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## **Probing organoid metabolism using Fluorescence Lifetime Imaging Microscopy (FLIM): the next frontier of drug discovery and disease understanding**

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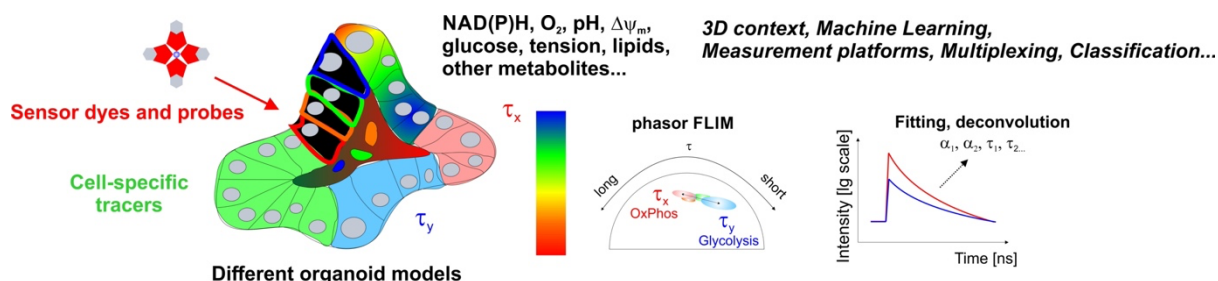
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## Abstract

Organoid models have been used to address important questions in developmental and cancer biology, tissue repair, advanced modelling of disease and therapies, among other bioengineering applications. Such 3D microenvironmental models can investigate the regulation of cell metabolism, and provide key insights into the mechanisms at the basis of cell growth, differentiation, communication, interactions with the environment and cell death. Their accessibility and complexity, based on 3D spatial and temporal heterogeneity, make organoids suitable for the application of novel, dynamic imaging microscopy methods, such as fluorescence lifetime imaging microscopy (FLIM) and related decay time-assessing readouts. Several biomarkers and assays have been proposed to study cell metabolism by FLIM in various organoid models. Herein, we present an expert-opinion discussion on the principles of FLIM and PLIM, instrumentation and data collection and analysis protocols, and general and emerging biosensor-based approaches, to highlight the pioneering work being performed in this field.

## Graphical abstract



**Keywords:** FLIM, hypoxia, metabolism, microenvironment, mitochondria, organoids, PLIM

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## 1. Introduction: organoids, FLIM and metabolism

The complexity imposed by the multicellularity, biomechanical properties, biophysical cues and 3D spatial and temporal organisation of tissue in development, homeostasis, injury and disease has instructed researchers to develop physiologically representative *in vitro* three-dimensional (3D) models [1-3]. The use of such ‘organ-on-a-chip’ and ‘organoid’ models has exploded in the past decade, becoming a multi-million dollar/euro industry and providing significant advancements in scientific discovery [4, 5]. These 3D models not only attempt to model human physiology or pathology conditions *in vitro* but also are an ethically viable alternative to animal testing, reducing the need for the use of animal models in drug toxicity testing and biomedical research (3Rs)[6, 7]. US-based Environmental Protection Agency (EPA) has recently announced its plan to end the animal experimentation by year 2035. While this ambition can be debated [8], developments in advanced on-chip, organoid, ‘assembloid’ and other alternatives to the animal experimentation are highly expected.

Organoids are stem cell-derived 3D microtissues sharing cell composition, organisation, and ability to recapitulate the physiological function of the tissue they represent [9]. Since being introduced over a decade ago, organoid research field has shown a rapid growth, from developmental and cancer biology to the cross-disciplinary fields of tissue engineering, environmental science, zoology, and virology [5, 10].

Organoids can be derived from adult, embryonic and induced pluripotent stem cells and have been shown to be capable to reconstitute intestinal, retinal, neural, liver, skin and many other tissues. They can be also produced from tumour biopsies, resulting in ‘tumour organoids’[4, 11]. Depending on the specific growth conditions (e.g. ‘embryonic’ EGF, Noggin, R-spondin, valproic acid, CHIR vs. ‘differentiated’ EGF, Noggin only-growth media with mouse intestinal organoids), the enrichment of different cell populations can be achieved, which can include rare cell types and modelling of a particular developmental state of a specific tissue [10]. Using bioengineering approaches, the morphology, topology and spatial organisation of organoids can be controlled to guide the differentiation of specific cell lineages, study interactions between the connected organoids, produce ‘assembloid’ models and enable studies of such inter-species communities such as co-culture with microbiota or parasites [1, 5, 12-14].

Importantly, these models can be used as minimal ‘tissue building block’ units enabling the study of organ / tissue cell biology and function in an accessible and dissectible formats [15-20]. Organoids are characterised by their variable growth rate, time required in culture and cell composition, cytoarchitecture, morphology and topology. Thus, organoids can display different levels of heterogeneity, depending on spatial, temporal, natural (physiologically relevant) and extrinsic culture conditions [5].

Not surprisingly, a plethora of experimental methodologies are being applied to address the appearance, biomass, growth rate, genetic and cell composition, biomechanics and dynamic changes in the physiological state of a wide variety of organoid models. Methodologies, including transcriptomics and proteomics (‘omics’), conventional and ‘fixed cell’ microscopies (widefield, high-content, confocal, light sheet, superresolution, expansion and other modalities), have provided an unprecedented level of insight into the physiology of the cells, grown in 3D, within different types of the organoid cultures [5, 21-23]. However, more informative and less destructive imaging methodologies are necessary to achieve deeper level of detail and more reliable quantification [24, 25]. Ideally, an imaging method would provide multiparametric, live and quantitative readouts, with the capability of performing long-term dynamic imaging experiments [26]. Thus, fluorescence lifetime imaging microscopy (FLIM) and related luminescence lifetime imaging modalities can enable such multiparametric and multi-dimensional 5D imaging readouts, and study all X,Y,Z,  $t$  and luminescence lifetime ( $\tau$ ,  $\tau$ ) dimensions for intra- and intercellular processes, biomarkers and physical-chemical parameters [25]. FLIM- and closely related luminescence lifetime imaging microscopies can improve image contrast, expand multiplexing capabilities, help decipher the physiological relevance of the cell and tissue autofluorescence, and leverage all types of the biosensor probes, from ratiometric to FRET, to quantitate cellular metabolic and signalling processes [25]. Tightly connected with cell growth and differentiation, live vs. death decisions and stem cell niche environment; cell metabolism is one the most important processes that can be recapitulated and tightly regulated in 3D organoid models. Analysis of cell metabolism is also one of the most exciting and well-established modality in FLIM applications and is the pertinent topic of this review.

## 2. Basics of FLIM microscopy, instrumentation, and analysis

FLIM is a group of imaging approaches for fluorescence, phosphorescence, and delayed fluorescence phenomena, which addresses measurement of the luminescence lifetime (decay time), of a wide variety of exogenous and endogenous luminescent molecules. The decay time ('lifetime', tau,  $\tau$ ), is an intrinsic characteristic of luminescent molecules, and quantifies the period a molecule remaining in an excited state, between the light absorption and emission events. Importantly,  $\tau$  is affected by the changes in the environment directly surrounding the luminescent molecule, its orientation, rotation, pH, presence of quenchers, temperature and other conditions [25, 27]. This can also facilitate the assessment of protein conformation and intramolecular interactions by FLIM, when for example, a luminescent molecule undergoes self-quenching due to the changes in the protein tertiary structure. FLIM can also provide quantitative measurements of the Förster Resonance Energy Transfer (FRET) events, in which the energy transfer occurs when a luminescent donor molecule is within 1-10 nm from the acceptor and has correct orientation [27-31]. The flexibility, reliability and adaptability of FLIM makes it a very useful quantitative (and qualitative) modality in numerous biological applications [25].  $\tau$  can vary and exist within dozens of pico- and nanoseconds to micro- and even milliseconds (delayed fluorescence of protoporphyrin IX). Three lifetime range 'domains', often requiring different types of detection equipment can be classified: 0.1~20 ns as 'a **conventional FLIM**', ~0.5-100  $\mu$ s as phosphorescence lifetime imaging microscopy (**PLIM**, mostly for O<sub>2</sub> and thermally activated delayed fluorescence temperature imaging) and 100~1000  $\mu$ s in macro-imaging, phosphorescence quenching microscopy (PQM) and **delayed fluorescence**-based O<sub>2</sub>-sensing [32-34]. Importantly, FLIM can be assayed in the visible as well as the near infrared range, permitting its application in microscopy as well as in small animal optical imaging [25, 27, 35, 36]. The required lifetime resolution, light source and speed of acquisition dictate different engineering approaches to construct a wide variety of FLIM microscopes.

Over the past decades, various technologies to measure fluorescence and phosphorescence decays in an image have been developed and applied to metabolic imaging of various endogenous and exogeneous metabolic markers. In short, time-resolved technologies, especially for FLIM, later applied also to PLIM and to time-resolved fluorescence anisotropy

imaging, encompass frequency-domain and time-domain approaches. As these technologies have been recently reviewed in detail elsewhere [37], here we focus on more recent developments that address the unique challenges imposed by metabolic imaging in organoids, especially with regard to signal loss due to scattering events (Table 1).

The main challenge imposed by metabolic FLIM, whether in frequency or in time domains, based on single-photon counting or on time gating, is effective photon management at high acquisition speed. The optimal type of FLIM technology is directly related to the adequate type of microscope used to image organoids, i.e. light-sheet microscopy or theta-microscopy for translucent samples [38, 39]. Importantly, these microscopy systems allow a geometrical separation of the emitted light and the excitation beam. Alternatively, two-photon microscopy using fs-pulsed near-infrared lasers [40], is adequate for highly scattering, cellularly dense organoids.

Light-sheet microscopy typically relies on continuous-wave excitation sources, e.g. diode lasers, which can be easily modulated, thus enabling frequency-domain FLIM [38, 41]. As frequency domain FLIM requires complex analysis strategies for multiexponential fluorescence decays, time-domain solutions based on single-photon counting [42, 43] or time-gating [44-46] have been proposed and successfully applied to perform light-sheet FLIM.

In multiphoton microscopy, time-domain approaches, either relying on single-photon counting or on time-gating, have prevailed as best solutions for both FLIM and PLIM. Highly sensitive single-photon counting approaches optimally take advantage of the time window imposed by the fs-pulsed excitation laser sources, with repetition rates of 80-100 MHz for FLIM and 100 kHz- 1 MHz for PLIM, respectively, (or also combined [47]) ensuring almost no photon loss, when very low signals are expected. In comparison, time-gated approaches allow for much faster image acquisition. To cope with the low acquisition speed of single-photon counting approaches, specifically time-correlated single-photon counting (TCSPC), without photon loss, multi-channel detection setups have been proposed, some currently being commercially available [48-50], also paralleled by multi-beam excitation [51-53]. Further increasing

sensitivity in a multi-channel setting, SPAD detector arrays in multi-photon time-resolved imaging allow for best photon management at high image acquisition speed [36, 54, 55].

Moreover, balancing spatial resolution and temporal resolution is central in FLIM acquisition and, later, data evaluation, as cellular or even subcellular information is requested to answer biological questions in a mechanistic manner [50]. As multiplexing has emerged as a necessity for biosciences, spectral FLIM (or even hyperspectral FLIM) evolved recently as a powerful tool [56, 57], with (multi)omics applications [58]. Finally, the limited acquisition time window imposed by the laser repetition rate limits the signal quality and the information, which can be extracted from it. While previously the instrument response function of the FLIM detectors and electronics needed to be considered when developing the analysis strategy, e.g. by the instrument response function (IRF) re-convolution, new detectors with temporally narrow and symmetric IRFs, with low jitter – high reproducibility of IRF position with respect to the laser pulse train – are now available, as well as solutions to cope with the limited acquisition time-window in FLIM [59].

For sustainable dissemination of FLIM and PLIM approaches as reliable tools in biosciences, specifically for metabolic imaging in model systems such as organoids, commercial accessibility to technology needs to be accompanied by accessibility to reliable algorithmic routines. Next to the quality of the time-resolved signal previously discussed, the challenge in interpreting FLIM data (both frequency-domain and time-domain), i.e. providing evaluation tools, is imposed by the complexity of the fluorescence decay curve, as the samples seldom contain only one state of a fluorophore, well characterized by a single exponential decay. Typically, two or more exponential components build the decay curve, with the additional contribution of detector electronic noise (in the best case, Poisson distributed) – a non-dampened multi-frequency oscillation. A multitude of algorithmic models have been proposed and successfully applied over the past decades, which are also readily accessible in open-source software applications such as FLIMfit, FLIMJ and others [60-62]. For the metabolic imaging of the endogenous coenzymes NAD(P)H and FAD, the data evaluation still represents a challenge, as the coenzymes have hundreds of enzymatic partners, they may bind to, which differently affect their fluorescence lifetime. To evaluate such data, typically bi-exponential

approaches are used [63-65], which have been further optimized to differentiate between NADH and NADPH in their enzyme-bound states [66, 67]. However, these FLIM analysis approaches require models to visualize the distribution of decays, and imply per se assumptions, which may limit their general applicability. Using the phasor approach to time-domain FLIM images allows model-free data visualization [68], however, for the interpretation of the results, models are also needed, similarly to the evaluation algorithms relying on exponential approximations in time domain [69-71]. Adding information from other sources than FLIM, e.g. knowledge about enzyme expression levels to NAD(P)H-FLIM data [72] evaluation, will allow to extract more detailed information on the biology from such complex data.

As the low number of photons is a central limitation for FLIM approaches, in general, leading to noisy signal, a solution is to filter the data in space-domain, reducing spatial resolution, to gain higher accuracy in time. Recently, Wang et al [73] proposed and successfully demonstrated the benefits of complex wavelet filtering in phasor approach evaluation of TCSPC FLIM data, which overcomes the trade-off between time and spatial resolution: the spatial resolution was preserved, whereas the width of the phase vectors distribution caused by signal noise was considerably reduced. The approach is now also commercially available. The development of improved statistics algorithms for omics technologies, relying of parameter clustering, has been successfully applied also for analysis of noisy or erroneous FLIM data, to distinguish between different fluorophores [74, 75].

Moving away from generating models of complex fluorescence decays to approximate FLIM data, towards classifying various biological states, machine-learning and deep-learning algorithms gain increasing relevance in FLIM data evaluation. In this way, the otherwise challenging FLIM evaluation is readily accessible also to FLIM non-experts and have been successfully applied either to distinguish specific (metabolic) states [76-80], or to improve the access to information in FLIM data [81-84]. However, the challenge when evaluating complex FLIM or PLIM data using machine- or deep-learning approaches is imposed by an appropriate algorithm training. To avoid misinterpretations, strategies to verify results reliability, in comparison to results of the same data using model-based evaluation approaches, are required.

### 3. Assessment of metabolism by FLIM: tools

The sensitivity of organoids to niche-specific and microenvironment conditions and controlled perturbations makes them amenable models for the study of the dynamic regulation of cell metabolism and bioenergetics processes in cancer biology, regulation of tissue development and production of physiologically relevant artificial tissues [17, 85-88]. The capability of FLIM to assay different metabolic parameters such as oxygenation, mass exchange and availability/utilisation of metabolic substrates (NADH, glucose, O<sub>2</sub> and others), and composition of extracellular matrix, is key to our understanding of the regulation of metabolism within the 3D context. Thus, increased attention to the 3D dynamics of mitochondria, forward and reverse Krebs cycle mechanisms, modified glycolysis pathways, dependent on the availability of substrates are seen [85, 89-91].

An important question is whether there is a minimum set of parameters, that enable a complete understanding of cell metabolism, while maintaining cell viability? The traditional positive answer, established long before the introduction of the organoid model, is based on analysing cell redox ratio, NADH/ FAD levels and fluxes of pH and O<sub>2</sub>. However, over the decade, a growing list of novel FLIM-aided parameters and biomarkers have been characterized and used to study cell metabolism, depending on the choice of the analytical method and broader compatibility with the tested experimental models. Studies of cell metabolism have evolved from respirometry and colorimetry to the mass spectrometry and quantitative fluorescence microscopy. As described below, depending on the context and research task, several additional parameters provided by FLIM-based assays can be used to probe cell metabolism in organoid models, using endogenously produced pigments, genetically encoded fluorescent biosensor proteins, chemically modified dyes, nanosensors and ECM-binding materials (Table 2, Fig. 1).

[Insert Figure 1 here]

### 3.1 Optical metabolic imaging

Cellular autofluorescence represents one of the intrinsic features, that is important for understanding cell function. In case of mammalian cells, a common source of blue and green cell autofluorescence is a population of the metabolic cofactors NADH, NADPH (collectively designated as NAD(P)H) and FAD, heavily involved in redox metabolism [92, 93]. Being present in free (e.g. cytoplasmic) or protein-bound (e.g. in mitochondria) forms these pigments display characteristic lifetimes, indicating their involvement either in glycolysis or oxidative phosphorylation (OxPhos)[94, 95]. Oxidised form of NAD(P)<sup>+</sup> (and some fraction of NADH [96]) and reduced form of FAD (FADH<sub>2</sub>) are not fluorescing thus making these pigments indicators of either cellular reduction (NAD(P)H) or oxidation (FAD) processes. Classically the ratio in fluorescence of NADH:FAD was designated as ‘redox’ and can be used either in one- (excitation at 340 and 460 nm) or two-photon (710-750 and ~800 nm) excitation modes. Almost two decades ago, Skala and co-workers demonstrated that the optical redox ratio can be also measured by two-photon FLIM, which subsequently evolved into ‘optical metabolic imaging’ (OMI) concept [92]. Overall, measurement of NAD(P)H and FAD-based autofluorescence enables for non-destructive and label-free (i.e. no external fluorescent probes or genetically encoded biosensors required) analysis of cell bioenergetics in tumour biopsies, organoids and other complex tissues. Due to such advantages of two-photon microscopy as deep light penetration and mild illumination to the cells, in principle, live 3D FLIM is possible with this approach. With further calibration experiments and analysis of endogenously expressed pigment-binding proteins, the lifetimes of NAD(P)H and FAD can be used to distinguish the biochemical processes in the cells in more detail [72, 97].

Typically, NAD(P)H shows short lifetimes when in its free form and longer lifetimes upon binding to the proteins, while FAD displays a reverse behaviour (i.e. longest lifetimes of the free form). Analysis of the classical double-exponential fitting provides deeper insight in the oxidation state of NAD(P)H, which is the most frequently measured autofluorescence parameter, often in the absence of FAD measurements. More recent approaches based on phasor FLIM require less computing power and are currently gaining in popularity (Fig. 1C). The fluorescence intensity of endogenous NAD(P)H and FAD is an intrinsic feature of cells that have active metabolism and high concentrations of these pigments. However, the

measurement and interpretation of these endogenous signals are complex and not always straightforward [94]. Thus, a number of fluorescent probes and biosensor proteins have been proposed to measure cellular redox status and even NADH:NAD<sup>+</sup> ratio such as fluorescent protein-based peredox or fluorescent dyes [98-101].

### 3.2 Alternative ‘label-free’ methods based on cellular and tissue autofluorescence

Other useful autofluorescent pigments can be encountered in specific cell types, tissues and organoid models [102-104]. In addition to the retinoic acid and retinol (see 4.2), which are relevant only to the photoreceptor cells, perhaps the most studied examples are tryptophan autofluorescence, delayed fluorescence of the protoporphyrin IX (PPIX), and luminal autofluorescence observed in live intestinal organoids. Naturally present **tryptophan** shows two-photon excitable autofluorescence and can act as a FRET donor for soluble NADH, an useful assay to determine the activity of glycolytic dehydrogenase enzymes [105] and to complement the optical imaging of redox ratio (see 3.1). In addition, tryptophan is involved in different metabolic pathways, including biosynthesis of serotonin, kynurenine, NAD<sup>+</sup> and indole, that are important for intestinal and neural tissues, and microbiota. Altogether, multiphoton FLIM of tryptophan, interacting proteins and respective metabolites should be key for future studies of cell-specific metabolism of small intestinal organoids targeting inflammatory bowel disease (IBD) [106].

Mik and co-workers found that endogenously produced mitochondrial **protoporphyrin IX** displays O<sub>2</sub>-quenched delayed fluorescence, which can enable direct measurement of mitochondrial O<sub>2</sub> and analysis of cellular O<sub>2</sub> gradients across mitochondria and extracellular space [107]. This method can be complemented by adding 5-aminolevulinic acid (ALA) to the cells, is compatible with *in vivo* measurements and has some clinical applications. Depending on the type and function, living tissues also display additional autofluorescence. For instance, intestinal organoids often show broad emission red autofluorescence of their lumen [103, 104, 108, 109] (Fig. 2), which can complicate their live microscopy and must be removed or segmented out of the images. The source of this autofluorescence is not clearly known but can be explained by the presence of porphyrin-based degradation products of cytochrome P450 3A

from dying enterocytes [110], connecting its lifetime with the viability and overall metabolic function of differentiating organoids.

Datta and co-workers identified a **ROS-oxidised lipid** fraction of the green (420~500 nm) autofluorescence in the white adipose tissue (WAT), visible upon two-photon excitation of NAD(P)H. This compound displayed a long monoexponential lifetime of 7.9 ns and characteristic Raman spectral (coherent anti-Stokes Raman spectroscopy, CARS) signature [111]. Similarly, a long lifetime autofluorescence component associated with lipid droplets was measured in the study of differentiating adipocytes by Sanchez-Ramirez and co-workers [112] and Niesner group [71]. Such long lifetime signatures were also detected in retinal organoids (see 4.2). In addition to NAD(P)H and oxidised lipid signals, two-photon NAD(P)H-FLIM can also detect intracellular protein-based autofluorescence, produced by keratin. Thus, in keratinocytes, such signals can contribute to ~1.5 ns lifetime component and significantly affect analysis of NAD(P)H [113].

As an organ with strong metabolic function, the kidney displays a diversity of autofluorescent components (NAD(P)H, FAD and red fluorescence) under two-photon FLIM [114]. For example, S1 and S2 tubules display different metabolic signatures and autofluorescence lifetime helps to discriminate between the interstitium, urinary lumen and glomerulus.

Connective tissue, such as white and red blood cells also display autofluorescence, due to their complex pigment composition. This can be also visualised and separated using phasor FLIM approach [115].

The ‘functional’ autofluorescence FLIM may also go well beyond different animal and human tissues. For example, **bacterial** cells can display striking patterns of autofluorescence [116]. Since their biological and metabolic state can depend on the presence of antibiotics, culturing time and growth rate and the recovery, phasor analysis enables for screening of their NAD(P)H-FLIM features. Metabolic ‘fingerprint’ phasor FLIM maps were reported for *E. coli*, *S. enterica* serovar Typhimurium, *P. aeruginosa*, *B. subtilis*, and *S. epidermidis*. Without a doubt, further FLIM studies would enable probing these features and metabolic heterogeneity within the inter-species host-microbiota interactions in a complex organoid setting [117].

### 3.3 Molecular oxygen (O<sub>2</sub>)

Molecular oxygen is of paramount importance for tissue physiology within 3D context, since it is an essential substrate for aerobic respiration and oxidative phosphorylation (OxPhos) activity [32]. Diffusion-limited O<sub>2</sub> supply to the tissues is crucial for the development of niche-specific regions and function of stem cell niche(s). Abnormal cell and tissue O<sub>2</sub> can result in oxidative stress, production of reactive oxygen and nitrogen species, but this O<sub>2</sub> level is unique to the tissue being studied as what may be abnormal for one niche-specific region can be the *de facto* level of oxygenation of another [118]. Not surprisingly, a variety of approaches to measure O<sub>2</sub> have been developed, ranging from applications in aquatic biology to biomedical science [33, 34]. Typically, phosphorescence lifetime imaging microscopy (PLIM) and related confocal fluorescence microscopy-based methods are applicable for O<sub>2</sub> measurements in organoids [103, 119]. For bulk measurements, microplate-based methods are often preferred [120-123](see 3.6). PLIM is normally used for imaging oxygenation with (sub)cellular resolution in live and 3D contexts [103, 108, 119]. This method is based on the use of a phosphorescent compound, such as supramolecular conjugates or nanoparticles, which display specific luminescence quenching by the molecular oxygen. Higher O<sub>2</sub> levels result in shorter decay times, while the lower or absence of O<sub>2</sub> lead to measurement of unquenched longer lifetime ( $\tau_0$ ). The phenomenon of the quenched phosphorescence-based O<sub>2</sub> detection is often described by Stern-Volmer relationship and its modifications such as ‘two-site model’ [32]. A number of O<sub>2</sub> probes have been described for 3D tissue models and organoids: typically they all display large Stokes shifts with red (610~670 nm) or near-infrared emission (730-770 nm), with the lifetimes in range of 1~70  $\mu$ s, showing mono- and double-exponential decay behaviour. Some of these O<sub>2</sub> probes can be efficiently excited using two-photon excitation and have been used in advanced intravital applications [124-126]. A number of alternative methods, based on fluorescent proteins, reduction reactions and other modalities were also recently proposed for semi-quantitative and end-point analysis of O<sub>2</sub> [33, 127, 128].

### 3.4 Redox sensors and reactive oxygen species (ROS)

Molecular oxygen (O<sub>2</sub>), as an oxidant, is also involved in the production of radical-harboring reactive oxygen, nitrogen and sulfur species. A large body of research in redox biology focuses

on detection of ROS and quantification of the redox status [129-131]. Generally, ROS represent short-lived radicals or radical donors such as superoxide anion  $O_2^{\cdot -}$ , hydrogen peroxide  $H_2O_2$ , hydroxyl radical  $HO^{\cdot}$  and peroxynitrite  $ONOO^{\cdot}$ . Most of these species are being produced by the cell via a multitude of processes involving superoxide dismutase, iron, mitochondrial complexes I and III and NADPH oxidases. Production and measurement of these different ROS species are therefore important to the analysis of cell metabolism and imaging of hypoxia (see 3.3.). A good number of genetically encoded (GEFI) and dye-based fluorescent indicators have been proposed to specifically sense ROS species. This is normally challenged by their short lifespan (half-life) in the active form, lack of means for specific detection, high reactivity (often resulting in end-point measurement, so the fluorescent probe is not recovered after binding ROS molecule), and lack of adequate control of the environmental  $O_2$  [33, 132, 133]. Reversible probes, sensitive to redox (i.e. can be oxidized by ROS and reduced by thiol compounds), such as HyPer, roGFP1, Grx-roGFP2, OxyFRET, PerFRET and dye-based systems, enable for ratiometric and FLIM detection of cellular redox status [100, 129]. However, often these probes experience issues with the rate of response, sensitivity, localization and compatibility with FLIM. Collectively, the search for an ideal ROS probe is ongoing.

### 3.5 Mitochondrial membrane potential

Mitochondrial membrane potential ( $\Delta\Psi_m$ ) is a hallmark of the activity of the mitochondria and, together with  $O_2$  consumption, can be used to quantify mitochondrial dysfunction [134]. In addition to generation of energy,  $\Delta\Psi_m$  is important for the processes of mitochondrial fission and fusion, production of reactive oxygen species, mitophagy, mitochondrial quality control and cell and mitochondrial motility [135]. Mitochondrial polarisation can be visualized and measured using fluorescent probes, e.g. tetramethylrhodamine methyl ester (TMRM), JC-1 and others, that accumulate in mitochondria in a potential-dependent manner. While traditionally their fluorescence intensity has been correlated with the mitochondrial membrane potential ( $\Delta\Psi_m$ ), we and others reported that the fluorescence lifetime of these probes can also be used for quantification of  $\Delta\Psi_m$ . Thus, TMRM, MTFPS and a number of Syto dyes can provide more quantitative context in the analysis of mitochondrial function [136, 137]. Dye self-quenching upon accumulation in the polarised mitochondria leads to shortening of its decay

time, in a concentration and time-dependent fashion. Syto dyes, initially reported for labelling of nucleus in live cells, normally can reside in both mitochondria and nucleus, but upon mitochondrial depolarisation (e.g. after application of mitochondrial uncouplers or in the stressed and dying cells) relocate to cell nuclei, remaining within the cell [137]. In contrast, when mitochondria decrease their polarisation, TMRM diffuses out of the cell, which makes analysis of its fluorescence lifetime more difficult in monolayer cell cultures. It is worth noting that accumulation of  $\Delta\Psi_m$ -sensitive dyes in mitochondria can result in mild uncoupling effect and if their working concentration was not optimised and tested initially, negatively influence the mitochondrial function. In general, these dyes display multi-exponential decay and are suitable for phasor-FLIM analysis of 3D tissue models such as intestinal and cancer organoids (see 4.1).

### 3.6 Extra- and intracellular pH

pH also regulates cell function and often displays heterogeneous distribution inside (cell organelles) and outside of the cell. Most striking is the difference between cytoplasmic- and extracellular pH gradient(s) between normal and tumor tissues [138]. Typically, glycolytic cells are expected to acidify the extracellular space. Therefore, pH can be viewed as an additional parameter informing on cell metabolism, with the preference of being sensed at the plasma membrane or outside of the cells, within a confined 3D volume. A great number of pH-sensing dyes, probes, nanosensors and fluorescent biosensor proteins have been described for sensing pH by conventional fluorescence microscopy, FLIM and by the microplate reader-based / Seahorse platforms, in an Extracellular Acidification Rate (ECAR) analysis [123, 139, 140]. In order to sense the extracellular pH, probes based on extracellular matrix proteins can be used, e.g. cellulose-binding ECFP or cell-impermeable probes [141, 142]. While technically not challenging, sensing of extracellular pH to understand organoids metabolism has not been explored fully yet. Various dye structures such as BCECF, DAOTA, perylene bisimide or fluorescent proteins such as mCherryTYG, ECFP and others can be used for this purpose [140, 143-146]. With wider use of FLIM, several dyes, conventionally used as organelle-specific fluorescent tracers, can be also applied to analysis of pH in the cell. For instance, LysoTracker Red displays strong changes in the lifetimes within intestinal organoids (Fig. 2).

[Insert Figure 2 here]

### 3.7 Glucose, pyruvate and other metabolites

Recent progress in research on the genetically encoded fluorescent indicators (GEFIs) enables quantifying cell metabolism in a real-time and non-destructive quantitative manner. Several FLIM-compatible biosensor probes based on circularly permuted fluorescent proteins (cpFP) and two protein-based Förster Resonance Energy Transfer (FRET) have been developed to measure glucose (iGlucoSnFR-TS, AcGFP-GBPcys) and pyruvate (pyronic) [147-149]. Some FRET-based GEFIs for sensing lactate,  $\alpha$ -ketoglutarate, citrate, amino acids, ATP, ADP, GTP, long-chain acetyl-CoAs [99, 150] were also described, but have not been reported in FLIM applications. Altogether, the available GEFIs already enable for multi-parameter analysis of the organoids metabolism, at least concerning the Krebs cycle and the availability of the energy and nutrients. This is especially important considering the recognised role of the growth medium composition on recapitulating of the physiological state of the cells in intestinal organoids and tumour spheroids [90, 119, 151] (see sections 4.1 and 5). Alternatively to the extracellular FLIM sensing, extracellular flux analysis (Seahorse XF) or dynamic flow (on-chip) systems with controlled and predictable concentrations of metabolites can be used [152]. Soluble glucose-based fluorescent tracers (such as 2-NBDG, 6-NBDG and others [153, 154]) were not reported in FLIM and normally may be not compatible with the use of some organoid models, displaying efficient barrier function [155]. They should also be used with caution, since they can utilise different intracellular transport, compared to unlabeled glucose and 2-deoxyglucose [156].

### 3.8 Other biomarkers and physical parameters

There are reports on cellular and intracellular **temperature** (T) gradients, produced and potentially sustained via cell metabolic activity, in different cell models, tissues or cancer models [157-160]. The field of nanothermometry addresses such measurements via FLIM, PLIM and dedicated small molecule and nanosensor probes [161-163]. While has not been studied with the organoids yet, nanothermometry can potentially help visualising mitochondrial function, polarisation and uncoupling in better details [164, 165]. Using multicellular spheroids produced from human colon cancer cells, we found that changes in T

gradient across spheroids (3-4 °C above the physiological 37 °C) were dependent on the activity of mitochondria and concomitant with changes in their oxygenation [161]. Currently, the nanothermometry field addresses staining, biodistribution and sensitivity of the sensor probes [157, 163, 166].

Cellular **viscosity** is yet another ‘physical’ parameter with the growing relevance in studies of cell metabolism, membrane tension, cell contractility and cancer metastasis [167]. So-called ‘viscous memory’ of cancer cells promoted their dissemination in the chorioallantoic membrane (CAM) assay. Interestingly, increased viscosity should also correlate with decreased cell oxygenation and their potential priming towards more glycolytic phenotype, although this was not studied by the authors. FLIM method can also be applied to measure cellular and membrane viscosity, using so-called fluorescent molecular rotors, such as Bodipy-C12 and others [168]. Although in 3D setting these dyes display strong concentration-dependent effects on the decay time [169], they are promising tools for cancer and organoid models. Recently, viscosity measurement was also used for assessing the hypoxic damage of the extracellular vesicles (EV) in placenta [170] and resulting in altered phospholipid composition.

Intra- and extracellular **Ca<sup>2+</sup> dynamics** is essential for tissue homeostasis, especially in neural and excitable tissues. Intracellular Ca<sup>2+</sup> handling is also tightly connected with the mitochondrial function [171, 172]. Various fluorescent dye- and protein-based probes (GECI) have been proposed, although their performance and usability in FLIM is not always addressed. Some of these include Oregon Green BAPTA-1 AM, Asante Calcium Green/ Red [173-175] and genetically encoded proteins, such as TN-L15, Twitch-2B or Tq-Ca-FLITS [50, 176-179]. So far, only few studies addressed measurements of intra- and extracellular Ca<sup>2+</sup> in the small intestinal organoid cultures and staining with fluorescent dyes [176, 180] (see 4.1).

Studying metabolism alone has its benefits but often adding other biosensors and sensing moieties helps integrating the biochemical processes and linking e.g. chromatin compaction and epigenetic regulation with the mitochondrial function, cell differentiation vs. death decisions, mitochondrial function and cell motility and extracellular matrix physical properties,

RNA dynamics and others [181, 182]. Many additional imaging probes and modalities can be integrated into multi-parameter FLIM of cell metabolism, such as probes informing on cell **mechanical properties**, e.g. ‘tension sensors’. Among these, dye-based Flipper-TR probe has been already demonstrated in sensing mechanical stress in organoids, gastruloid and mouse embryo models [183, 184]. Similarly, many genetically encoded fluorescent protein based fluorescent FRET-FLIM probes can be also used to sense membrane tension [185-188]. Such FRET biosensors can be potentially inserted into the larger proteins (e.g. collagen, spectrin, vinculin) or tagging, and enable probing a multitude of molecular tensions experienced by cells, cytoskeleton, and tissues. However, many of such biosensors are limited by their intrinsic inability to measure forces normally higher than 7 pN and would ideally require single molecule measurements rather than analysis of a complex cell mixture, unless more complex imaging approaches (e.g. PIE-FLIM [189]) are applied.

#### **4. Progress so far: organoids and some spheroids application areas**

Organoid models come in a number of different experimental setups: static and semi-static flow systems such as spheroids (and tumour ‘avatars’), simple monolayer organoids (e.g. intestinal and endometrial organoids) and large organoids (e.g. brain organoids), most often grown within basement membrane matrix, e.g., Matrigel, related hydrogels [10, 12, 151, 190-192] or air-lift cultures [193]. Due to the importance of shear stress, improved nutrient and mass exchange and flow dynamics: several downscaled (e.g., to spheroids), microfluidic and bioreactor-based systems have been increasingly used [151, 192, 194, 195]. Microgravity and lab-on-a-chip platforms can pose some challenges for integration with imaging setups and, in some cases, this is being addressed e.g. in MOAB or tumour-on-a-chip models [152, 196, 197]. While conventional lab-on-a-chip models benefit from a huge number of electrode-, colorimetric and optical sensing methodologies, most of the already performed FLIM studies of organoid metabolism are still focused on conventional static models [21, 25]. Below we discuss and highlight the major studies focused on FLIM of metabolism using adult, embryonic stem cell (ESC) and iPSC-derived and cancer organoids.

#### 4.1 Intestinal organoids

Being one of the first described models of the intestinal epithelium development, adult stem cell-derived small intestinal organoids have been analysed by several FLIM-based approaches. Perhaps the first stem cell-derived organoid application using FLIM imaging was the labelling of cell proliferation by incorporating BrdU into the cell nuclei in Hoechst 33342-stained organoids, grown under differentiating conditions. Using this method, the regions enriched with cell proliferation (crypts) could be easily visualised in live adult mouse-derived organoids. In addition, general compatibility with one-photon excitation (pulsed 405 nm laser) and live fluorescence imaging were demonstrated. Interestingly, luminal autofluorescence was also observed in this study [104]. Further studies from our group focused on biosensor-aided analysis of the organoid metabolic function: thus, using phosphorescent O<sub>2</sub>-sensing metalloporphyrin-based small molecule probe Pt-Glc, we showed efficient staining of mouse intestinal organoids and subsequently measured their steady-state oxygenation under regular growth conditions, within the ‘Matrigel domes’, using phosphorescence lifetime imaging microscopy (PLIM) [103]. Surprisingly, we found that differentiating mouse organoids grown within the Matrigel at ambient O<sub>2</sub>, displayed rather deep and heterogeneous deoxygenation (40~73  $\mu$ M O<sub>2</sub>), independent on their location within Matrigel. This finding was later confirmed independently in a study of apical-out organoids [13]. Such strongly ‘hypoxic’ phenotype could be caused by the active oxidative metabolism in organoids. We observed rather delayed responses in cellular oxygenation after adding mitochondrial activator (uncoupler FCCP) and inhibitor (potassium cyanide), which could be explained by the slower diffusion of drugs. We also looked at the effect of metformin on oxygenation of organoids, in a passage-dependent manner: interestingly, its inhibitory effect on cell respiration increased with the passage number, reflecting the change in organoid composition during culturing. With our methodology, we also demonstrated the presence of intracellular O<sub>2</sub> gradients between basal and apical membranes although at that stage PLIM analysis in 3D was limited to measurement of different optical planes. Furthermore, our team focused on designing new FLIM assays to address and explain this strong metabolic heterogeneity. Thus, we analysed oxygenation with labelling cell proliferation and used transgenic Lgr5-GFP (*Lgr5-EGFP-ires-CreERT2*) culture of the mouse intestinal organoids, to visualize Lgr5-positive stem cells [109, 119]. With the help of Lgr5-GFP live tracing we could distinguish between regions enriched

in stem (and Paneth) cells and ‘differentiated’ compartments (no Lgr5-GFP expression) and measure oxygenation and NAD(P)H in these regions. We did not find significant differences in oxygenation, protein-bound NAD(P)H lifetime and fraction of protein-bound NAD(P)H between Lgr5<sup>+</sup>/<sub>-</sub> regions under conventional measurement conditions. However, when we changed the glucose concentration in the medium, we could observe that while enterocytes had generally higher O<sub>2</sub> consumption and deoxygenation than the stem cell niche regions, Lgr5<sup>+</sup> cells could quickly upregulate their OxPhos at 0.5 mM glucose concentration. This finding raises important question on the correct use of the measurement medium and the nutrient composition in studies of intestinal organoids metabolism and stem cell regulation. For instance, some *ex vivo* studies used nutrient-poor HBSS buffer to map metabolic trajectories in the live intestinal crypt [69]. While we also showed that frequently used Seahorse XF analysis method normally fails to grasp such cell-specific differences in the organoids and is inferior to a multi-parameter FLIM, we also emphasised the need of a proper 3D analysis and more advanced live cell segmentation to correctly interpret cell-specific metabolic changes in live organoids.

Subsequent work also introduced and demonstrated additional biosensors, addressing studies of cell metabolism in intestinal organoids. Thus, staining with dyes and probes enabled labelling mitochondrial polarisation in the stem cell niche of live organoids by using TMRM, Syto 16 and 24 dyes, together with Lgr5-GFP and cell proliferation FLIM [137]. Such method, compatible with one- and two-photon excitation modes helps visualising dynamic changes in proliferation and link it to the mitochondrial polarisation and morphology in the intestinal crypt. Biosensors, confined to the extracellular space, such as cell-impermeable probes or binding to an extracellular matrix (or scaffold material), can help visualising extracellular pH, relevant for observing glycolytic function in cancer cells and organoids [142] and extracellular Ca<sup>2+</sup> [180]. In the latter case, extracellular Ca<sup>2+</sup> at the basal membrane of mouse intestinal organoids was linked to the dynamics of lipid droplets, visualised by FLIM of the Nile Red dye. This method was shown to be compatible with measurement of organoids oxygenation and mitochondrial polarisation. Kook Chun and co-workers used primary mouse intestinal organoids to see the effect of circadian rhythm-involved genes *Apc* and *Bmal1* in transition to the colorectal cancer (CRC) [198]. Here, FLIM of NAD(P)H autofluorescence helped to reveal

the activation of the glycolytic metabolism to sustain cell proliferation. However the imaging medium composition and glucose concentrations were not reported. Collectively, the work performed on intestinal organoids helped reveal the importance of imaging their metabolism, highlighting the issues with heterogeneity and stability in the culture, and role of the growth and experimental medium compositions. At the same time, its important to emphasize the growing need of implementing 3D analysis of these complex tissues to correctly interpret data on cell metabolism. Strong luminal autofluorescence, while potentially useful for studies of the enteroid (differentiated organoids) function, is normally being ignored or not reported.

## 4.2 Retinal organoids

Retinal organoids represent another well-established model, that can benefit from the use of FLIM. They are produced from the human pluripotent stem cells, recapitulating formation of embryonic retinal development, including appearance of a bilayered optic cup, photoreceptor layer and positioning and migration of the retinal neuron layer. These relatively large (up to mm size) organoids are typically cultured for long time in culture (46-151 days), which would require application of ‘label-free’ two-photon microscopy FLIM analysis, relying mostly on the cellular autofluorescence. In case of retinal organoids, cellular autofluorescence would consist of NAD(P)H, retinol and retinoic acid signals, which can be separated spectrally and using phasor FLIM. Pioneering study of Browne and co-workers [199] reported temporal changes in glycolytic metabolism in retinal organoids and observed the highest glycolytic metabolism in the layer of differentiated photoreceptor cells. The same team and co-workers further studied autofluorescence (morphological and metabolic changes) of retinal organoids during the differentiation, using two-photon FLIM and hyperspectral imaging [200]. Authors reported tissue heterogeneity in metabolism, especially at the early stage of differentiation. Subsequently, to address organoids heterogeneity and more controlled differentiation, this team engineered a bioreactor platform for retinal organoids. In this work, they used NAD(P)H-FLIM and tracing with GFP-expression in photoreceptor cells to validate the differentiation of the neurons in this model and compare it with conventional static-grown retinal organoid model [201]. In this study, in addition to NAD(P)H autofluorescence, authors also measured long lifetime species ( $\sim 7.89$  ns) of lipid droplet attributed to the oxidative stress [111].

The group of M. Skala further improved the multiphoton FLIM analysis of retinal organoids [202] by performing simultaneous two- (760 nm) and three- (1040 nm) photon excitation. The team found that this would improve the ability to resolve the autofluorescence lifetimes of retinal pigments. This method also enabled the capture of visual cycle dynamics in retina explants and 200 days differentiated retinal organoids. While less efficient response to the white light was observed with organoids, this study introduced a new method of functional characterisation and validation of retinal development in the organoid system. Additionally, in retinal degeneration, metabolism-dependent mechanisms of degeneration have been clarified using FLIM whereby such analysis of retinal explants from a rhodopsin knockout ( $\rho^{-/-}$ ) mouse model of photoreceptor degeneration enabled the assessment of reduced NADH pools in the photoreceptor areas, which was attributed to SARM1 engagement of NADase activity. Such work points towards added opportunity in discovering pathways of degeneration and disease for finding new avenues of therapeutic intervention [203].

### 4.3 Polycystic kidney organoids

Hiratsuka and co-workers produced patient-derived organoid-on-chip platform to study polycystic kidney and hepatic disease gene PKHD1. One of the key features of this model would be formation of cysts (observed only under flow conditions), which depend on cilia function. To determine if the cell membrane tension and tubule stretching in organoids is affected by the fluidic flow, authors used Flipper-TR membrane probe and FLIM [204].

### 4.4. Pancreatic islets

Pancreatic islets of Langerhans are a well-established 3D in vitro model with potential of transplantation into diabetic patients. However, this model still requires detailed characterisation of maturation. Metabolic NAD(P)H-FLIM and O<sub>2</sub>-PLIM with IrBTP and related phosphorescent probes [205] are among the methods, proposed to study the maturity and functional state of the islets. Thus, Azarello and co-workers used NAD(P)H-FLIM for the multi-parameter study of the human islets [206], where they correlated cell metabolism with distribution of alpha and beta-cells. Gregg and co-workers used this method for analysis of the impaired mitochondrial function in type 2 diabetes aged mice [207]. Zbinden and co-workers used NAD(P)H-FLIM to study effects of hypoxia on the functional quality of the pseudo-islets

grown on a chip [208]. In addition, pancreatic islets were probed by FLIM-FRET biosensor reporting the RhoA GTPase activity, involved in mechanosensing, growth and motility of cells [209] and Akt-FRET biosensor [210]. Such biosensor tools can complement studies of the pancreatic islet metabolism, quality, and functional state.

#### 4.5. Tumour and cancer organoids

Use of FLIM for studying the metabolism and redox status of the tumour tissues was pioneered by the Skala group as an ‘optical metabolic imaging’ (OMI) concept to study the drug response by breast cancer cells and *ex vivo* tissues [211]. The same team proposed production of organoids from the frozen cancer tissues and couple it to OMI [212]. Subsequently, OMI was applied for studying metabolic heterogeneity between the samples from different tumours and patients with breast and pancreatic cancers [213], neuroendocrine tumour organoids [214] and other works. In a more simplified form, limited to NAD(P)H-FLIM, such metabolic label-free imaging approach was successfully used for analysis and optimisation of treatment of the patient-derived human glioblastoma organoids [215] and bladder cancer organoids multiplexed with Raman imaging [216]. A bit surprisingly, very few other FLIM methods were used to study tumour organoids. Among them, the study of Sun and co-workers, who took advantage of FLIM-FRET to understand interaction between SOX8 and Aurora-A [217] in regulation of glucose metabolism in the patient-derived ovarian cancer organoids. Lakner et al used NAD(P)H-FLIM to study lumen-forming Caco-2 tissue aggregates and their metabolic responses to epidermal growth factor (EGF) as a physiologically-relevant influencer of cell metabolic behaviour [218].

On the other hand, many drugs can fluoresce or be modified to display changes in fluorescence lifetime upon their internalisation, interactions with intracellular environment, and biotransformation. This can be utilised for monitoring of their pharmacokinetics and action. For instance, paclitaxel conjugates were used to understand drug delivery process by FLIM by cultured cells [219], while intrinsic fluorescence of doxorubicin was used to study its action by FLIM in multicellular spheroids [220]. In addition to such drugs as doxorubicin [221, 222], interactions of drug inhibitors with FRET-FLIM biosensors such as Src (dasatinib) have been also exploited [223].

Some work on tumour spheroids can potentially also find its way in the analysis of organoids. Thus, oxygen-dependent maturation of spheroids was performed using FRET-FLIM in combination with the hypoxia-tolerant fluorescent-protein UnaG fused to mOrange2 fluorescent protein [128]. Other authors have analysed the micro-viscosity of the plasma membrane of live cancer cells during chemotherapy with cisplatin using specific viscosity-sensitive fluorescent molecular rotors and FLIM [224]. Recently, tumour spheroids were labelled with environment-sensitive dyes and subjected to phasor S-FLIM methodology to achieve rapid four-color FLIM, simultaneously with a single excitation wavelength [56]. The authors also integrated the multiplexed FLIM data acquired from spheroids labelled with JC-1 and Nile Red to spatially map a series of metabolic parameters of interest, such as lipid droplet concentration, the mitochondrial polarisation, the lipid order, and the hydrophobicity.

These examples demonstrate the wealth of information that can be acquired from FLIM in the study of tumour metabolism in 3D models. There are also caveats to organoid tumour models: a microenvironment which is rich in complexity and dynamic cell and ECM behaviour. One must consider the tumor cells, originally resident cells, the population of cells providing the supporting stroma of the tumor and the invading and newly resident tumor cells, which also include associated macrophages (TAM), tumor associated fibroblasts, T-cells, mast cells, natural killer (NK) dendritic cells and cells of myeloid origin [225, 226].

## 5. Outlook

Collectively, the field of organoid metabolism imaging using FLIM has shown a rapid development, benefitting from advances in the 3D cell culture models, bioengineering co-culture systems and bio-assembled structures, biomaterials science, mechanobiology and development of novel biosensor probes, addressing the mitochondrial function and cell physiology. Based on this, we suggest a roadmap in planning the next generation of experiments and outline exciting new research areas for the field.

**5.1 Next generation FLIM experiments roadmap.** Overview of proposed biosensor and autofluorescence-based approaches, together with already reported pioneering studies (sections 3 and 4), suggests a typical plan for experiments on the organoids using FLIM (Fig. 3).

[Insert Figure 3 here]

Prior to the beginning of an experiment, it is essential to understand the critical features of the selected organoid model, especially its **compatibility** with staining (if required), **imaging and microenvironmental setup** (static or fluidic flow, type of the microscope, e.g. compatibility with available objectives and working distance), and the **imaging / growth medium** composition. Even practical aspects such as phenol red presence (and obviation) in the growth medium to avoid absorbance, scattering and fluorescence quenching artifacts; timing of media replenishment/ replacement (which refreshes the potential availability of glucose, pyruvate and other factors) and maintenance of oxygen tension control [26, 227]. For instance, small intestinal organoids may not require two-photon excitation for increased depth penetration due to their 3D ‘monolayer’ topology, which is compatible even with a 405 nm pulsed diode laser excitation [108, 109]. However, their normal topology with basal membrane facing the outward space can pose limitations to a complete dye staining. More complex multiple cell layer and dense cultures of neural organoids would require either analysis in the form of live tissue slices, use of light sheet or two-photon microscopies and potentially complex imaging chamber designs. To analyse cell metabolism in organoids, the use of specific live cell tracers that are compatible with multiplexing, is recommended. These would include fluorescent biosensor ‘tags’ (e.g. fluorescent protein derivatives or ligand-binding Halo tag [119, 228-230]), specific functional dyes (e.g., tracers for mature neurons) or characteristic autofluorescence features. After successful staining or visualisation of the organoid model with specific metabolic biomarkers, e.g., NAD(P)H autofluorescence in combination with O<sub>2</sub> imaging, it is essential to perform control and calibration experiments, including inhibition and activation of electron transport chain experiments, measurement of lifetimes in response to the analyte (e.g. O<sub>2</sub>), and analysis of the instrument response function, essential for molecules with  $\tau < 1$  ns [231, 232]. More complex models, e.g., co-cultures of organoids with microbiota, may require additional ‘simplified’ calibration experiments, and separate analysis of their components. Importantly, analysis of cell responses and FLIM data may have to be performed in 3D, e.g. in case of mechanobiology and tension sensors or studies of mitochondrial structure and activity in multicellular aggregates, such as tumour spheroids or organoids.

## 5.2 Organoids for studies of immunometabolism

The topic of metabolism is timely and important given the burgeoning activity in the fields of immunometabolism, single-cell analysis and the need to capture information both spatially and temporally in models of development and disease. As mentioned above, to truly translate and accept organoids as reliable and translatable models to study biochemistry and disease we must capture as close as possible the big players in these tissues and those that we can engage with as clinical targets. Immunology is a vast and intricate field spanning from the innate to the adaptive extensions of this field and emerging heterogeneity in between. The subject area of immunology in organoid research is vast, and richly covered elsewhere [233], but somewhat overlooked is the appreciation of immunometabolism in organoid research. Immunometabolism is a field which has exploded in recent years and has demonstrated key and potent regulation of immune cell fate and behaviour across almost all inflammatory diseases [234]. As the organoid models can already recapitulate some aspects of immunometabolism and cell death [181], we will focus below on metabolism of macrophages, key players of the innate immunology.

Macrophage metabolism is closely linked to its phenotype and function [234-236]. This adaptation of macrophages is based on intrinsic or extrinsic stimuli such as physical and biochemical changes in tissue environment, including cytokines, growth factors, fatty acids, prostaglandins, and pathogen-derived molecules [237]. Long accepted paradigm postulated that macrophage polarisation exists at two extremes: M1 macrophages with a pro-inflammatory phenotype and M2 macrophages with an anti-inflammatory phenotype [238, 239]. However, human macrophage studies have demonstrated that these polarisation routes are not limited to two phenotypes [240, 241]: M1 macrophages are typically found in an inflammatory environment and are characterised by increased signalling of Toll-like receptors and interferon. They have high levels of phagocytic activity and the ability to induce an acute inflammatory response through the production of pro-inflammatory cytokines and chemokines, which in turn activate T-cell responses and phagocytosis [242, 243]. M1-like macrophages are also capable of antigen presentation and phagocytosis of microorganisms and matrix debris in the early stages healing. M2 macrophages are involved in the resolution phase of the inflammatory response and promote tissue repair through the release of molecules like endothelial growth

factor, TGF-beta, and fibroblast growth factor [244, 245]. M2-like macrophages require a sustained energy supply and thus utilise oxidative phosphorylation in order to achieve this. Interestingly, forcing glycolysis in M2 macrophages shifts them to a M1-like state, and forcing oxidative phosphorylation in a M1 macrophage shifts them to a M2-like state [246, 247]. The importance of cellular metabolism in both stem cell biology and immunology is clear, with metabolic shifts occurring to meet the energetic and biosynthetic demands of cell maturation, phenotype activation, and cellular function [234, 248]. There is an increasing interest in exploring cellular metabolism, with potential applications in biomedical and medical research [249, 250]. Moreover, metabolic engineering offers the possibility of tailoring cellular metabolism and function by means of metabolic chemical inhibitors or extracellular metabolites [251]. Macrophages have only very recently started to be incorporated (or at least reported) in organoid research, specifically in alveolar [252], enteroid [253, 254], and squamous cell carcinoma [255] models.

### 5.3 AI and ML-based approaches in organoids research

Machine learning (ML) is a field of computer science where computers learn without instruction, stemming from pattern recognition and computational learning theory in artificial intelligence [256]. Recent advances in computing and imaging systems have created a new research dimension with large datasets from single experiments, increasing the complexity of the data with higher numbers of conditions and dependent variables [257-259]. FLIM can benefit from ML with improved analysis and calculation of fluorescence lifetime (ideally addressing the photon budget issues), clustering and segmenting imaged species, help with tagging when combined with other analytical methods, or even to predict the data [25, 58, 84, 260, 261].

To address these challenges, precise machine learning-based data mining algorithms are necessary. Machine learning tasks are divided into two categories: unsupervised and supervised learning, where unsupervised tasks are related to data clustering, and supervised machine learning algorithms are used to predict a categorical outcome (classification) [259]. Examples of unsupervised machine learning algorithms are PCA and UMAP. PCA and UMAP analysis use the dependent variables of experimental samples to establish relationships and orthogonal

coordinates at lower dimension plots (2D or 3D). To further analyze FLIM data without losing sight of significant trends, such methods are used to reduce the data's dimensionality. This has been successfully implemented in life sciences and biomedical research where large datasets from genomics, transcriptomics and metabolomics are generated [262-264] and have moved into the pipeline of FLIM data analysis [97]. Supervised machine learning methods are used to predict or classify an outcome of interest. These methods are used in prediction tasks where the objective is to forecast or classify a specific outcome of interest [265]. As an example, supervised machine learning algorithms can be used to determine the activation state of T-cells [97], differentiation and progeny state of cells [266, 267], or the presence of a disease [268]. In considering multiparametric analysis and machine learning approaches, one must be acutely cognisant that while such tools are powerful in their classification capacity to uncover unique routes of interrogation; appreciation and delineation of cell type and heterogeneity must be captured, and in sufficient numbers of cells. Organoid approaches can realise this through high-throughput analysis, scaling up of organoid numbers and the availability of modes to segregate cell populations through morphology, genetically encoded markers, exogenous identifying compounds and specific surface marker utilisation [108, 119, 269].

While Machine Learning is AI that can automatically adapt with minimal human interference, a subset of machine learning is 'Deep learning' that uses artificial neural networks to mimic the learning process of the human brain. This too is firmly setting its position in the next generation of FLIM pipelines. Notable and very recent mentions include FLI-Net [84]; a deep learning neural network using deep learning, to quantify fluorescence decays simultaneously over a whole image and at fast speeds and flimGANE [270] to profile cells using deep learning and generate accurate and high-quality FLIM images even in the photon-starved conditions. Furthermore; another extension of this AI subset; convolutional neural networks (CNN) have demonstrated applicability using FLIM data and micrographs with accurate cell tracking and classification performed [261]; and have been able to predict FLIM images based on fluorescent data when trained using a small subset of FLIM images; with a high degree of accuracy. Such applications are rapidly gathering momentum and are at the cutting edge of the field [97, 271, 272].

Biomedical scientists will always employ correlative techniques with FLIM assessment (include that of organoids) and ensuring multimodal or at least spatial tracking between modalities is key to deriving clear correlations. Such correlative techniques are extensively reviewed elsewhere [273, 274], and Machine learning will undoubtedly play a significant role in the future studies. A recent example includes MOSAICA; a lifetime-based imaging platform for profiling mRNA based on fluorescent properties (notably the lifetimes) of mRNA probes with an automated machine learning-powered spectral and lifetime phasor segmentation software that has been developed to spatially reveal and visualize the presence, identity, expression level, location, distribution, and heterogeneity of each target mRNA in the 3D context [58]. The somewhat complex marriage between computer science, informatics and microscopy heralds a new era in biomedical imaging and also poises FLIM as a contender to lead this field in assessment of cell and tissue behaviour when reproduced in organoid cultures.

## 6. Concluding remarks

Without doubt, the work summarized and discussed in this expert review points toward FLIM being a key tool for the acquisition of metabolic information in organoid research. Its adoption by biomedical engineers and interdisciplinary reserachers has accelerated its usage and applicability over recent years. The recent expansion in organoid research, and the every growing number of different organoids being developed, provides novel opportunities to capture and dissect human physiology in an improved and non-invasive manner. We have discussed the modalities of FLIM imaging and also the tools at hand to non-invasively, and in a spatio-temporal manner extract a multitude of parameters to study the question at hand in 3D. Of course, it does not stop here. With the roadmap to the future experiments we can utilise the emerging technologies such as the correlative techniques in imaging, increasing complexity of the organoid systems; and managing the current, and envisaged complexity; using *state-of-the-art* artificial intelligence. Already, we are at the cusp of FLIM ‘breaking through’ to become a steadfast tool of all biomedical science laboratories. More affordable, accessible, and adaptable emerging platforms will facilitate this, and accelerate advancements in basic science, cancer and stem cell research, tissue repair and transplantation, biomaterials development, and indeed

organoid research in discovering life-changing therapeutics for our friends, colleagues and fellow citizens of the world.

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**Table 1.** Summary of the FLIM-based microscopy systems recommended for different sample types

| Sample classification (according to scattering)             | Type of imaging (excitation sources)                                | Type of lifetime measurement / emission decay measurement | Detector type                           | Spatial resolution | Acquisition speed                             | Straight-forward analysis of complex emission decays | References                                                |
|-------------------------------------------------------------|---------------------------------------------------------------------|-----------------------------------------------------------|-----------------------------------------|--------------------|-----------------------------------------------|------------------------------------------------------|-----------------------------------------------------------|
| Translucent samples (low scattering)                        | Light-sheet / Theta-microscopy (typically diode lasers)             | frequency-domain                                          | SPAD array / modulated camera detection | ++                 | +++                                           | --                                                   | [38, 41]                                                  |
|                                                             |                                                                     | Time-gating                                               | e.g. GOI / HRI camera                   | ++                 | ++                                            | +                                                    | [44-46]                                                   |
|                                                             |                                                                     | TCSPC                                                     | SPAD-based                              | ++                 | --<br>(SPAD or PMT arrays +)                  | +                                                    | [42, 43]                                                  |
| Densely packed organoids / small animals/ highly scattering | One or Two- / multi-photon microscopy (typically, fs-pulsed lasers) | TCSPC                                                     | PMT / SPAD                              | +                  | --<br>(Parallelized detection / multifocal +) | +                                                    | [108, 275, 276]                                           |
|                                                             |                                                                     | time-gating                                               | GOI/HRI camera                          | --                 | ++                                            | +                                                    | recently less used, see the recent comparison study [277] |
|                                                             |                                                                     | frequency-domain                                          | modulated camera detection              | +                  | +++                                           | --                                                   |                                                           |
| Small animals / highly scattering                           | Macroscopy (NIR (also fs-pulsed) laser)                             | time-gating                                               | SPAD-based                              | --                 | +++                                           | +                                                    | [27, 36]                                                  |
| Small animals / highly scattering                           | Mesoscopy (NIR (also fs-pulsed) laser)                              | time-gating                                               | SPAD-based                              | +                  | ++                                            | +                                                    | [278]                                                     |

**Table 2.** Some of the available FLIM methods to probe cell energy production pathways.

| Cell energy production pathway | Method                                  | Principle                                                                                                                   | Comments and requirements                                             |
|--------------------------------|-----------------------------------------|-----------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------|
| Glycolysis                     | Optical metabolic imaging: NAD(P)H-FLIM | “Label-free”. Phasor or fitting decay-based analysis of free and protein-bound forms of endogenous NADH and NADPH [71, 94]. | <b>Two-photon FLIM</b> microscope. Alternatively, genetically encoded |

|                                    |                                                                  |                                                                                                                                                                                                                                 |                                                                                                           |
|------------------------------------|------------------------------------------------------------------|---------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------------|-----------------------------------------------------------------------------------------------------------|
|                                    |                                                                  |                                                                                                                                                                                                                                 | NADH:NAD <sup>+</sup> peredox biosensor can be alternatively used [98].                                   |
|                                    | Analysis of extracellular pH                                     | Requires extracellular pH probes, such as based on fluorescent proteins ECFP, mCherryTYG or dyes and nanoparticles [123, 140].                                                                                                  | Transfection or staining optimisation may be required. Virtually <b>any FLIM</b> platform.                |
|                                    | Redox FLIM                                                       | Fluorescent protein-based probes (HyPer, roGFP1, Grx-roGFP2, OxyFRET, PerFRET or dyes such as Ebselen-ADOTA)[100, 131]. Optical redox ratio (ORR) can be also measured with label-free NAD(P)H-FLIM and FAD-FLIM combined [37]. | Transfection or staining optimisation may be required. Virtually <b>any FLIM</b> platform.                |
|                                    | Tryptophan autofluorescence FLIM                                 | <b>“Label-free”</b> . Trp can act as a FRET donor for NADH and to probe function of glycolytic enzymes.                                                                                                                         | <b>Two-photon FLIM</b> microscope [105].                                                                  |
|                                    | Genetically encoded biosensors for glucose and other metabolites | Biosensor probes for glucose, pyruvate, lactate and other metabolites [99, 149].                                                                                                                                                | Transfection optimisation may be required. Virtually <b>any FLIM</b> platform.                            |
|                                    |                                                                  |                                                                                                                                                                                                                                 |                                                                                                           |
| Oxidative Phosphorylation (OxPhos) | O <sub>2</sub> -PLIM                                             | Probes, dyes and nanosensors based on <b>quenched phosphorescence</b> phenomenon. Absolute and real-time quantification of molecular oxygen (O <sub>2</sub> ) [33, 34].                                                         | Optimisation of staining may be required [127]. One- and two-photon <b>PLIM microscopes</b> are required. |
|                                    | Mitochondrial membrane potential                                 | Dye accumulation-based changes in fluorescence lifetime. Observed with TMRM and some other dyes [108, 135, 137].                                                                                                                | Has been demonstrated on small intestinal organoids but can display                                       |

|  |                                         |                                                                                                                                                       |                                                                                                                                    |
|--|-----------------------------------------|-------------------------------------------------------------------------------------------------------------------------------------------------------|------------------------------------------------------------------------------------------------------------------------------------|
|  |                                         |                                                                                                                                                       | concentration-dependent effects with spheroids [108].<br>Virtually <b>any FLIM</b> platform.                                       |
|  | Optical metabolic imaging: NAD(P)H-FLIM | See above in ‘Glycolysis’. Protein-bound form lifetimes of NADH and NAD(P)H can be used for deeper analysis of OxPhos activity [66, 72].              | Can be influenced by the growth (and measurement) medium composition [119].<br><b>Two-photon FLIM</b> microscope.                  |
|  | Optical metabolic imaging: FAD-FLIM     | <b>“Label-free”</b> . Phasor or fitting decay-based analysis of free and protein-bound forms of endogenously produced FAD (indicator of OxPhos) [37]. | Strength of the signal is cell-specific.<br>Virtually <b>any FLIM</b> platform.                                                    |
|  | Intracellular temperature               | Mitochondrial heat production probes and nanosensors [157, 163].                                                                                      | So far, reported only with fat and cancer cells [160, 161, 164, 279]. <b>One- and two-photon FLIM</b> and <b>PLIM</b> microscopes. |

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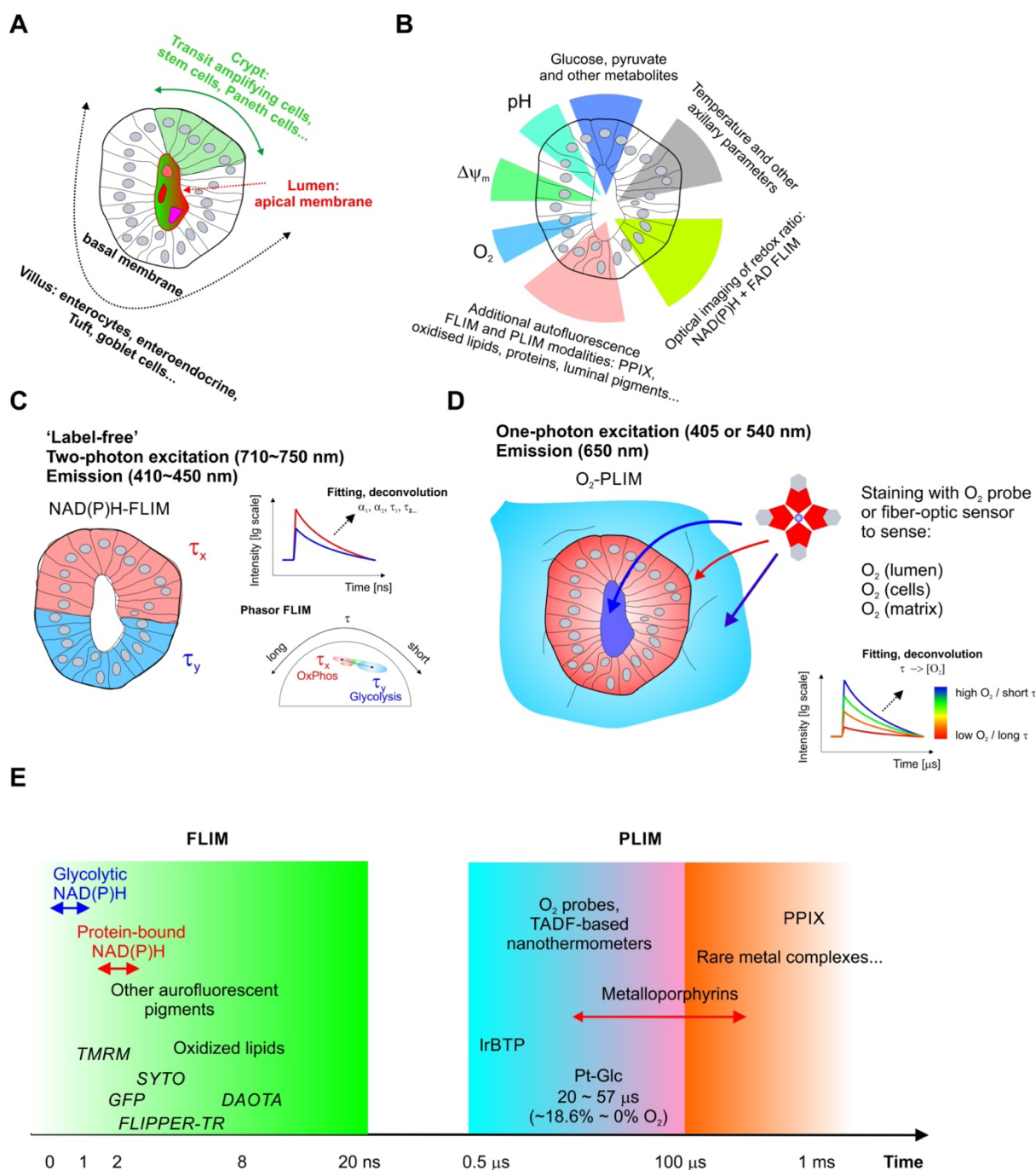
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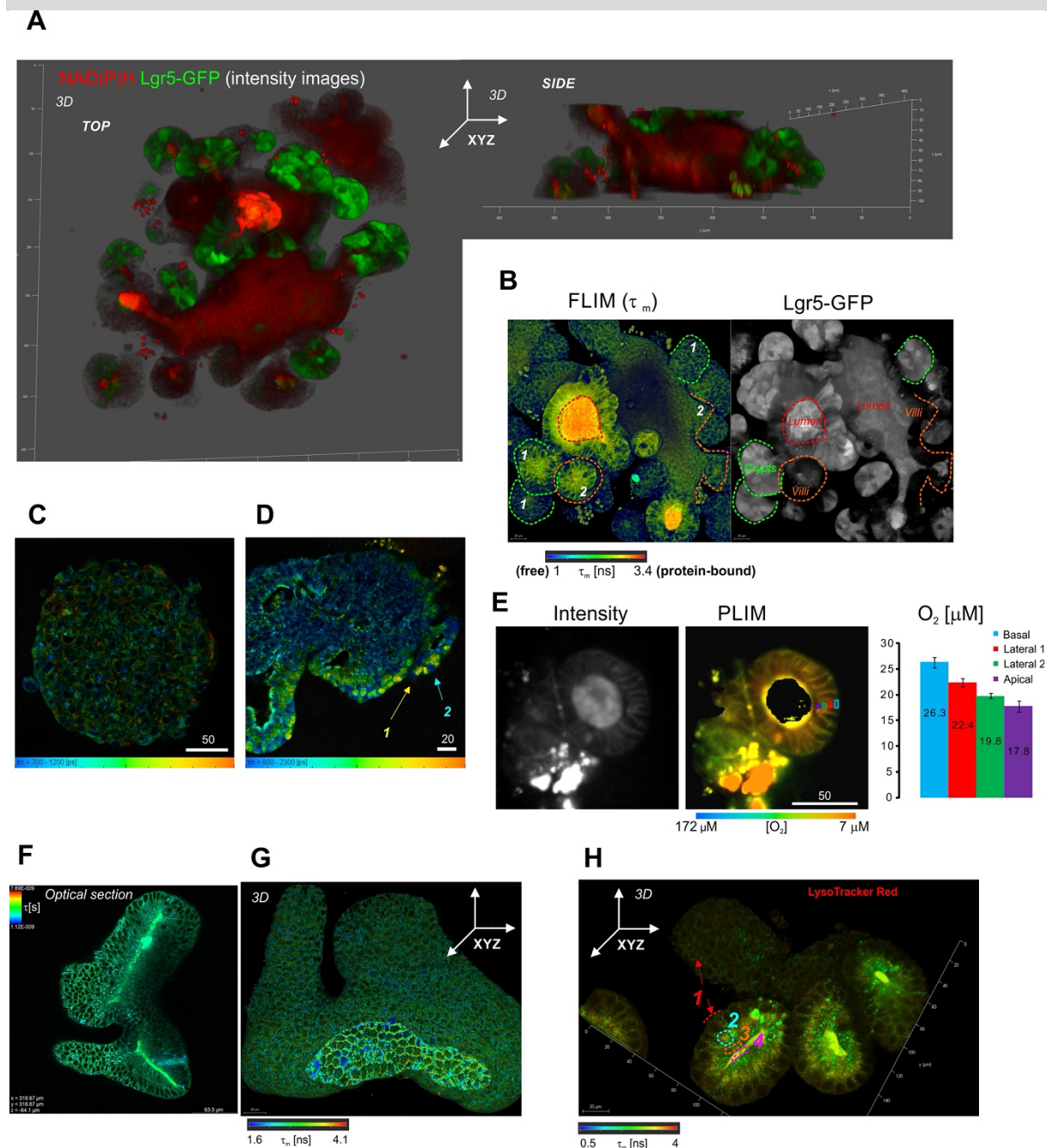
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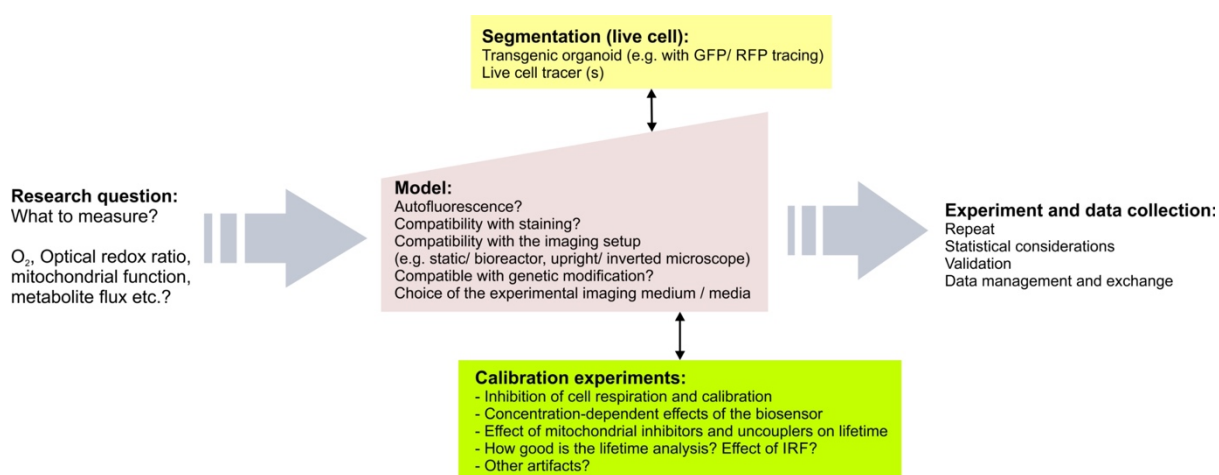
**Figure 1. Schematics of FLIM of organoids metabolism on example of small intestinal organoids, probed with NAD(P)H-FLIM and  $O_2$ -PLIM assays. A:** representation of a differentiating small intestinal organoid, with lumen, crypt and villus compartments. Lumen may contain products of cell metabolism, dying and dead cells and injected microbiota. **B:** overview of FLIM methods applicable to

studies of the organoids. **C:** Principle of the NAD(P)H-FLIM of intestinal organoids. The lifetime ( $\tau$ ) for 2 different regions (blue and red, displaying  $\tau_x$  and  $\tau_y$ ) can be calculated by different ways such as double-exponential fitting of the decay curves, yielding amplitudes  $\alpha_{1,2}$  and lifetime  $\tau_{1,2}$  or phasor FLIM, where the ‘absolute’ lifetime is to be found within the universal circle (multi-exponential) or at its edge (mono-exponential decay). Typically, longer lifetimes indicate protein-bound state of NAD(P)H lifetime and higher activity of OxPhos or other enzymes. A number of different intermediate lifetimes and ‘metabolic trajectories’ can be also observed within the phasor space. **D:** Principle of measurement of molecular oxygen by PLIM method.  $O_2$  probes can stain or be targeted to different compartments of the organoid, including extracellular matrix (e.g. Matrigel), solid supporting substrate, live cells or the lumen. Mono-exponential fitting is normally applied to see the effect of  $O_2$  quenching on the phosphorescence lifetime, calibration and subsequent direct quantification of real-time oxygenation. A number of different probes can be used simultaneously, and methods C and D can be used together. **E:** Relative timescale showing luminescence lifetimes and ‘time domains’ of commonly used biosensors.



**Figure 2. Typical examples of FLIM and PLIM microscopy images, produced with live organoid models. A-B:** two-photon excited autofluorescence FLIM of live mouse intestinal Lgr5-GFP organoid. **A:** 3D general appearance of organoid (414x414x105  $\mu m$  image size), shown as fluorescence intensity of NAD(P)H (720 nm exc., red) and GFP (920 nm exc., green). **B:** Mean (intensity-weighted, double exponential fitting)  $\tau_m$  (FLIM) and intensity of Lgr5-GFP (gray, also shows autofluorescence of the lumen) helps revealing differences in cell metabolism within crypts (1) and villi (2) regions. **C:** Example

of cell metabolic heterogeneity (NAD(P)H-FLIM,  $\tau_m$ , excited at 760 nm) in live multicellular spheroid, produced from colon cancer HCT116 cells. **D:** Combined NAD(P)H-FLIM (exc. 760 nm) with labelling of cell proliferation by Hoechst 33342 and BrdU quenching FLIM in mouse lung organoids. Yellow (1) nuclei indicate decreased proliferation. Blue (2) nuclei indicate proliferation. **E:** Confocal O<sub>2</sub>-PLIM of primary mouse intestinal organoid, grown at 5% O<sub>2</sub>, pre-treated with sodium butyrate and stained with Pt-Glc O<sub>2</sub> probe (exc. 405 nm, emission 650 nm). Intensity (gray), color-coded PLIM and measured O<sub>2</sub> gradient across the epithelial layer. F-G: Combined FLIM of mitochondrial polarisation (TMRM) and membrane tension (Flipper-TR) in porcine intestinal organoids. **F:** ‘TauContrast’ FLIM preview image of one optical section. **G:** live 3D FLIM of co-stained organoid (318 x 318 x 74  $\mu$ m image size, ‘fast FLIM’). Both TMRM (1 nM, 24 h) and FLIPPER-TR (2  $\mu$ M, 24 h incubation) probes were excited with 490 nm (white light laser, 7.6%), with emission collected at 503-701 nm. **H:** Live 3D ‘fast FLIM’ of porcine intestinal organoid, stained with LysoTracker Red (1  $\mu$ M, 2 h), visualising differences in lysosomal pH. Image size 204x204x42  $\mu$ m. Excitation at 572 nm (0.7% white light laser power), emission 585-750 nm. Numbers 1-4 indicate difference in lifetimes observed for lysosomes across basal to apical membranes and the lumen. Images A-B were collected using Dive Falcon SP8 (Leica), C-D using LSM780 (Zeiss, Becker& Hickl), E using Axio Examiner-DCS-120 (Becker& Hickl, Zeiss) and F-H using Stellaris 8 Falcon (Leica) microscopes. All scale bars are in  $\mu$ m.



**Figure 3.** Suggested workflow of the experiment on FLIM of organoids metabolism.