high resolution contrast-free technologies include orthogonal polarization spectral imaging, side-stream dark-field imaging, CytoCam incident dark-field imaging, and allows structures & flow assessment using the endogenous contrast caused by the absorbance of hemoglobin in red blood cells (RBC). Laser speckle imaging is another method which is based on the interference pattern that forms when a diffuse surface such as animal organ is imaged using a laser. As blood cells move, they produce fluctuations in the speckle pattern and this can be correlated to RBCs velocity. Laser Doppler microprobe imaging reported by Chvojka et al. is equally able to measure cortical $\mu c$ in septic ARF. However, these methods are limited to blood flow, and do not allow for the assessment of the biophysical processes underlying blood flow reduction.

Contrast-enhanced technologies provide additional information on $\mu c$. High-sensitive volumetric optical microangiography (UHS-OMAG) can provide quantitative high-resolution imaging of kidney $\mu c$ using iodinated agents. Intra-vital fluorescence microscopy (single or multiphoton) allows the micro-assessment of glomerular and cortical peri-tubular capillary perfusions. The renal intra-vascular space and circulating cells velocity are visualized by injection of fluorescent imaging agents combined or not with macromolecules (e.g., dextrans or albumin) enabling longer contrast retention in bloodstream. Circulating cells, mostly RBCs, can then be detected by negative contrast because impermeable to the fluorescent dyes. Bringing microscopy to the clinic through pCLE will allow us to acquire such data from various organs in humans.

4.3 FP-CLE as a novel imaging method of renal microcirculation in a model of ARF

FP-CLE can be used to image kidney $\mu c$ directly in animals through a laparoscopic (trans-parietal) access or by capsule exposure. Herein, we present preliminary results concerning a novel method we are currently developing for renal function assessment in a rat model of ARF – more specifically the cecal-ligation puncture (CLP) model. CLP in rodents is the most frequently used model of experimental sepsis and is currently considered as the gold standard of polymicrobial sepsis. Systemic inflammatory burst is ensuing, followed by septic shock, multiple organ dysfunction and death. This systemic inflammatory burst is associated with enhanced interaction of white blood cells (WBCs) and endothelial cells with intrusive presence and spill-over of the former cells in vital organs. WBCs infiltrate the kidney, causing local damage by release of oxygen radicals, proteases, and further production of inflammatory cytokines. WBCs-endothelial interactions result in physical congestion of the kidney $\mu c$ and a further decrease in regional blood flow with some degree of vasodilation. This $\mu c$ dysfunction-alteration of perfusion, observed in glomerular and peri-tubular areas, is contributive to renal hypoxia.
4.4 Material and methods

**Rat CLP model preparation.** CLP was performed in Sprague-Dawley male rats through a midline laparotomy followed by the ligation below the ileo-cecal valve and puncture of the cecum with a 21G needle. Cecum perforation was followed by a ~50-100 μL of fecal material milking from the puncture site. Animals were then returned to their cage for 18-24 h.

**Imaging agent injection and pCLE.** Rats were anesthetized and positioned as described before, and a PE50 was inserted in the right jugular vein. The kidney surface was exposed through an incision, and the S1500 probe positioned directly against this surface. The μc was highlighted using Evans Blue optical stain (EB) injected intravenously (i-v) in the jugular (300 μL at 20 mg/mL in saline). EB remains in the intra-vascular space by binding to albumin, and is imaged in the 660 nm channel (red) of the Cellvizio®. In parallel, 5-(and 6)-carboxyfluorescein diacetate succinimidyl ester (CFSE) is injected i-v. 5 minutes before imaging (1 mL at 0.5 mg/ml in saline). CFSE is naturally non-fluorescent and diffuses through intact cell membranes. It becomes fluorescent when taken up by circulating cells where esterases can remove acetate groups from its native form to yield a fluorescent carboxyfluorescein derivative, reacting with primary amines and cross-linking to intracellular proteins 120.

4.5 Results and Discussion

Figure 3 shows the kidney μc in a healthy (A) and CLP (B) rat. In the vasculature, RBCs appear as dark moving globules (negative contrast) because they do not convert CFSE to its fluorescent derivative and are impermeable to EB. CFSE+ cells are more frequent in CLP rats, which present a slower capillary blood flow, display possible extravasated WBCs outside the μc, and several larger or clusters of CFSE+ cells. The advantages of FP-CLE are its relatively high space and time resolutions and the possibility to observe WBCs eventual margination and/or diapedesis. While not shown here, several parameters can be extracted from these images, such as the average vessel diameter and the number of adhered cells, which can provide further insight on the state of the μc. However, limitations compared to other methods include the need to inject dyes and to expose the kidney (or at least to reach the cortical surface by a trans-parietal pathway access) which is somewhat invasive. In addition, the “touch & see” mandatory procedure of FP-CLE as well as the average limited imaging depth (~250 μm), hinder the exploration of medullary tissue μc.

5. Conclusion – future perspectives of pCLE

FP-pCLE has tremendous potential as a surrogate tool to invasive biopsies of the lung, mainly due to its versatility and ease of use. In our opinion, the major hurdles faced by the field lie in the difficulty with which small animal models can be used to perform proofs-of-concept. This, in turn, is due to challenges in the local delivery of fluorescent probes, navigation limitations in the small animal lung, and lack of wide-spread and validated quantitation methodologies. The community is currently tackling these issues, and translation of some methodologies, mostly qualitative or semi-quantitative methodologies, toward the clinic is already initiated.
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