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# Chapter 3

# Design of optical instruments to image subcellular, cellular, tissue and organ structures

**Marcelo Saito Nogueira**

**This chapter is part of the thesis "Optical spectroscopy for biological and biomedical applications: potentially impacting future of healthcare with research, clinical translation and education" by Marcelo Saito Nogueira**

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# 3. Design of optical instruments to image subcellular, cellular, tissue and organ structures

Chapter 3 shows an overview on the main categories of optical/photonic techniques for biomedical applications and provides a comprehensive guide on the selection of these categories by matching them with application requirements. This overview has the aim of clarifying the advantages of optical technologies and how they can be used for biophotonics applications. By having the big picture of optical technologies available and their corresponding advantages and disadvantages, this chapter introduces the rationale for the optical technologies used in this thesis. Details on the use of such technologies and how to extract tissue microstructure and biochemical information from them are introduced in the subsequent chapters.

Optical/biophotonic technologies have provided solutions to global challenges in energy, education, agriculture, and healthcare. In particular, optical technologies have the largest share on the medical imaging market, surpassing the radiological imaging sector with a 64% of the total medical imaging marketing (i.e., 2015-2017 estimated valuation of $72 billion per year). The market valuation does not include the home healthcare (mobile health market valuation of $13.2 billion in 2016) and wearable devices market (valuation of $5.5 billion in 2016) [1]. Part of the commercial success of optical technologies is due to its cost effectiveness. In terms of small-animal molecular imaging systems, optical systems offer the lowest cost-per-animal by far (US$2- US$13) compared to X-ray/CT, positron emission tomography and MRI (US$100-US$400) [2]. A similar scale of cost difference is expected upon translation to human healthcare systems.

Numerous methods of disease detection and monitoring rely on observing structural changes of biological tissues by using radiological imaging. Biochemical features have been conventionally evidenced by expensive exams based on magnetic resonance imaging (MRI) and/or by using contrast agents for specific tissue biomarkers. With the advance of optical diagnostics, the use of contrast agents has enabled real-time tissue identification for surgical guidance and clinical diagnostics. However, early-stage disease detection still remains a challenge. In order to overcome this challenge, current advances in medical devices have focused on label-free (i.e., no use of contrast agents) optical technologies capable of visualizing metabolic and immunologic features in the micromolar to nanomolar ranges [2]. Optical technologies include optical spectroscopy and imaging, which are used for non-invasive interrogation of biological tissues and high-throughput analysis of biofluids. The collected data can be used to directly identify tissue characteristics through multivariate analysis or to extract the tissue molecular fingerprint by referring to well-established biomolecular features described in the literature. These features are absorption and scattering variations for reflectance and transmission spectroscopy [3], emission peaks for fluorescence [4,5], Fourier-transform infrared spectroscopy (FTIR) [6], and Raman spectroscopy [7,8], decay times for fluorescence lifetime measurements [4,5,9] and calibrated intensities for imaging systems.

The success of medical optical techniques is not only restricted to only commercial applications, but both basic and applied research in biophotonics and biomedical optics, including the generation of technologies which advance other research fields and resulting in a number of Nobel prizes in the recent years. In fact, optical techniques have a number of advantages compared to conventional methods in medical applications including:

- optical photons consist of non-ionizing radiation and are safe as long as light is not directly shone in the eyes or lead to heating effects on operators or treated patients
- optical techniques are molecular sensitive (tissue biochemistry related to absorption, fluorescence, and Raman scattering)
- molecular sensitivity ranges from picomolar to millimolar ranges, which allows optical techniques in the micromolar and nanomolar ranges to detect diseases earlier (through alterations in metabolic rates, oxygenation, blood content during angiogenesis, lipid droplets in cancer cells, etc) than conventional medical methods based primarily on changes on tissue microstructure.

- microstructural information (associated with light scattering properties) can be obtained with respect to size cell nuclei, mitochondria, lysosomes, vesicles, striations in collagen fibrils, macromolecular aggregates, membranes, etc
- information on tissue asymmetric structures (including collagen and muscle fibers/fibrils) and or cell flow can be obtained via anisotropy measurements based on optical polarization.
- blood flow can be obtained through optical frequency shifts due to the optical Doppler effect
- possibility of using contrast agents (e.g., exogenous fluorophores or nanoparticles) for high sensitive diagnosis and treatment
- upon increased sensitivity with contrast agents, contrast for molecular imaging of gene activities is feasible
- simultaneous detection of multiple contrast agents (easy to multiplex signals).
- high-resolution imaging of the retina is possible due to the optical transparency in the eye
- possible to make miniaturized devices to be integrated into medical instruments
- cost-effective technologies
- real-time data analysis
- Non-invasive or minimally-invasive *in vivo* tissue interrogation for diagnosis or disease monitoring
- green technology when made label-free (no contrast agents needed)
- possible to do diagnosis and treatment with the same equipment and possibly under the same clinical procedure (repeating the procedure fewer times, minimizing patient discomfort and side effects, potentially increasing patient compliance)
- laser surgeries with dry and clear field of surgery, continuous sterilization (no contact with mechanical instruments), higher precision, less postoperative pain (sealing of nerve ends), possibility to perform surgery within the body without openings and microsurgery under microscopes.
- better cosmetic effect of optical treatments such as photodynamic therapy (drug accumulates in the areas of diseased tissue and cell death occurs only on these areas upon illumination, which can be controlled in real-time)
- possibility of combination with other optical and non-optical techniques (Table 3.1)

**Table 3.1: Comparison between optical and non-optical methods, according to Wang and Wu** [10]

| General features | Optical Imaging | X-ray Imaging | Ultrasonography | MRI |
|---|---|---|---|---|
| Soft-tissue contrast | Excellent | Poor | Good | Excellent |
| Spatial resolution | Mixed | Excellent | Good | Good |
| Maximum imaging depth | Good | Excellent | Good | Excellent |
| Function | Excellent | None | Good | Excellent |
| Nonionizing radiation | Yes | No | Yes | Yes |
| Data acquisition | Fast | Fast | Fast | Slow |
| Cost | Low | Low | Low | High |

Optical techniques can be divided into many categories (Tables 3.2 and 3.3 (continuation of table 3.2)). The choice of optical techniques can be made by selecting categories of Tables 3.2 and 3.3 according to the flowchart of figure 3.1. This selection was performed for optical techniques used in this thesis.

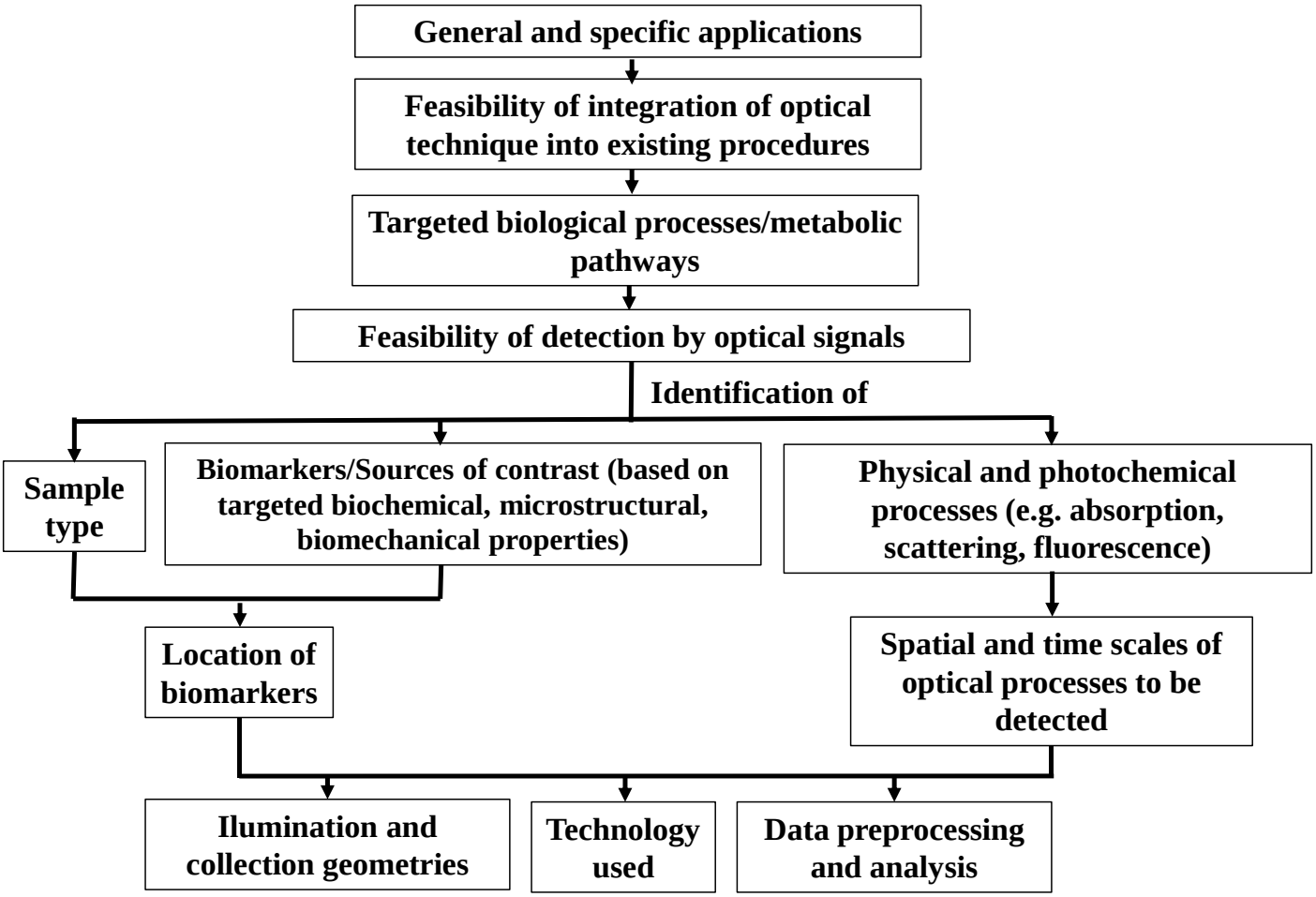



**Figure 3.1: Steps of selecting categories for choice of optical techniques to be used for specific applications.**

**Table 3.2: Categories of optical techniques for biological tissue applications. Instrumentation, methods, applications involve combinations of the categories described in each column. Continuation of columns on the right side is given on the next pages.**

| General Application | Clinical procedures | Specific application | Information collection | Non-optical associated methods | Type of sample | Location of biomarkers | Targeted biomarkers (organic, metallic, organometallic, diverse geometries) | Physical/photochemical processes (detection/diagnosis) | Physical/photochemical processes (treatment) |
|---|---|---|---|---|---|---|---|---|---|
| • Understanding origin and progression of diseases | • Physical exams (skin, oral cavity, eyes, nails, ears, nose, throat) | • Disease risk estratification (prediction of cumulative risk of death and biopsy guidance) | • Point | • Ultrasound (e.g. photoacustics, acousto-optical tomography) | • Biofluid | • Biofluid | • Chromophores (includes IR active molecules) | • Absorption + light emission | • Heat generation |
| • Screening | • Oftalmology exams | • Tissue identification | • Single-pixel imaging | • X-ray (Cherenkov imaging/spectroscopyor x-ray scintillation) | • Cell culture | • Endogenous (tissue) | • Fluorophores | • Absorption + sound (pressure wave) emission | • Generation of reactive oxygen species |
| • Diagnostic | • Endoscopy procedures (Anoscopy, Arthroscopy, Bronchoscopy, Colonoscopy, Colposcopy, Cystoscopy, Esophagoscopy, Gastroscopy, Laparoscopy, Laryngoscopy, Neuroendoscopy, Proctoscopy, Sigmoidoscopy, Thoracoscopy) | • Delineation of tumor margins | • Scanning imaging | • Microfluidics | • 3D cell culture | • Exogenous (tissue) - contrast agents | • Raman-active molecules (change in polarizability ≠ 0) | • Mie scattering | • Generation of singlet oxygen |
| • Disease monitoring | • Surgery (multiple organs) | • Surgical guidance | • "Simultaneous-collection" imaging | • Lab-on-a-chip technology | • Tissue | • | • Tagged-antibodies (i.e. labeled antibodies) | • Rayleigh scattering | • Photobiomodulation |
| • Palliative care | • Sentinel lymph node removal | • Tumor staging | • Scanning tomography | | | | • Nanoparticles | • Fluorescence | |
| • Treatment | • Skin resurfacing | • Metastasis detection | • Light-sheet tomography | | | | • Quantum dots | • Phosphorescence | |
| • Antimicrobial disinfection | • Psoriasis treatment | • Prediction of treatment outcome | • Holography | | | | • Photosensitizers | • Upconversion | |
| • Photobiomodulation | • Acne treatment | • Light dosimetry | | | | | | • Multiphoton excitation/emission | |
| • Assessment of organ/tissue viability and organ function | • Stain/tattoo removal | • Drug dosimetry | | | | | | • Raman scattering | |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| | • Laser prostatectomy<br>• Laser surgery (e.g. eye surgery)<br>• Laser ablation<br>• Laser lithotripsy | • Seal or treat blood vessels (e.g. vitrectomy, treatment of varicose veins, and dry field of surgery on laser surgeries)<br>• Sealing nerve ends (laser surgery)<br>• Precise cutting andsterilization (laser surgery)<br>• Monitoring athletic performance<br>• Improvement athletic performance<br>• Photobiomodulation (dermatology and aesthetics, e.g. skin antiageing and hair growth)<br>• Photobiomodulation (pain relief, disease management and healing)<br>• Photodynamic therapy<br>• Photothermal therapy<br>• Optical micromanipulation with optical tweezers (rotors, particle binding, cell sorting, levitation, optical traps, determination of visco-elastic properties and cell motility)<br>• Elimination of vectors of infectious diseases (e.g. dengue, zika, chikungunya)<br>• Optogenetics | | | | | | • Doppler effect (present in e.g. laser flowmetry, acousto-optical tomography)<br>• Light polarization and anisotropy | |

**Table 3.3: Categories of optical techniques for biological tissue applications. Instrumentation, methods, applications involve combinations of the categories described in each column (right-side columns continued)**

| Measurement spatial scale | Measurement time scale | Illumination geometry | Light collection geometry | Measurement geometry | Technology used | Data analysis (Modeling of light/sound propagation and machine learning) |
|---|---|---|---|---|---|---|
| • Nanometers-micrometers (e.g., optical micromanipulation)<br>• Micrometers (microscopy)<br>• Milimeters-centimeters (macroscale) | • Picoseconds-nanoseconds (e.g. time-of-flight spectroscopy, time-resolved fluorescence and Raman spectroscopies)<br>• Miliseconds-seconds (e.g. continuous wave modalities and photobleaching evaluation)<br>• Miliseconds-minutes (phosphoresnce decay evaluation) | • Point (single-pixel)<br>• Confocal (microscopy)<br>• Ring area illumination<br>• Defocusing (overlap between illumination and collection areas)<br>• Spatial sinusoidal pattern<br>• Spatial ring pattern<br>• Spatial binary pattern<br>• Spatial triangular pattern<br>• Spatial trapezoidal pattern<br>• Spatial Moiré pattern<br>• Stripe boundary code pattern<br>• N-ary code pattern<br>• Hadamard pattern illumination<br>• Light sheet illumination/scanning<br>• Fiber beam shapes (e.g., Bessel, Laguerre-Gaussian, and circular Airy beams) | • Point (single-pixel)<br>• Confocal (microscopy)<br>• Ring area collection<br>• Defocusing (overlap between illumination and collection cones) | • Contact reflectance<br>• Non-contact reflectance<br>• Contact transmittance<br>• Non-contact transmittance<br>• Interstitial transmittance | • Continuous-wave methods<br>• Spatially-resolved methods (a.k.a differential pathlength or spatial frequency domain techniques)<br>• Time-resolved methods<br>• Wavelength-resolved methods<br>• Frequency-domain methods (based on wave modulation and phase shift)<br>• Polarization methods<br>• Interferometry<br>• Fourier-transform-based methods<br>• Computational imaging (for lens-free cameras and lens-free microscopes)<br>• Fiber optic imaging<br>• Transmission-matrix-associated illumination/collection<br>• Laser speckle imaging | • Analytical or semi-analytical models using electromagnetic theory, (radiative or sound) transport theory, adding-Doubling method or diffusion theory<br>• Probabilistic methods (e.g. Monte Carlo)<br>• Tissue classification (e.g. supervised machine learning methods or diagnostic indexes)<br>• Quantitative analysis (e.g. regression and correlation methods)<br>• Exploratory analysis (unsupervised machine learning methods such as data clustering, data interpretation through loadings of principal component analysis or partial least-squares)<br>• Reinforcement machine learning methods<br>• Convolutional neural networks (image reconstruction, light pattern projection, selection of fiber mode propagation, light focusing from fibers)<br>• Deep learning<br>• Feature extraction (principal component analysis (PCA), partial least-squares,<br>• Data sampling (e.g., Competitive Adaptive Reweighted Sampling Method, Kennard-Stone Sampling, random sampling)<br>• Mathematical transforms/expansion series/wavelet analysis (e.g., Fourier, Laguerre, Laplace, etc)<br>• Signal preprocessing (Background removal, smoothing, filtering, convolution, deconvolution)<br>• Normalization techniques (e.g., normalization by intensity, area under the curve, rescale of each variable between -1 and +1) |

## 3.1 Biomedical optics at micro- and macro-scales

Optical techniques can be largely divided into those applied to the scale where changes on biological processes occur. With the development of biophotonics over decades, advances on optical technologies have enabled the investigation and manipulation of biological systems at subcellular, cellular, tissue, and organ levels. Biological tissues can be studied from:

- nano-to-microscales (1-1000 nm): focused on (1) finding biomarkers over several disease stages, (2) potential identification of those specific early-stage, (3) identification of single molecular events which are the sentinel origin of diseases, (4) understanding biological processes via co-localization of biomarkers or signal co-registration from different probes and/or optical processes.
- meso-to-macroscales (100 µm - 20 cm): focused on (1) understanding origin and progression of diseases, (2) development of screening tests, (3) disease diagnosis, monitoring and treatment, (4) surgical guidance, (5) antimicrobial disinfection, (6) photobiomodulation, (7) optogenetics, (8) assessment of organ/tissue viability and organ function.

In this thesis, most of the applications explored were looking for biological systems at meso-to-macroscales. Also, optical systems have been built for future studies looking for applications in nano-to-microscales (especially in Raman microscopy discussed in chapter 18 section 18.1.2).

### 3.1.1 Microscopy and micro-spectroscopy techniques

Most microscale optical techniques intrinsically need some form of imaging in order to guide the signal collection from the desired location or to manipulate particles within subcellular structures, cells and tissues. Then, most of these techniques are essentially imaging techniques themselves or are coupled to imaging techniques to work. Recent advances in super-resolution microscopy and micro-spectroscopy enabled imaging below the diffraction limit of light, which allowed the study of a range of biological processes occurring in normal and pathological cells/tissues at single-molecule level (e.g., lipid peroxidation of myelin membranes culminating in neurodegenerative disorders, genetic mutations leading to cancer). In fact, microscopy and micro-spectroscopy techniques have largely progressed with the development of light sources, detectors, high-speed electronics and the evolution of data processing and storage capabilities. Moreover, these techniques have been used in a number of applications of newly created and emerging research fields such as discovery chemistry and synthetic biology [11], while still incorporating data science and machine learning methods for further development.

Selecting between microscopy/micro-spectroscopy and macroscopic techniques to design new optical systems require taking into account the opportunities and limitations provided by techniques of each category. This includes understanding the opportunities and limitations of state-of-the-art technologies available in the market, along with their aims, challenges and associated technology needs, and potential solutions. Optical techniques used and system developed in this thesis took such aspects into consideration. A summary of the aims, advances and opportunities, challenges, needs, and potential solutions of microscale optical techniques can be found in Table 3.4:

**Table 3.4: Aims, advances and opportunities, challenges, needs, and potential solutions of microscale optical techniques.**

| Aims | Advances and opportunities | Challenges | Need | Potential solutions |
|---|---|---|---|---|
| • Finding biomarkers over several disease stages | • Visualization through super-resolution imaging | • Low specificity to detection of single molecular events which originate diseases | • Increase sensitivity and specificity | • Improved light excitation sources and techniques (e.g. multiphoton excitation) |
| • Potential identification of those specific early-stage biomarkers | • Reproducibility and repeatability: optimized and well-defined sample handling | • Dynamic counter-regulatory mechanisms: Biochemical reactions occur in parallel and molecular events/metabolic pathways are interlinked | • Improve signal-to-noise ratio | • Brighter probes (e.g. luminescent, phosphorescent, and fluorescent proteins, enzymes, and chemical probes) |
| • Identification of single molecular events which are the sentinel origin of diseases | • Availability to powerful data processing | • Technical and computational costs | • Enable data collection without computational memory overflow | • Sensitive detectors |
| • Understand biological processes via co-localization of biomarkers or signal co-registration from different probes and/or optical processes | • Time-resolved techniques in picosecond, nanosecond and microsecond scales | • Lack of ground-truth for novel medical light-based technologies: Optical/photonic technologies progress faster than the standardization of measurements between research centers, hindering the reproducibility of results among these centers | • Distinguish and multiplex overlapping time- and wavelength-dependent signals captured with each particular optical technique | • Data processing to extract the desired signal from imbued in the biological background |
| | • Enhanced contrast: combination of wavelength- and time-resolved spectroscopy and imaging | • Lack of multicenter validation: machine learning algorithms are specific to the research setting of a particular study and do not work with datasets of other studies | • Optical measurement standards | • Instrument adaptation for enhanced light collection and sample contrast |
| | | • Lack of determination of citotoxicity, dosimetry, pharmacokinetics, and pharmacodynamics (especially in terms of long-term effects) of contrast agents, probes, and other chemicals used | • Multicenter studies | • Application of exogenous biomarkers (especially those with signals which are reduced or not present in biological systems e.g. photonic crystals) |
| | | • Complexity of signal on novel advanced techniques may require time-consuming training for system operation and data interpretation | • Display of user-friendly and reduced volume of information to end-user system operators (e.g. clinicians) | • More computing power and data storage |
| | | | | • Simultaneous measurement of wavelength- and time-dependent cell/tissue optical properties |

## 3. 1.2 Macroscopic optical techniques

Macroscopic optical techniques are applied to analysis of biofluids, tissues and organs of small (e.g., Drosophila species, C. elegans, zebrafish) and medium/large animals (rodents, pigs, dogs, horses, etc), as well as human subjects [11]. Tests on small animals are typically performed for proof-of-principle of novel techniques providing enough resolution to image subcellular and cellular at small tissue depths, whereas those performed in medium/large animals and human subjects tend to focus on translational research concerning either proof-of-principle under controlled conditions (animal models), or proof-of-principle and increase the technique maturity for certain applications in terms of technology readiness levels (TRL) over multiple clinical trials and multicenter studies. The aims of such studies include the (1) understanding origin and progression of diseases, (2) development of screening tests, (3) disease diagnosis, (4) monitoring and treatment, (5) surgical guidance, (6) antimicrobial disinfection, (7) photobiomodulation, (8) optogenetics, (9) assessment of organ/tissue viability and organ function. The advantages of optical techniques including:

- Sensitivity to a range of biomolecules
- Possible to miniaturize and integrate into medical tools
- Cost-effectiveness
- Real-time data collection and display
- In vivo interrogation
- Label-free technology (non-invasive and green technology)

make these techniques attractive for medical applications such as those explored in the colorectal cancer studies and studies to monitor organ function/viability for transplantation covered in this thesis. A summary of the aims, advances and opportunities, challenges, needs, and potential solutions of macroscopic optical techniques can be found in Table 3.5:

.

**Table 3.5: Aims/applications, advances and opportunities, challenges, needs, and potential solutions of macroscale optical techniques.**

| Aims/applications | Advances and opportunities | Challenges | Need | Potential solutions |
|---|---|---|---|---|
| • Understanding origin and progression of diseases | • Availability to powerful data processing | • Dynamic counter-regulatory mechanisms: Biochemical reactions occur in parallel and molecular events/metabolic pathways are interlinked<br>• Trade-off between optical imaging depth and spatial resolution due to light scattering (e.g. obtaining high-resolution images of cellular and subcellular is restricted to a few hundreds of microns from the tissue surface, whereas diffuse optical tomography measurements can be performed a few centimeters deep into tissue at low-resolutions - resolution dependent on the optical properties of the medium). | • Increase sensitivity and specificity | • Improved light excitation sources and techniques (e.g. multiphoton excitation) |
| • Development of screening tests | • Time-resolved techniques in picosecond, nanosecond and microsecond scales | | • Improve signal-to-noise ratio<br>• Combination of fast scanning of large tissue areas/volumes and computational processing and image reconstruction for meso-to-macroscopic images | • Brighter probes (e.g. luminescent, phosphorescent, and fluorescent proteins, enzymes, and chemical probes) |
| • Disease diagnosis, monitoring and treatment | • Enhanced contrast: combination of wavelength- and time-resolved spectroscopy and imaging | • Connecting images and data across large spatial scales, from sub milimeter to tens of centimeters | | • More sensitive and faster detectors |
| • Surgical guidance | • Novel optical designs for improved image resolution | • Optical dispersion and continual motion and bending of optical fibers hinder endoscopic high-resolution nonlinear imaging (degradation of femtosecond laser pulses for nonlinear microscopy and nanoscopy). | • Enable data collection without computational memory overflow<br>• Display of information useful for real-time decision making, especially in clinical settings (e.g. disease diagnosis and treatment planning) and discovery of fundamental biological processes | • Extraction of selected information on images/data containing the most important content for the targeted application |
| • Antimicrobial disinfection | • Improved image processing algorithms for coherence-based optical methods | • Data volume, analysis, and management in micro/nanoscopy. | | • Data processing to extract the desired signal from imbued in the biological background |
| • Photobiomodulation | • Rapid advance of imaging speed and faster detectors, especially with swept-source OCT (SS-OCT) systems (MHz axial scan rates) | • Technical and computational costs of advanced optical methods<br>• Lack of ground-truth for novel medical light-based technologies: Optical/photonic technologies progress faster than the standardization of measurements between research centers, hindering the reproducibility of results among these centers | • Distinguish and multiplex overlapping time- and wavelength-dependent signals captured with each particular optical technique | • Instrument adaptation for enhanced light collection and sample contrast |
| • Optogenetics | • Expansion of computational imaging with progress on processing and display hardware, high-performance computing, and parallel computing. | | • Optical measurement standards | • More computing power and data storage |

| | | | | |
|---|---|---|---|---|
| • Assessment of organ/tissue viability and organ function | • Higher sensitivity of detectors (enhanced signal) and detection systems (improved signal-to-noise ratio)<br>• Extraction of multiple tissue and cellular properties (e.g. biomechanical, biochemical, microstructural) with multimodal optical imaging combining information of diverse physical processes (e.g. Linear and non-linear optics, photoacoustics, elastography).<br>• Probing diverse tissue depths with geometry of illumination and collection (e.g. reflectance, transmittance, reemittance), use of non-linear optical phenomena (multiphoton excitation and upconverting nanoparticles), and configurations of beam delivery systems<br>• Simultaneous diagnostic and treatment upon use of targeted and activatable molecular probes (e.g. photosensitizers, nanoparticles, antibody-conjugated probes)<br>• Simultaneous collection and coregistration of images from multiple contrast mechanisms over space and time.<br>• Machine learning techniques for extraction of both spectroscopy and imaging features<br>• Non-invasive imaging methods feasible in most parts of the body except internal organs, whereas minimally invasive tools can be used during surgery and endoscopy<br>• Advancement of highly multiplexed detection/imaging with integration of nanotechnologies in surface-enhanced spectroscopies (e.g. surface-enhanced Raman scattering (SERS))<br>• Combined optical and radiological imaging (e.g., positron emission tomography and MRI) is now also becoming possible through the use of multimodal nanoparticles.<br>• Combination of wide applications in the same procedure (e.g. optogenetics and theranostics) | • Lack of multicenter validation: machine learning algorithms are specific to the research setting of a particular study and do not work with datasets of other studies<br>• Lack of determination of citotoxicity, dosimetry, pharmacokinetics, and pharmacodynamics (especially in terms of long-term effects) of contrast agents, probes, and other chemicals used<br>• Complexity of signal on novel advanced techniques may require time-consuming training for system operation and data interpretation | • Multicenter studies<br>• Display of user-friendly and reduced volume of information to end-user system operators (e.g. clinicians) | • Simultaneous measurement of wavelength- and time-dependent tissue optical properties |

## 3.2 Point spectroscopy versus imaging methods

Essentially, all optical techniques are limited by the (1) efficiency to generate the desired photons, (2) efficiency of collection of desired photons, (3) photon detection at signal-to-noise ratio levels sufficient for finding contrast between the desired samples. (1) and (2) can be obtained with brighter light sources, optimized configuration of beam delivery systems and more sensitive detectors (i.e., detectors able to detect more photons) to achieve sufficient signal-to-noise ratio for detection of sample molecular characteristics (e.g., absorption) in the captured optical signals. (3) is highly dependent on the detector sensitivity and how the detected photons are distributed over space, time, wavelength, polarization, and other photon characteristics which allow the determination of targeted cell/tissue properties. For instance, wavelength-dependent spectroscopy enables the determination of sample absorption, scattering and luminescence properties and is highly explored as part of this thesis.

Imaging methods either scan signals at each position (scanning beams) or plane (light-sheet microscopy) of the sample, or detect signals from all positions at once and distribute the collected photons over each pixel of the light detector in case of detector arrays such as charge coupled device (CCD) or complementary metal–oxide–semiconductor (CMOS) sensors. After sending light (photons) within samples, point spectroscopy methods are either based on (1) sending mono-or multi-chromatic light, collection of photons from a specific position on the sample by using position a detector on or close to the sample (with the relevant optical components, i.e., lenses, mirrors, etc) or sending light to the detector via optical fibers, and distribution of these photons over positions of a detector array (e.g., wavelength-resolved by using a dispersive element such as a prism or diffraction grating and a charge-coupled device (CCD) for detection), or (2) based on sending illumination signals at every unit of certain light characteristics (e.g., light at one wavelength) at a time and detection of all collected photons generated at specific wavelengths (e.g., measurement of extinction coefficient at a particular wavelength without interference of fluorescence and Raman signals) and times (time-gating techniques and time-correlated single photon counting (TCSPC) techniques; Chapter 6, section 6.3 and Chapter 8, section 8.1.4) for each type of emission events of interest (e.g., Raman or fluorescence photons). Therefore, the choice of imaging or point spectroscopy is highly dependent on the trade-off between spatial resolution and precise optical signals. Furthermore, designing imaging instruments have to consider this trade-off and the spatial scale where targeted biological changes occur in order to define the required image resolution for a certain application, whereas selecting point spectroscopy equipment would need to take into account the distribution of the photons not only over wider or narrower ranges of characteristics (wavelengths, times, polarization, etc), but delivering more photons to (wavelength, space and time) ranges where detectors are less sensitive or making detectors more sensitive for (wavelength, space and time) ranges where it is not possible to collect photons due to unlikely photon generation events (e.g., Raman scattering, second harmonic generation, multiphoton processes). Finally, hybrid and multimodal spectral imaging techniques (i.e., techniques combining several optical methods among themselves or with non-optical methods) would need to consider all aforementioned aspects.

Imaging and point spectroscopy also differ in terms of features extracted from each modality. As pointed above, the identification of these features is highly dependent on the instrument sensitivity, photon collection and ability of highlighting these features after data processing with computational imaging algorithms. Due to the limitation of the photon distribution over space (pixels in imaging and on spatially-resolved spectroscopy), colors (wavelengths on wavelength-resolved spectroscopy), times (on time-resolved spectroscopy), signals have to be prioritized over the dimension with highest image contrast (e.g., highest spatial oxygen saturation differences if the objective is looking for blood oxygenation). In general, images may prioritize spatial features while keeping enough sensitivity and specificity to wavelengths, times and other variables to detect the desired structures, whereas spectroscopy prioritizes wavelengths, times and other variables for accurate determination of tissue biochemical, microstructural properties. A list of image and spectroscopy features which can be extracted and used for biophotonics applications is included in Appendix 2: List of image and spectroscopy features.

A number of imaging and spectroscopy techniques can be used either in purely imaging or point spectroscopy or in hybrid technologies. These techniques can be applied to a number of optical processes including scattering (elastic and ineslatic), absorption, fluorescence, phosphorescence (chapter 4 Light-tissue interactions), and are divided into wavelength-resolved, spatially-resolved, time-resolved (or time-domain) and frequency domain techniques (chapter 8. Optical techniques).

Take home messages

- The superiority of optical techniques compared to others is specific to every application. Therefore, each optical instrument should be designed to meet particular application requirements and available funds for optimal cost-effectiveness.
- This chapter shows an overview on what can potentially be improved by optical techniques (advantages) upon their implementation, steps to select and optimize optical techniques, and what should be taken into account when studying biomedical optics in micro- or macroscales, and when using spectroscopic and/or imaging methods.
- In this thesis, the selection and optimization optical techniques were performed for future applications in endoscopy, cancer microsurgery, cancer screening, and monitoring organ function and viability. This includes macroscopic/macroscale techniques for tissue identification and biofluid analysis, as well as future microscopic/microspectroscopic applications on analysis of tissues, cells and structures in biofluids for increased understanding on the origin of diseases [12–102].
- Optical parameters and data analysis were optimized for the accuracy of tissue identification and interpretation of changes in tissue biochemistry and microstructure via point-spectroscopy methods. Finding on this optimization are relevant for future applications in imaging, as parameters such as source and detector wavelength sensitivity need to be selected after probing many wavelengths with spectroscopic or hyperspectral imaging methods.

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