Optical toolkits for *in vivo* deep tissue laser scanning microscopy: a primer

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Abstract: Life at the microscale is animated and multifaceted. The impact of dynamic *in-vivo* microscopy in small animals has opened up opportunities to peer into a multitude of biological processes at the cellular scale in their native microenvironment. Laser scanning microscopy coupled with targeted fluorescent proteins has become an indispensable tool to enable dynamic imaging *in-vivo* at high temporal and spatial resolutions. In the last few decades, the technique has been translated from imaging cells in thin samples to mapping cells in the thick biological tissue of living organisms. Here, we sought to provide a concise overview of the design considerations of a laser scanning microscope that enables cellular and subcellular imaging in deep tissue. Individual components under review include: long working distance microscope objectives, laser scanning technologies, adaptive optics devices, beam shaping technologies and photon detectors, with an emphasis on more recent advances. The review will conclude with the latest innovations in automated optical microscopy, which would impact tracking and quantification of heterogeneous populations of cells *in-vivo*.

Keywords: Multiphoton, laser scanning, Microscopy

1. Seeing is believing

Ever since Antonie Leeuwenhoek and Robert Hooke opened our eyes to the world of microbes using polished lenses, microscopy has become an indispensable tool in life science. Due to the immense complexity of biological processes, the progress of microbiology is largely guided by experiments, and in the field of microscopic imaging, the capabilities of the latest microscopy technologies available (Editorial, 2009). Optical microscopy has evolved into a powerful scientific tool that is indispensable to the study of biological systems on excise tissue samples or on cell cultures (Wollman *et al.*, 2015). Only in the last two decades has there been a surge of high performance *in vivo* microscopy systems designed for deep tissue imaging (Pittet and Weissleder). The increasing accessibility of high numerical aperture (N.A) microscope optics, compact laser systems, tunable ultrafast lasers, adaptive optics devices, high speed laser scanners, photon counting devices, and a growing library of fluorophores and digital imaging and computing resources, have catalysed a myriad of advanced *in vivo* microscopy systems (Ntziachristos, 2010). Many of these advanced optical microscopy systems have begun to be able to quantify biological events such as cell division (Orth *et al.*, 2011), cell death (Spencer and Sorger, 2011), homing(Bixel *et al.*, 2017) and tissue reorganisation (Friedl and Alexander, 2011). The key contributing factors to the success of modern optical microscopes are: minimal invasiveness, millisecond acquisition speed, sub-cellular resolution and the flexibility to record biological events at different length scales (nanometres to millimetres). The current generation of *in vivo* optical microscopes are routinely answering some outstanding questions relating to individual cells' responses to pathological stimuli within specific microenvironments, in living animals *in vivo* by accessing the superficial layers of entire organs.

This paves the way to the next generation of *in vivo* microscopes that will unpick subcellular changes (on a sub-micrometre scale) over a large imaging volume (on a millimetre scale). Such a multiscale imaging approach will make possible the ability to correlate and

understand the interconnectivity between different homeostatic processes and quantify complex pathways that govern the organisation and proliferation of cells in living tissue (Pittet and Weissleder, 2011). The exponential growth in the use of confocal laser microscopy in microbiology has been expedited by the advances of coherent laser sources that can be tightly focussed due to high spatial coherence and monochromaticity (i.e. low chromatic aberration), as well as efficient and versatile fluorescence excitation due to controlled wavelength bandwidths. The recent progress in deep tissue *in vivo* imaging is made possible by technological developments in advanced ultrafast lasers, optomechanical devices and new computational tools. Here, we first discuss the principles of laser scanning microscopy, which is then followed by a review of the existing technologies, lasers, optomechanical systems, photon detectors and optics that are powering the existing *in vivo* laser scanning microscopes. After which, we will deliberate on the next generation technologies that will enable a "smart" *in vivo* microscope.

2. Basics and challenges of *In vivo* laser scanning microscopy (LSM)

a. Fluorescence Imaging

Fluorescence light microscopy has a unique set of advantages that lends itself to success in *in vivo* imaging, namely: non-invasiveness, compatibility with the existing library of fluorophores, rapid measurements, high resolution (cellular and subcellular) and the flexibility of laser scanning microscope systems (Pittet and Weissleder, 2011). There are increasing numbers of reporter fluorophores (Treynor *et al.*, 2007; Grimm *et al.*, 2015) and transgenic mice (Hadjantonakis *et al.*, 1998) being made available to researchers that are continually enhancing the experimental usefulness of fluorescence microscopy. The combination of new biochemistry of fluorescence agents also supports new ways of interrogating and measuring biological responses *in vivo*, for instance through optogenetics (Williams and Deisseroth, 2013). Fluorescence imaging is primarily based on Jablonski's theory of energy transfers

(Jabłoństski, 1933). Figure.1 a) shows a simplified Jablonski energy diagram (excluding phosphorescence) demonstrating the steps that a molecule would undertake after 1-photon or 2-photon excitation from S0 to S1; i.e. vibrational relaxation and fluorescence. Whilst the absorption events occur in the order of femtoseconds, the fluorescence emission events are orders of magnitude slower, in the range of 10^{-9} to 10^{-7} seconds. Transitional states (10^{-12} s), such as internal conversion and vibrational relaxation, that can occur as excited photons return to ground states, are where the energies are likely to dissipate or be lost. The fluorescence photon possesses energy that is equivalent to the excitation energy remaining after the aforementioned transitional states, which results in much lower energy than the photons used to excite the sample. Although single photon absorption typically requires narrow linewidth lasers that are continuously emitting a stream of coherent photons, there are multiple variations to the number of vibrational levels during transition between electronic states. 1-photon excitation does not require a laser. In single photon excitation, the emitted fluorescence comprises of photons of wavelengths longer than those of the incident photons. Apart from single photon absorption, there is the possibility of accommodating a higher number of photons at longer wavelengths - and therefore lower energy levels - during the process of absorption, as proposed theoretically by Mayer (Göppert-Mayer, 2009) and verified experimentally by Kaiser and Garret (Kaiser and Garrett, 1961). Following a similar excitation process, it is possible for a fluorophore to undergo simultaneous absorption of two less energetic photons (consequently often in the infrared spectral range). Figure. 1b) i) shows the laser wavelength used to excite eGFP fluorescence photons via 1 photon and 2 photon absorption. The absorption cross-section of an eGFP fluorescence molecule with 2p is typically larger than 1p (Zipfel *et al.*, 2003; Xu and Webb, 1996). Figure. 1b) ii) demonstrates other available visible fluorescence proteins that have been used in 2p microscopy. such as Blue, Cyan, Yellow and DsRed. By the selection of multiple fluorophores with distinct excitation and/or emission

spectra, these signals can be multiplexed to reveal information about the relative positions, interactions and concentrations of the marked features within a heterogeneous sample. While fluorescence labelling is vital to much biomedical imaging, both *in vitro* and *in vivo*, it is important to consider how the addition of the fluorophore itself may impact the feature to which it is bound, as well as biological interactions as a whole in the locality of the labelling.

Two photon (2p) microscopy relies on the efficient generation of short laser pulses to maximise multiphoton excitation. A typical pulse shape from ultrafast lasers represented by $sech^2$-shape profile and is limited by time-bandwidth product of ≈ 0.315. The shortest duration of time that a single laser pulse (Δt) is expressed as $\Delta t \geq 0.315\ (\lambda_o^2/\Delta\lambda.c)$, where c is the speed of light (m/s), λ_o is the central wavelength and $\Delta\lambda$ is the spectrum (Wollenhaupt *et al.*, 2007). Hence to achieve the shortest possible laser pulse, an increased spectral bandwidth is required. Figure. 1c) illustrates how the eGFP molecules are excited in 1-photon and 2-photon relative to the focus of the beam. 2 photon absorption events are highly nonlinear, as they involve the simultaneous absorption of double the number of photons by an individual fluorophore over a short time-scale. This means that the probability of two photons to be simultaneously absorbed by a fluorescent molecule is no longer linearly dependent on the excitation intensity, but rather exhibits a quadratic dependence, as these photons must be highly coincident both spatially and temporally. To achieve this, laser energies are concentrated spatially by high numerical aperture objectives and temporally by short laser pulses, as depicted in the inset of Figure 1c). Multiphoton imaging requires pulsing (ultrafast) sources that generate a train of laser pulses with high peak power (Zipfel *et al.*, 2003; Spence *et al.*, 1991). The laser pulses for 2p imaging typically operate at a few hundred femtoseconds as illustrated in figure 1. c), and so also satisfy the time scale of the photoabsorption event, as shown in Figure. 1a). Pulsed lasers also open a variety of label-free techniques such as optical coherence tomography (OCT), second harmonic generation imaging (SHG), stimulated Raman spectroscopy (SRS).

b. Light-tissue scattering

The depth within a sample that is able to be imaged by a laser scanning microscope is limited by optical scattering as illustrated in figure 1. d). In general, the output intensity of light after undergoing optical scattering is inversely proportional to the fourth power of the excitation wavelength (Helmchen and Denk, 2005; Kobat *et al.*, 2009). Hence, at the same incident laser power, the use of a longer wavelength would enable imaging at greater depths because of the reduction in light scattering (Theer and Denk, 2006). As a result, multiphoton microscopy, which operates at integer multiples of the wavelengths used by single photon systems (NIR), has taken over as the primary *in vivo* imaging tool for the majority of biological experiments in living animals (Pittet and Weissleder, 2011).

Another difficulty in *in vivo* imaging is the ability to actively track individual cells at real time in all three dimensions. In neuronal networks, the ability to measure calcium responses between neurons allows one to track decision making processing within the brain. These networks are highly interconnected, and these calcium signals typically rapidly propagate in all 3 dimensions. There is therefore a benefit to imaging at least two separate axial planes to locate dendritic segments over a 3-dimensional ensemble of neurons. This enables the examination of 3-dimensional neural communications over the given depth with sufficient axial resolution to resolve individual neurons. Only recently have the computational power, high speed electronics, and axial laser scanning systems become capable of demonstrating the possibility of conducting optically-sectioned 3-dimensional imaging at high speeds (Kong *et al.*, 2015).

3. Optical tools in LSM from current to next generation

The most important question for researchers is whether to construct your own microscope system or to just purchase a commercial system. The construction of a laser scanning microscope requires significant time investment and expertise in electronics and optics,

however, this in return offers greater control, flexibility and potentially more efficient signal collection than an equivalent commercial system (Zinter and Levene, 2011). More importantly, the ability to tailor a microscope system for a specific disease model such as eye or colon will greatly benefit longitudinal studies, as various suggested optimisations, specific sample mounting requirements and software controls can be built into the system. (Veilleux *et al.*, 2008).

In this section we shall discuss the tools used to construct a laser scanning microscope. It is important to consider how the microscope system plays into the larger experimental workflow, particularly when high throughput is required. To facilitate this discussion consider Figure. 2.a), that describes a modern approach for live animal imaging. In the modern biological experiment, the imaging system helps to validate a proposed hypothesis, shown here, as an example on neuroscience. Once the hypothesis is proposed, the researchers develop a surgical method to expose the internal organs of interest under a laser scanning microscope. After the imaging session is completed, a series of image processing steps are required. Then, additional analysis of cellular level activities is carried out to correlate these activities to an external stimulant. The limitation of the existing imaging experiment is that most of the correlations are obtained after the experiment has completed and not during the experiment. Also, many of these processes require human intervention and thereby reduces experimental throughput. Ideally, the microscopy system should have built in components that can provide direct analysis with real time image processing capabilities. However, to achieve that there needs to be considerable improvement to the imaging microscope. In the following sections, we shall explain the purpose of each of the components of the laser scanning microscope and introduce new optical instruments (beam shaping, adaptive optics etc) that can enhance automated imaging workflow. We have also detailed the advantages and limitations of various design choices relating to each component. Figure. 2b) details the key components required (laser

systems, beam scanners, adaptive optics devices, beam-shaping techniques, photon detectors and automated image analysis) and their respective positions within a laser scanning microscope. A unique feature of a deep tissue *in vivo* microscopy system is the large working space after the objective lens that is designed to accommodate instruments for live animal preparation (heating stage, stereotactic mounts, anaesthesia equipment, electrophysiology etc.). Commercial *in vivo* microscope objectives are designed to achieve long working distance (~ 1-2 mm), high numerical aperture (water immersion with numerical aperture from 0.8 to 1.05) and wide field of view (~ 1 mm). Commercial systems offered by Nikon, Olympus, Zeiss and Leica deliver standard imaging rates (1 Hz to 30 Hz). In addition to turnkey microscope systems, there is now an increasing range of OEM microscope platforms developed by optomechanical instrumentation companies (Scientifica, UK; Bruker, USA; Thorlabs, USA; Sutter, USA; Femtonics, Hungary; LaVision, Germany) that focus on achieving a greater degree of flexibility, such as random-access scanning and holographic photomanipulation capabilities (Ronzitti *et al.*, 2017). We have listed the different commercial provider and the associated companies in Table. 1. For freely moving animals, there is a need to accommodate large motion and this is where miniature microscopes have become increasingly applicable for wide field one-photon (Ghosh *et al.*, 2011) and two-photon imaging (Zong *et al.*, 2017; Helmchen *et al.*, 2001).

a. Laser sources (CW, Ultrafast)

Most modern confocal microscopes use compact diode pumped solid state (DPSS) laser sources that are increasingly replacing ion lasers and flash-lamp pumped lasers(Gratton and Van De Ven, 2006). DPSS lasers operate by pumping a solid gain medium with a semiconductor laser diode, with the capacity to operate at higher efficiency (10%) than previous technologies, and with a life span of over 20,000 hours. The main advantage of DPSS over conventional diode lasers is the higher quality TEM_{00} Gaussian beam mode that they

produce. A standalone laser diode is side-emitting and possess an asymmetrical beam mode. DPSS lasers use external optics for efficient light coupling that tends to yield a higher quality transverse beam profile (Hulicius and Kubeček, 2013). The quality of the beam intensity gradient defines the diffraction limited performance of the corresponding high-resolution laser scanning microscope system. In addition, the wavelength of the laser also defines the scattering coefficient that scales to the power of 4 with the wavelength. As such, shorter wavelength CW lasers may produce a more localised fluorescence signal, but cannot penetrate beyond the first few cell layers (typically around 50 μm) (Jacques, 2013).

Apart from continuous wave laser sources, the invention of "self mode-locked" laser systems paved the way to a new generation of practical and high power ultrafast laser systems. The observation of high levels of nonlinearity in Ti:Sapphire crystals made it possible to enable a large collection of modes to be phase-locked and used to generate a pulse of less than 100 femtoseconds (Spence *et al.*, 1991). The appeal of the mode-locking process is the ability to generate peak optical pulse powers greater than 5 MW by temporal focusing. The combination of these ultrafast lasers and modern high numerical aperture objective lenses made it possible to realise multiphoton microscopy (Denk *et al.*, 1990). However, femtosecond lasers add an additional dimension of complexity to the system. Due to the need for a coherent temporal pulse front, there needs to be extra attention paid to ensuring that each laser pulse undergoes minimal temporal dispersion i.e. remains transform limited. A transform limited pulse ensures maximum coherent addition of intensity from all of the spectrum and leads to the maximum possible peak power. Pre-compensated methods using prism pairs, dispersion-compensating mirrors and active pulse-shaping schemes are available tools to reduce this dispersion (Field *et al.*, 2010).

In deep tissue imaging, there are an increasing number of strategies being employed to reach beyond the first few hundred micrometres and into the millimetre range. Some of the

tactics employed to increase intensity include: increasing the energy of the excitation pulse by reducing the repetition rate using a pulse picker before the laser amplifier, and increasing the wavelength range of the excitation lasers (up to 1200- 1700 nm) to evoke 3 photon fluorescence microscopy (Horton *et al.*, 2013). The added risks of increasing energy density include the potential for: photodamage - damaging the biological system under investigation, photobleaching - irreparably damaging the fluorophores used for imaging, fluorescence quenching - shown in Figure. 1.a) whereby an excited fluorophore returns to S_0 without photon emission, and exciting out of focus fluorescence signals, thereby reducing the imaging contrast (Hoover and Squier, 2013). Furthermore, for excitation wavelengths above 1.3 µm, there is a need to include a special immersion medium (Wen and Qiu, 2017) to reduce light absorption for water immersion objectives.

b. Relay optics

Laser scanning microscopy allows a system to concentrate all optical energies onto a single point for more efficient nonlinear fluorescence excitation, while generally still being able to cover useful areas over a single camera exposure. At the heart of laser scanning techniques lies the concept of optical conjugate planes and telecentricity, to ensure a diffraction-limited beam scans across the sample after the microscope objective lens. Most microscope objective lenses are designed with telecentricity, whereby its entrance and exit pupil are both at infinity space (object and imaging). Relay optics are carefully designed to fulfil telecentricity so that all the beams passing through the telecentric plane at an angle are relayed onto the sample plane with minimal beam distortion. This is achieved by matching conjugate image planes. Any modifications to a laser scanning microscope for the purpose of achieving high speed laser scanning, axial scanning and adaptive optics would therefore need to be inserted in the appropriate conjugate planes (Stelzer, 2006).

c. Scanners

There are a wide range of scanning devices developed for laser scanning. For laser scanning fluorescence imaging, the strength or intensity of the excited signal is primarily determined by laser dwell time and fluorophore characteristics. The imaging field of view (FOV) and speed requirements are often determined by the type of biological model of interest, so for these discussions we assume a 512 by 512 pixel scan over a $500\mu m$ FOV. A standard galvanometer scanning system has an imaging speed of a few hertz, and is sufficient to track a large population of motile cells *in vivo* that occurs over several minutes to several hours. For transient calcium signals and blood flow, it is necessary to make use of high speed imaging techniques (> 15 Hz) (Veilleux *et al.*, 2008; Li *et al.*, 2017). Another advantage of using a high speed imaging system is that it is also very useful when removing large motion artefacts, caused by natural movements of the organism. In laser scanning microscopy, the scanning rate may be adjusted to suit the imaging conditions within the limitations of the scanners used. Figure 2.c) i) shows the performance of scanning line rate of different scanners and the corresponding scan angle. An ideal scanner should have both a faster scanning line rate and higher scan angle. From our comparison, it is clear that MEM scanners are the best. However, they are not adopted by most microscope companies due to limited range of apertures. Commercial microscope systems try to expand their range of scanning speeds by switching between a fast and a slow mirror scanning system; termed as "tandem scanners" so as to accommodate a wide range of scanning speeds. Tandem scanners typically consist of a pair of galvanometer mirror scanners (maximum line rate ~2 kHz) and a resonant scanner (of a fixed line rate of maximum ~15 kHz) that are selected independently from each other. Apart from resonant scanners, there are a wide range of scanning technologies that can be used for high speed imaging. Acousto optic deflectors (AOD) are shown to offer the widest dynamic range of scanning speed (up to 20 MHz), albeit with high dispersions and aberrations (Nadella *et al.*, 2016; Kirkby *et al.*, 2010). Rotating polygon scanners provide a scan angle that is over two orders of magnitude (2-15^o) larger than that of AODs

(0.2^{o}). They also operate over a large dynamic range of scanning speeds (2 kHz to 120 kHz) and retain superior optical efficiency over AODs. Increasingly, MEMS-based micromirror scanners are being used, which are able to offer high speeds and high scan angles due to their low inertia. As shown in figure 2.c) ii) Mirror scanners (MEMS, galvanometer, resonant and polygon) have an obvious advantage over diffractive AODs due to their high optical efficiency. Recently, the use of oblique scanning methods such as swept confocally-aligned planar excitation (SCAPE) have offered a rapid 3D scanning system akin to lightsheet microscopy through a single objective (Bouchard *et al.*, 2015). Such architectures remove the need for point-by-point scanning, by leveraging the axial properties of the excitation beam to excite entire planes or lines (in a digitally scanned light-sheet) simultaneously. However, the drawback of such a scanning system is the non-telecentric arrangement of the scanning conjugates that can create significant aberrations during scanning as well as increased difficulty of implementing nonlinear excitation.

d. Microscope objectives

Microscope objective lenses represent an important element in the microscope. The characteristic of *in vivo* microscope objective lenses is that they have low magnification, 10X - 20X, with relatively high numerical aperture (N.A) of 0.8 – 1.0, working distance from 1 mm to 3 mm and water immersion. The working distance of *in vivo* imaging systems is an important factor because of extensive surgical windows (Makale *et al.*, 2009) constructed to access different parts of an organism.

For 2p microscopy, there is always a need to accommodate for longer wavelengths and objective lenses would also need to be coated to optimise for improved light transmission (Keatings *et al.*, 2008). Existing microscope objective lenses are limited to << 1 mm field of view, which covers only a small fraction of an organ with diffraction limited performance. Lately, customised microscope objective lenses have emerged as an alternative to reach a field of view over 5 mm (Tsai *et al.*, 2015; Sofroniew *et al.*, 2016; Stirman *et al.*, 2016). A large

field view increases the number of cells that can be imaged concurrently. This means that distinct cellular activities distributed heterogeneously can be mapped out at much higher throughput. With a larger field of view, emerging issues pertaining to image sampling (space bandwidth product (Lohmann *et al.*, 1996a)) need to be addressed either computationally or with high rates of sampling. Another important aspect of beam scanning is the control of the beam waist illuminating the back aperture of the microscope objective lens to reduce optical scattering. As pointed out by Helmchen and Denk (Helmchen and Denk, 2006), a slight under-filling of the objective lenses reduces the optical resolution slightly, resulting in a lower scattering in biological tissue and can achieve an improved imaging depth.

e. Detector

Due to the multiple scattering interfaces in organic tissues, the *in vivo* fluorescence signal is generally weaker, resulting in a reduced 'photon budget'. Single photon counting devices such as photomultiplier tubes and charge coupled devices are routinely used to collect the available fluorescence photons emerging from the tissue. Whilst confocal microscopy only collects in-focus fluorescence photons, multiphoton microscopy relies on the collection of all exiting fluorescence photons. This is typical for nonlinear microscopy, where the exiting photons are the result of the previously discussed nonlinear interactions at the focus of the beam (Helmchen and Denk, 2006). Optical sectioning is thereby preserved by this nonlinear dependence, as excitation intensity (and consequently fluorescence signal) quickly drops-off outside of the focal plane.

f. Adaptive optics

Adaptive optics has been a well-established field in astronomy that aims to retrieve the optical aberrations in a system and reverse them, thereby increasing the signal to noise ratio. Although adaptive optics aberration correction has been previously implemented in confocal microscopy

(Booth *et al.*, 2002), the technique is now increasingly gaining widespread use in deep tissue. The popular adaptive optics tools include deformable mirrors (DM) (Zheng *et al.*, 2017), spatial light modulators (SLM) (Neil *et al.*, 2000) and digital mirror deflectors (DMDs) (Hanley *et al.*, 1999). These tools allow one to reverse optical distortions patterns onto the incidence beam and thereby pre-compensate for optical aberrations. Figure.3a) i) illustrates how a distorted wavefront emerging from the microscope objectives lead to an enlarged excitation volume. By shaping the wavefront of the beam, it is then possible to reverse the distortion and increase the fluorescence signal as shown in Figure 3a) ii). We demonstrate this effect using our AO multiphoton microscope in in figure 3.a) where we introduced an additional layer of glass so as to create additional spherical aberrations in the sample. By using our deformable mirror, we observed the increase in fluorescence signal (fluorescence dyed paper). More elaborate phase reversal methods (Kim *et al.*, 2015) have been introduced lately to recover highly scattering non-living tissue. The key issue is that the phase retrieval for a living biological tissue is often complex and time consuming due to unpredictable light scattering. Recently, the development of time reversal acoustic probe (Ruan *et al.*, 2017) and speckle decorrelation methods (Qureshi *et al.*, 2017) aims to measure the dynamic events so as to provide phase correction at the appropriate time intervals where random scattering is low.

g. Shaping and axial scanning

Laser scanning microscopies (confocal and multiphoton) provide depth resolved images which collectively add up to form a volumetric reconstructed image of the sample. While mirror scanners (MEMs, galvanometer, resonant, polygon) provide high speed raster scanning, they typically do not provide any axial scanning capabilities. On the other hand, two pairs of AODs can be combined to perform raster and axial scanning(Nadella *et al.*, 2016) for random access imaging and photoexcitation. The scanning pair used for axial scanning is termed as acousto-optic lenses (AOLs). The generation of counter propagating chirped acoustic

waves (~ 400 kHz) within a pair of AOLs is capable of producing a dynamic spherical lens where each single pre-chirped AOD produces a tunable cylindrical lens. However, temporal dispersion in AOLs can significantly stretch laser pulses from femtoseconds into the picosecond range and thereby lower multiphoton excitation efficiency. AOLs operate by producing an acoustic standing wave within a crystal, which is proportional to the refractive index changes within the crystals, thereby creating a dynamic optical grating, with the deflection angle determined by the frequency of the acoustic wave. The shape of the laser beams exiting the sample undergo diffraction in one direction, which accrues significant phase aberrations (Kirkby *et al.*, 2010). Such technical difficulties prohibit dynamic AOLs from being widely integrated into existing multiphoton microscope systems. Femtonics has been one of the few commercial providers that offers complete AOD and AOL scanning solutions designed for multiphoton applications. Lately, tunable liquid lenses with low inertia, driven at high speed electrically(Grewe *et al.*, 2011) or ultrasonically (Duocastella *et al.*, 2014; Kong *et al.*, 2016) can generate scanning speeds from kHz to MHz. The advantage of these lenses is their ease of integration into existing laser scanning microscope systems, as well as control systems that are fairly robust due to their similarity to traditional scanner controllers. However, special care must be taken to ensure mechanical resonance of these liquid lenses, which results in unstable beam pointing, is avoided. In terms of phase curvature, the AOLs and tunable lenses impose a parabolic phase function that changes the axial position of the focal point. However, this is a sub-optimal approach because the defocusing phase curvature at each imaging depth varies from the parabolic phase of the axial scanners. A simple approach to compensate for this wavefront inconsistency is to create a direct optical conjugate axial scanning system. Botcherby *et al* (Botcherby *et al.*, 2007; Botcherby *et al.*, 2012) simply replicated the axial scanning profile by using a pair of identical microscopy objective lens, where optical properties of the imaging microscope objective is replicated and reversed. Using this scanning geometry, it

becomes possible to compensate for wavefront errors as the beam is focused axially. Apart from scanning a focussed beam, it is also possible to shape the input beam to collect fluorescence along the axial plane using a sheet of light (scanning light sheet) (Engelbrecht *et al.*, 2010) or needle beam (Bessel beam). More specifically, as figure 3.b.i) shows, an elongated Bessel beam is able to image with an increased depth of field, and thereby image an extended volume in a single 2D snapshot (Lu *et al.*, 2017). The advantage of this technique is the ability to compress sparsely distributed neurons along the imaging volume into a single image i.e. a maximum projection in the z-axis. Figure 3.b.i) illustrates the resultant maximum projections in the z-axis generated using this technique. This approach drastically reduces computational capacity and increases imaging speeds but fails to provide depth resolved images. This means it is only suitable for sparsely populated samples, as high density samples will have unacceptable levels of overlap and obfuscation within the imaged volume.

h. Data handling and software

In multiphoton microscopy, volumetric imaging is vital to the analysis of cell to cell interaction within the local microenvironment of an organ, and is typically implemented by the capture of multiple 2D image slices at different depths within the sample. For example, calcium transients in mouse cortical neurons *in vivo,* provide the required volumetric imaging rates (Duemani Reddy *et al.*, 2008) . The acquisition rate of the imaged volume increases the load on data transfer and storage components. This increases the space bandwidth product (SBP) (Lohmann *et al.*, 1996b), a metric that aims to measure the amount of raw information captured by a system, and it is simply measured in pixels. The SBP is defined as the number of pixels required for diffraction-limited imaging at a given N.A. and FOV, $SBP = 4 * field\ of\ view / (lateral\ imaging\ resolution)^2$ .The rate of digital data generation for the system is defined by $Detector\ dynamic\ range\ (in\ bits) * 2D\ frame\ rate * SBP$. Various trade-offs can be made within these relationships to decrease the data overhead imposed by a microscope system,

for instance, the FOV may have to be reduced. This means that the microscopy is data-limited. Figure 3. b) ii) shows the data-rate generated by a diffraction-limited, single channel, 1.3 N.A. microscope system operating at a standard FOV of $500\mu m$ by $500\mu m$. This system has an SBP of approximately 8.85 Megapixels where each pixel is made up of 2 bytes. Figure 3. b) ii) also compares this to various data transfer standards (PCI, USB, camera-link) commonly used by scientific image sensors and data acquisition cards. We can see that the commonly used detector interface standards are quickly eclipsed at 40fps, and the fastest solid-state data storage technologies available at the time of writing are only applicable at up to 110fps. While the SBP of this system is beyond that commonly used *in vivo* today, existing AOD scanners and photodetectors (PMT, APDs) possess greater bandwidth. Therefore, it is logical to assume that the logistical challenges of data-transfer and storage will play a more significant role in future high-performance imaging systems.

There are many methods by which the amount of information required to be generated, stored and analysed may be significantly reduced, while still retaining the core information needed to satisfy experimental requirements. Attempts to maximise the usefulness of captured information (and thereby reduce the need to capture more of it) have been made through the emerging field of machine learning. One such implementation is CSBDeep, developed by M. Weigert and colleagues (Weigert *et al.*, 2017), and it is shown in figure 3.c). Their technique of content-aware image restoration works by training their deep learning algorithm on a set of high signal-to-noise ratio (SNR) images (termed 'ground truth') as in figure 3.c) i). After being trained on a particular biological structure, the system is then presented with an image of a similar sample with a significantly lower SNR. The algorithm can then retrieve its structure and features based on its experience with the higher quality training data as in the application step figure 3.c) i). This technique is clearly applicable to improving the quality of images taken *in vivo*, however it does only provide an interpretation of data, rather than an increase in

information recovered. An example of CSBDeep's performance is shown in figure 3.c.ii) for Drosophila. The algorithm has been fed training data as in the ground truth, and when given the low quality input shown, has been able to retrieve a significant level of detail as indicated in the 'network' image. While it has been demonstrated for low SNR imaging, the technique is equally applicable to images of a lower SBP, as once the system has been trained, imaging SBP could potentially be lowered to alleviate the data-based pressures that we have discussed.

Apart from the increasing amount of data being collected in modern microscope systems, the workflow of modern microscopy is increasingly reliant on well-designed image processing frameworks with sufficient control feedback to tailor the microscope to each imaging experiment (Carpenter *et al.*, 2012). As such, there is a surge in customised microscopy systems. The program interface should also allow users to tune the laser and scanning parameters for each experiment and avoid undesirable photobleaching or photodamage. Microscope developers are also releasing their own open-source software available for high throughput cell tracking and 3D reconstruction. These include micromanager (Ashcroft and Oosterkamp, 2010), ScanImage (Ashcroft and Oosterkamp, 2010), HelioScan (Langer *et al.*, 2013) and PScan (Li and Lee, 2016). These custom built microscopy systems allow users to control and amend imaging program parameters and therefore provide a higher degree of freedom than a closed commercial software (Carpenter *et al.*, 2012).

4. Volumetric Intelligent microscopy

Biology aims to provide a dynamic quantitative overview of complex biological functions from cells to tissues and organs. Existing quantitative microscopy is often hampered by time-consuming manual selection of large numbers of cells to gain new insights into complex biological networks. The next generation of in-vivo microscopes need to be able to autonomously image, highlight and track large populations of cells over long periods of time. This new breed of intelligent imaging tools combine interdisciplinary approaches in laser

scanning technology, real-time computer vision tracking and genetic engineering that aim to revolutionize systems biologists' approaches. The automated platform needs to be ready to work across a multitude of biological systems and answer key questions in immunology, neuroscience and stem cells. This approach will unpick a hundred-fold more biological processes in living organisms than ever before and precisely map out cell communication channels that have not been seen before. Here, we aim to draw from a current approach in histocytometry (Gerner *et al.*, 2012) and envision how an automated intelligent microscope would perform.

Histocytometry (shown in Figure. 4a)) aims to address the two main crucial factors to satisfy in large scale *in vivo* imaging and mapping. These are (1) throughput and (2) cell locality (location of cells at the micro-anatomical level). Existing high content screening systems extend the throughput of an optical microscope by combining it with robotic handling, and automated image processing. The idea behind histocytometry is to combine histological immunostaining methods and conventional confocal laser scanning microscopy with data analysis methods from flow cytometry. This stems from the need for large scale high throughput automated detection of cell populations while retaining information on the local distribution of individual cells in living organs. The success of optical clearing (Zhu *et al.*, 2013) and expansion microscopy (Chen *et al.*, 2015) techniques would enhance histocytometry methods. However, these are *in vitro* microscopes and so do not fully recapitulate cellular events in their microenvironment.

The next generation of deep tissue optical microscopes need to provide sufficient visualisation for multiple cell population tracking. Lightsheet microscopy has made significant progress in generating dynamic interaction maps with quantitative data supporting developmental biology. Udan and co-workers (Udan *et al.*, 2014) quantified interactive information of cell during morphogenesis and added new insights into mouse embryogenesis

as shown in Figure. 4b). Pioneering work by Keller and co-workers (Royer *et al.*, 2016) has recently realised an automated "smart" lightsheet imaging system, using a customised framework named "Autopilot". At the core of its design, the smart microscope needs to be fully adjustable, with multiple degrees of freedom through digital controls and imaging feedback to detect and analyse imaging performance in all dimensions. Their method was shown to improve signal to noise ratio and imaging resolution and recovers even sub-cellular features in real-time. An intelligent microscopy system must be able to quantify large imaging (volumetric) datasets in thick living tissues. Only then can a heterogeneous distribution of cells around a given organ be accurately mapped and critical responses that cannot be replicated in an in-vitro setting be extracted (Tang *et al.*, 2013). In figure. 4c), we have provided a work flow that could be govern future intelligent microscope systems. Once the biologists have identified an organ or cell population of interest, they would prepare the appropriate imaging window for either a single or longitudinal experiment. During imaging, the microscope would continuously provide image correction on-the-fly for motion and optical aberrations. The automated optical correction requires the effective combination of precision robotic handling and optimal aberration correction methods. Once the correction is optimised, the microscope would then initiate its automated image acquisition to achieve either large area or volume images. During imaging, the microscope system would also include real-time image selection capabilities whereby classifications of cells can be continuously tracked and monitored. Additional image enhancement through machine learning approaches would be used when required. This step is likely to be limited by the current data transfer rates. If active tracking is needed, the biologist should also be able to use photoconvertible fluorescence genes with an automated microscopy system that allows "active" tracking of a specific population of cells in living mice. Once data is being computed, the microscope system would automatically generate a correlation map that associates the interactions of the different cell locations within different

regions of the organs. This will then provide a detailed map of cell-cell interactions at different microenvironments that is crucial in answering many questions in systems biology. The new automated volumetric workflow proposed in Figure. 4c is to essentially integrate most of the post processing and analysis steps, previously shown in Figure. 2a, into the microscope imaging step. By doing so, it has the potential to reduce the overall time taken for each experiment and thereby increase the overall throughput.

Deep tissue microscopy is currently the only method that provides clear, direct and immediate spatiotemporal functional information at a cellular/subcellular level in an intact mammalian immune system. This multi-parameter imaging that has brought a new understanding of mammalian biology of neutrophils, tissue-specific immune cells that cannot be easily be studied ex vivo because of their short life spans. Another important factor is the difficulty that one faces when replicating a complex microenvironment that is essential for functions which are also not yet well understood. *In vivo* imaging studies are essential to test particular hypotheses and avoid the reductionist approach taken in *in vitro* systems. Since the last decade, it has already become evident that paradigms established in some organs may not apply in others. The invention of *in vivo* multiphoton microscopy was made possible from an interdisciplinary research collaboration that enabled the combination of the state-of-the-art in ultrafast lasers, laser-scanning microscopy techniques and new photochemical tools (e.g. dyes/fluorophores). We anticipate that the next generation of deep tissue *in vivo* imaging tools will combine the latest in laser scanning methods, automation technologies, computer vision and machine learning techniques.

Acknowledgements

W. M. Lee acknowledges funding and fellowship support from the FERL program and Australian Research Council Early Career Researcher Award (DE160100843).

Table 1

Company	Location	Model series	website
Scientifica	East Sussex, UK	Hyperscope	http://www.scientifica.uk.com/products
Bruker,	Massachusetts, USA	Ultima	https://www.bruker.com/products/fluorescence-microscopes/ultima-multiphoton-microscopy.html
Sutter,	Novato, USA	DF-SCOPE™	https://www.sutter.com/MICROSCOPES/dfscope.html
Thorlabs,	New Jersey, USA	Bergamo® II	https://www.thorlabs.com/navigation.cfm?guide_id=2189
Femtonics,	Budapest, Hungary	FEMTO	http://femtonics.eu/
LaVision,	Bielefeld, Germany	TriM Scope II	http://www.lavisionbiotec.com/

Figure.1

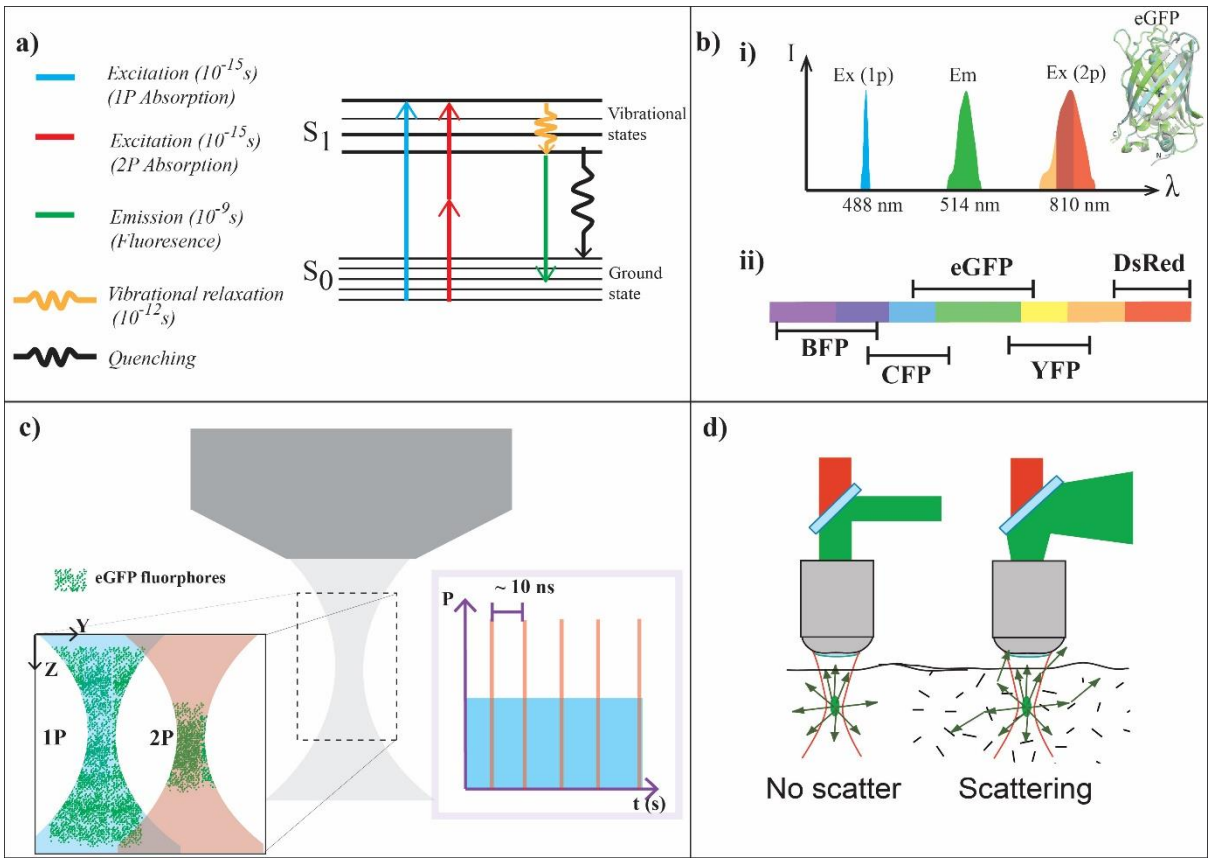

Figure. 1 Fluorescence and optical scattering.

a) Shows a simplified Jablonski energy diagram illustrating the energy interactions that occur during 1 photon and 2-photon excitation. For direct comparison between 1p and 2p fluorescence process, only the energy transitions between S0 to S1 are shown. **b)** i) depicts a sketch of the spectral bandwidth of two types of lasers (continuous wave, CW) used in single photon (1p) imaging and broadband lasers (pulse) used in two photon (2p) microscopy for eGFP (inset) and b) ii) describes a range of standard fluorescent proteins used in laser scanning fluorescence microscopy. **c)** Describes the excitation wavefront used in confocal and multiphoton microscopies and illustrates the resultant fluorescence emission of each modality. The high number of coincident photons required to achieve strong nonlinear excitation are shown to be achieved through both spatial focusing by the objective lens, and temporal focusing through the use of a mode-locked femtosecond laser pulse. **d)** Optical paths taken by photons as they travel through a scattering medium (biological tissue) (reproduced with permission (Zipfel *et al.*, 2003)).

Figure.2

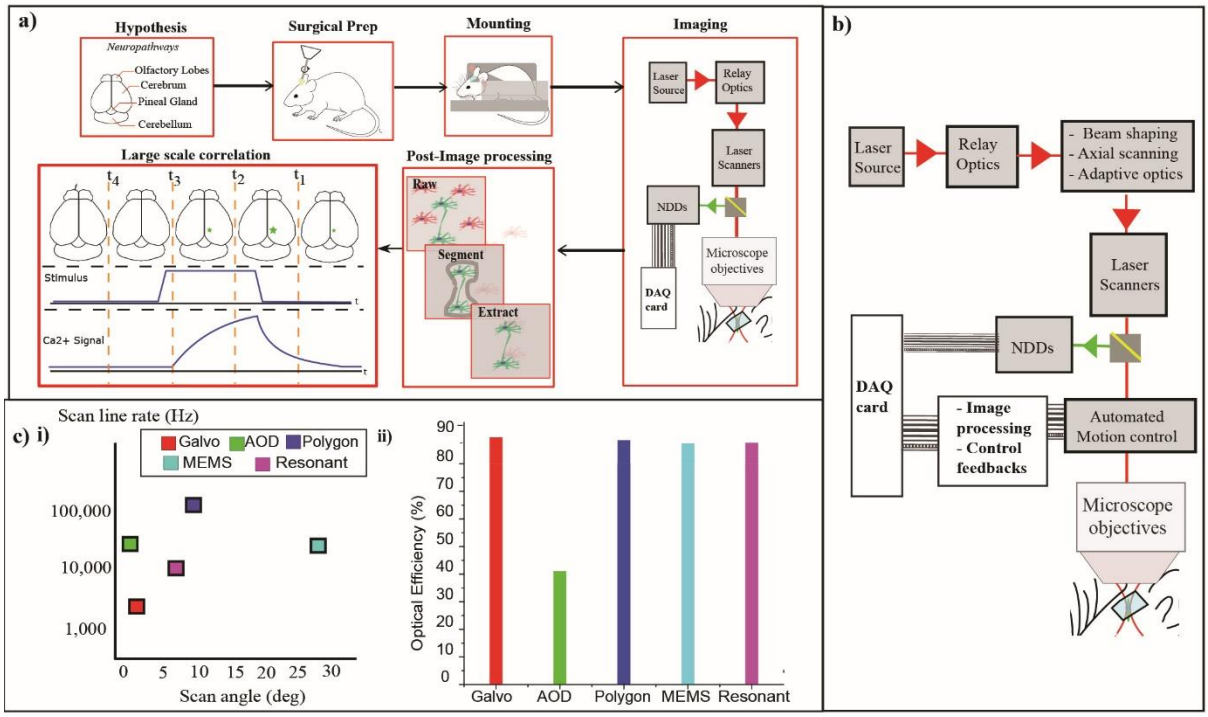

Figure. 2 Laser scanning microscopy workflow and construction

a) Typical workflow of an *in vivo* imaging experiment applicable to multiphoton microscopy, with the schematic of a basic commercial microscope described. Components of microscope are identical to the headings of the sections of which they are discussed. **b)** Multiphoton microscope containing enhancements to improve optical, experimental and data handling performance. As in 'a)' the labelled components match with the sections in which they are discussed. **c)** Comparison of various performance characteristics of beam scanning options commonly used in both commercial and homebuilt multiphoton systems. The scanning speed and angles are obtained from commercial scanner datasheets. Galvo and Resonant (Cambridge Technology Bedford, MA, USA) , MEMS (Innoluce BV Nijmegen, Netherlands), Polygon (Lincoln Laser, Phoenix, USA). Note that the usefulness of an increase in scanning speed reaches a limit at the repetition rate of the laser pulse. For instance, a scan rate of 2MHz is not able to be implemented on a 1MHz femtosecond source as only every second scanned point will undergo excitation. NDDs denotes non-descanned detectors.

Figure.3

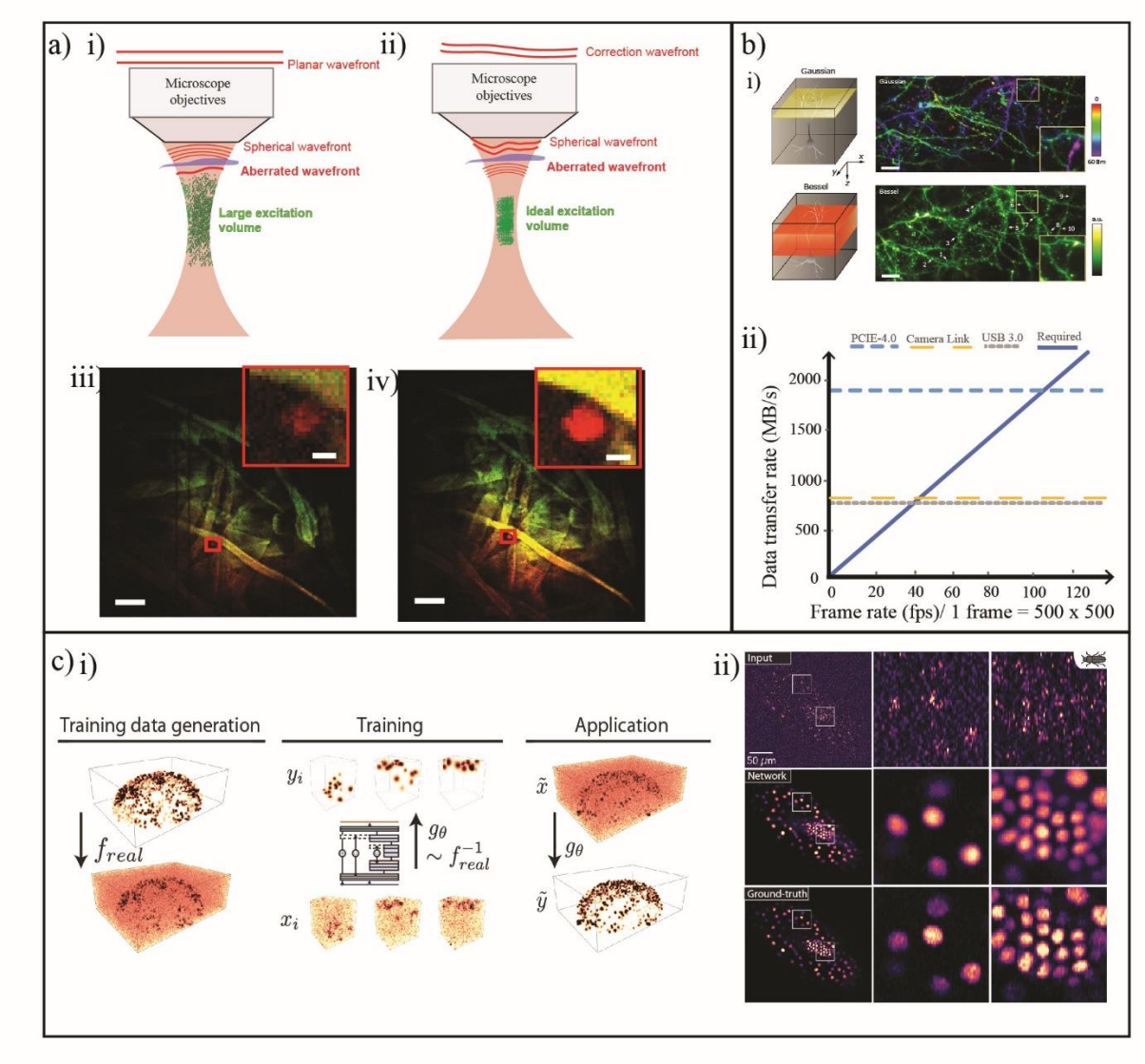

Figure. 3 Enhancements to laser scanning microscopy systems

a) Adaptive optics principle and applications. a)i) shows how aberration occurs in a normal microscope system, leading to reduced imaging performance as shown in a)iii). a)ii) shows how adaptive optics can be applied to compensate for the aberrations caused by the sample by pre-compensation of the excitation wavefront, leading to improved imaging performance as shown in a)iv). **b)** emerging issues in handling of generated data. b) i) volumetric snapshot imaging by Bessel beam illumination, image used with permission. A large volume is recorded in a single image, but is not depth resolved (Lu *et al.*, 2017). ii) The data handling requirements of an optical system (500*500μm FOV, 1.3NA, single channel, Nyquist sampling) at different imaging rates. Standard data communications protocols are also indicated, showing that limitations are reached at approximately 40fps for common detector interfaces, and 110fps for the fastest acquisition and storage systems available today. **c)** Content-aware image restoration by CBSdeep. Fgure reused under CC-BY-NC-ND 4.0 International license. (Weigert *et al.*, 2017). i) outline of the technique workflow. ii) reconstruction of low SNR input to higher quality data that closely resembles the high SNR ground-truth image.

Figure.4

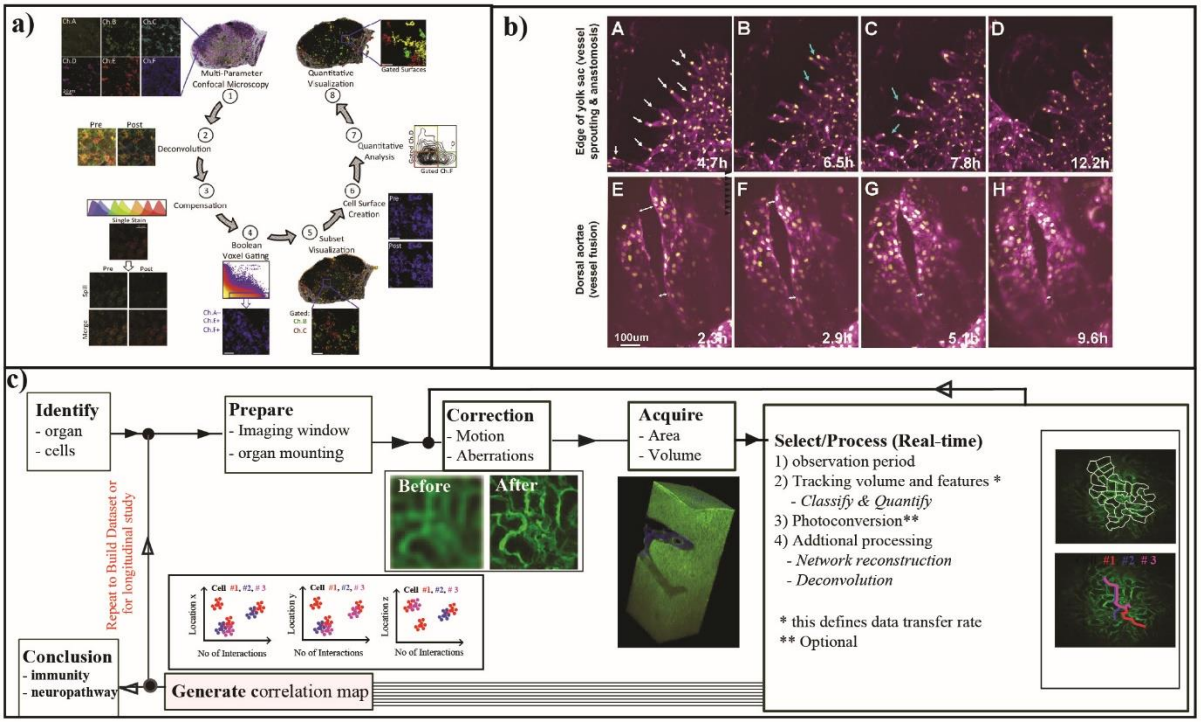

Figure. 4 Automated Volumetric Imaging

a) Work flow of histocytometry (reproduced with permission (Gerner *et al.*, 2012)) by combining volumetric imaging with conventional confocal microscope and flow cytometry representation. **b)** show active tracking of cells using lightsheet microscopy over several hours, Reproduced with permission from (Udan *et al.*, 2014). **c)** Proposed workflow of automated volumetric imaging technique. Once an organ or cell population is identified, the user will need to prepare the imaging chamber. A self-correcting feedback loop allows the system to compensate for motion and aberrations (inset illustrates before and after correction with mice vasculature images). Once the images are improved, the imaging system will acquire large area or volume (inset image shows reconstructed volume image of liver). With the full volume reconstructed, user will be able to select and process the imaging sites at real-time. This is includes observation period so as to track large cell population based on specific markers, which leads to automated classification and quantification. Additional image processing could be achieved directly. Finally a correlation map will be generated depending on the locality of the cells and their relative interactions. This will provide necessary empirical evidence to validate a model for immunity studies or identifying a neuropathway.

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