Design and commissioning of a directly coupled *in-vivo* multiphoton microscope for skin imaging in humans and large animals

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ABSTRACT

The application of near infrared multiphoton excitation to the laser-scanning microscope was first conceived by Denk, Strickler and Webb in 1990. Since then, advances in design have seen the multiphoton laser scanning microscope (MPLSM) applied to a wide range of biological research areas, including skin imaging and vaccine delivery. The technique has the attributes of low phototoxicity, high-resolution functional imaging to depths in scattered tissues. These characteristics have encouraged engineers and scientists to develop *in-vivo* imaging systems. For these applications, laser excitation pulses can be delivered to the sample through optical fibers. Although this solution provides a number of advantages relating to movement and flexibility of the site of interest relative to the laser source, the peak powers that can be delivered down the fiber are limited. We report on the design and commissioning of a directly coupled *in-vivo* MPM system, optimised for the imaging of epidermal vaccines delivered to a range of biological models and humans. Specifically, we seek to apply the system to visualise *in-vivo*, the influence of hand-held, helium powered needle-free systems on skin cells. A standard Nikon E600FN microscope, dissected above the optical plane was cantilevered from a vibration isolated table using rigid support arms. The modified microscope was coupled to an infrared optimised Bio-Rad Radiance 2100MP, multiphoton dedicated laser scanning control and image acquisition system. Femtosecond laser pulses were provided by a 10W Verdi pumped Mira Ti:Sapphire laser, from Coherent Inc. The microscope was modified such that the transmission half may be selectively attached for conventional imaging with *ex-vivo* and cell culture samples, or removed for *in-vivo* imaging of skin sites on the body of humans and large animals. Optical performance of the system, and aspects of its design and commissioning are discussed in this paper.

1. INTRODUCTION

Powdered epidermal needle-free vaccine delivery technology offers a safe and efficient means of vaccination against disease [1]. This novel drug delivery method incorporates a small, hand-held, gas powered device, to accelerate powdered micro-particle vaccines to speeds sufficient for ballistic penetration into lower layers of the skin [2, 3]. By selectively controlling delivery system conditions such as the micro-particle size, density and delivery velocity to the skin, an exclusive population of immune cells resident at the suprabasal layer of the epidermis can be targeted for vaccination [4-6]. This has been successfully proven in clinical trials immunizing against Hepatitis B and influenza and shows immense therapeutic potential for the treatment of many currently incurable diseases [7, 8].

Further enhancement of the reliability and efficacy of this promising vaccination method can be provided with ongoing biomedical research focused at understanding the physical and biological response of skin cells at the delivery site following administration [9, 10]. Amongst the investigative techniques currently available to researchers in this field, one of the most promising has emerged with the development of non-linear fluorescence microscopy [11]. Near infrared (NIR) multiphoton laser scanning microscopy (MPLSM) is based on non-linear fluorophore excitation by simultaneous absorption of two or more near infrared (NIR) photons within the sub-femtoliter focal volume of a high numerical aperture (N.A.) objective lens [12]. This microscopy technique has a number of qualities that include an inherent ability to optically section in three dimensions, as well as efficient simultaneous excitation of multiple fluorophores. Highly effective penetration of NIR photons deep into scattering tissue enables sub-cellular NIR multiphoton imaging to depths beyond ~ 1 mm in living biological specimens [13]. The low risk of photo-damage during NIR-MPLSM has increasingly encouraged scientists to utilise the technique for vital imaging of biological tissue and organs, including skin [13-17].

In recent studies we reported the application of multiphoton microscopy to image powdered micro-particles delivered to excised skin of human and porcine models [18, 19]. These investigations revealed the technique's utility to acquire detailed qualitative and quantitative data pertaining to the dynamics of powdered vaccine delivery. A number of key parameters

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associated with the efficiency of powdered delivery could be measured with a sub-cellular spatial resolution multiphoton imaging system. These include the number of vaccine particles delivered into the skin, the average depth of particle delivery and the distribution of particles across the target "footprint". *In-vivo* multiphoton microscopy offers further opportunity to quantify the cellular and immunological efficiency of powdered epidermal vaccines in a clinically relevant model. Further parameters to be examined include the proportion of Langerhans cells (LC) transfected at the skin site following delivery, the number of transfected LC to migrate from the tissue to draining lymph nodes, as well as the regeneration of peripheral skin cells at the delivery site following particle injection. With clear benefits to developing an *in-vivo* MPLSM system for the study of biolistics and epidermal vaccines, this study was undertaken to design and commission a multiphoton microscope for *in-vivo* imaging of humans and large animal models.

Currently, there are two distinct microscope configurations for *in-vivo* multiphoton imaging. The first employs fiber optic technology to guide excitation pulses from a femtosecond laser to the imaging site of interest [21, 22]. The optical fiber tip can be mounted directly above the imaging site. Alternatively, the optical fiber may be coupled to a high N.A. objective lens and mounted with a cover glass and/or immersion medium against the tissue. A method of scanning the beam across the sample is employed to acquire and reconstruct three-dimensional optical sections of the tissue. Fiber coupled MPLSM systems provide flexibility for animals or patients to move in the laboratory during imaging. The potential use of this technology to image complex biological processes *in-vivo* in an uninhibited setting has numerous applications across the rapidly developing fields of neuroscience, cancer therapy and immunology [16, 21- 23].

There are limitations to the use of fibers in delivering femtosecond laser pulses to the focal plane of a multiphoton microscope [21, 22, 24]. The effects of Group Velocity Dispersion (GVD) and Self-Phase modulation (SPM) contribute to temporal broadening of short laser pulses traveling down a fiber, which in turn restricts the peak-power levels that can be delivered to the focal plane of a fiber-fed multiphoton system. As an alternative, a directly coupled multiphoton microscope can provide superior beam conditioning at the focal plane [25].

In the second MPLSM configuration for in-vivo imaging, the pulsed laser beam is directly coupled to the objective lens with far fewer components in the optical path. This design exhibits significantly improved beam conditioning at the focal plane. Small animal models and human limbs can be stabilized for in-vivo imaging by fixing the image site against the stage of an inverted microscope [16, 17, 26]. Alternatively, confocal imaging of many sites across the body including the scalp, face and back has been demonstrated using a custom built microscope in an upright configuration [27]. This design incorporates steering mirrors mounted into a flexible housing assembly to guide the laser beam to an exposed objective lens coupled to the skin.

In this paper we report on the design of a modified upright directly coupled multiphoton microscope with a modular transmission half that can be selectively attached below the plane of the objective lens. When the transmission half is attached to the microscope, cell culture samples, *ex-vivo* samples and small *in-vivo* animal models can be imaged. Removal of the transmission half of the microscope exposes the objective lens for coupling to skin sites on the body of large animal models and humans. With such a configuration, users are provided with flexibility to acquire high-resolution images *in-vivo* in clinically relevant biological models of powdered vaccine delivery. A description of the mechanical and optical design of this specific MPLSM system is presented. Details and results of system commissioning tests are reported. Important imaging system specifications and a set of acquired images relevant to the investigation of powdered vaccines are also shown.

2. MATERIALS AND METHODS

2.1 Initial design considerations

Table 1.1 summarises the design considerations specific to this problem. These considerations were used as inputs to the development of the optical system. Major factors included the choice of biological models for epidermal vaccines research and performance and practicality of various optical components and imaging system configurations. Time and monetary constraints associated with purchase of a commercial MPLSM system and the construction of a custom-built microscope were also considered. A solution that addresses these issues is presented in section 2.2.

Table 1: Important design considerations in the design of a multiphoton microscope for *in-vivo* imaging of epidermal vaccines delivered to the skin of large animal models and humans.

Major system component	Quality desired for *in-vivo* imaging of powdered epidermal vaccines	Practical qualities/limitations of commercial system components
NIR mode-locked laser source	Reliable output beam pointing and mode-lock over many hours. Necessary for time dependant studies of biological events.	Fluctuations in laser output determined by temperature and relative humidity control within laser cavity and laboratory.
	Tunable output wavelength for excitation of endogenous fluorphores of the skin and various exogenous fluorescent probes.	Ti:Sapphire lasers offer up to 300 nm tuning range within a band 690 nm-1100 nm (selection of specific mirror sets necessary)
	Compact laser size to provide space in laboratory for patients, large animals and associated monitoring equipment.	Compact laser sources with automated tuning via computer control are available, larger sources with manual tuning offer greater selection of output wavelength, but require larger space on table. Both require mounting on vibration isolation table for maximum performance and reliability.
	Ease of use in laboratory.	
Beam alignment, conditioning, diagnostics and control	Stable beam alignment. Monitoring and recording of pulse condition for study replication. Power adjustment with increase in depth of focal plane within tissue.	Pulse width monitoring, power monitoring and automated continuous laser control are all readily available.
	Power and pulse width to achieve multiphoton excitation at depths beyond the epidermis. Required for studies of cell migration from outer skin layers.	No high performance commercial fibre systems available. Femtosecond directly coupled lasers provide > 500 mW power, 200 fs pulsewidth at back aperture of objective.
	Optimal conditioning of beam for use with varying magnification lenses.	Automated beam collimation modules available.
Detector Systems	High sensitivity detection of fluorescence signal providing information pertaining to cell condition. Identification of particles and cell types based upon fluorescence signature.	High sensitivity non-descanned direct detectors available for fluorescence lifetime and spectral imaging. Can be applied in transmission and epifluorescence.
Microscope Systems	Direct coupling of infrared laser to objective lens.	Objective focussing microscope required.
	Coupling of objective lens to large and small animal models and human patients. Delivery sites of interest include volar forearm, stomach, breast and shoulder of patients. Back, ear and inguinal region of porcine models.	Residual epifluorescence microscopes available, but not with objective focussing.
		Transmission systems offer space above an objective lens, but difficult to access imaging site. Configuration requires strong support table above microscope.
		Upright microscopes available with long working distance dipping lenses. Very limited space for mounting large samples below objective lens. Posibility of removing transmission half for *in-vivo* imaging.
	Imaging with in-vitro gel test systems, cell culture samples and small animal models.	Transmission system is an ideal configuration. Upright microscope might also be considered.
	Automated mapping of effector particle and target cell position within skin delivery site.	Automated x-y stages available.

2.2 A multiphoton microscope for *in-vivo* imaging of powdered epidermal vaccines.

2.2.1 Major system components

Figure 1 shows a schematic of the multiphoton system. Commercially available optical components were used throughout. These components include a near-infrared multiphoton excitation laser source, a fluorescence laser scanning and detection system and an upright microscope dissected into an upper scope head and modular transmission half. The laser excitation source consisted of a 10 W solid-state pump laser coupled to a tuneable, pulsed Ti:Sapphire crystal laser (Coherent Inc, Ely, UK). The laser output NIR laser pulses with a pulsewidth approximately 150 fs at a repetition rate of 76 MHz [28]. Coupled to the laser source was a Bio-Rad Radiance 2100 multiphoton dedicated (MPD) laser scanning and

detection system (Bio-Rad Cellscience, Hemel Hempstead, UK). The Bio-Rad 2100MPD consisted of a beam conditioning unit (BCU) that incorporated steering mirrors and alignment sights, power meter, spectrum analyser and Pockels cell laser power controller [29]. At output of the BCU, the laser beam was directed through a periscope and an automated beam collimation module to a Nikon E600FN upright objective-focusing microscope (Nikon UK Ltd, Surrey, UK). The beam collimation module expanded the beam to fill the back aperture of the microscope objective lens, ensuring efficient, uniform excitation of the sample specimen across the image plane; it also ensured parfocality between the viewing eyepiece (in widefield imaging) and focus of the NIR laser beam (in multiphoton fluorescence imaging). Scanning in the x-y plane was achieved with galvanometer mirrors that provided up to video-rate image acquisition. Stepper motor control of the objective lens focus enabled scanning along the optical z-axis with a minimum step size of 150 nm. In the multiphoton dedicated system provided by Bio-Rad, there was only a single 670UVDCLP dichroic mirror placed in the excitation path within the infinity focus of the microscope head. This dichroic mirror directed fluorescence emission signal in the UV to visible wavelength range towards direct detectors positioned adjacent to the microscope head. The direct detection system consisted of two bi-alkaline photomultiplier tubes (PMTs) interfaced with filter blocks to capture fluorescence emissions in the UV-blue wavelengths, as well as two multi-alkaline PMTs to acquire fluorescence signal in the green-red wavelength range.

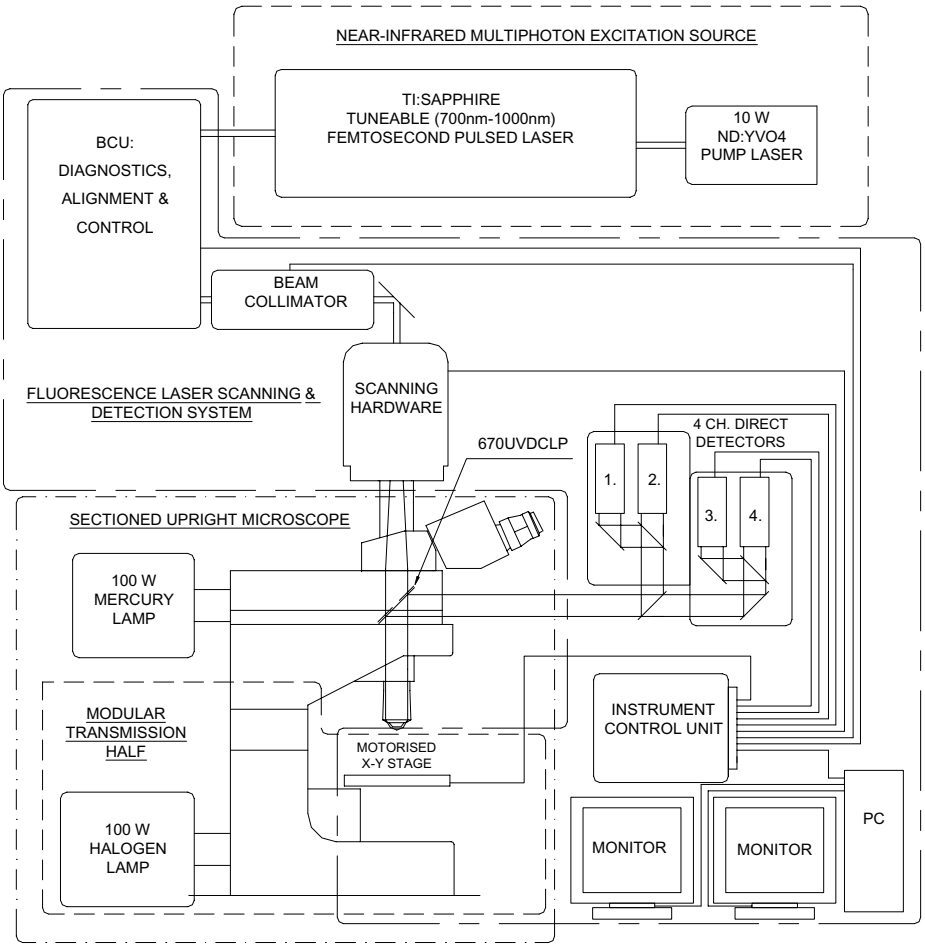

Figure 1: A schematic of the MPLSM system for *ex-vivo*/ *in-vivo* imaging. A conventional upright fluorescence microscope with motorised x-y stage was dissected above the optical imaging plane, providing modular attachment of the transmission half. Removal of this half exposes the objective lens for coupling to large specimens for *in-vivo* imaging. A Ti:Sapphire laser provided femtosecond infrared pulses directed into the microscope via diagnostics and control hardware. A motorized collimation module expanded the laser beam to fill the back aperture of a high N.A. lens. The beam traversed across the sample using galvanometer scanning mirrors. The placement of a dichroic mirror in the infinity focus of the microscope body directed fluorescence emission towards four high-sensitivity direct detectors. Fluorescence emission was split into component bands using interchangeable filter blocks.

When imaging small animal samples, cell culture samples and *ex-vivo* skin samples with the system, the target tissue could be mounted onto a motorised x-y stage (Marzhauser, Wetzlar, Germany). This stage can be configured to map regions of interest within the delivery site "target footprint". Imaging of large animals and humans *in-vivo* can be achieved by removing of the lower half of the microscope and coupling of the live specimen to the objective lens.

2.2.2 Sectioned upright microscope

To provide access under the objective lens for *in-vivo* imaging of large animals and human patients, the microscope was dissected at a plane approximately 25 mm above the mid range focus of a 60x water-dipping lens (Fig. 2). Prior to complete separation of the two scope halves, a 7.50 mm x 3.25 mm (±-0.05 mm) deep recess centred at the dissection plane was machined into the four external sidewalls of the main body. This recess provided reference alignment faces for fitting of a precision alignment adaptor, the purpose of which was to provide simple, accurate re-alignment of the two halves as required. Webbings within the cast aluminium body of the upper half of the microscope were left in place to retain strength. Removal of material from the lower scope webbing provided space for a machined aluminium bracket to be inserted. This bracket enabled rigid clamping of the upper and lower halves as required.

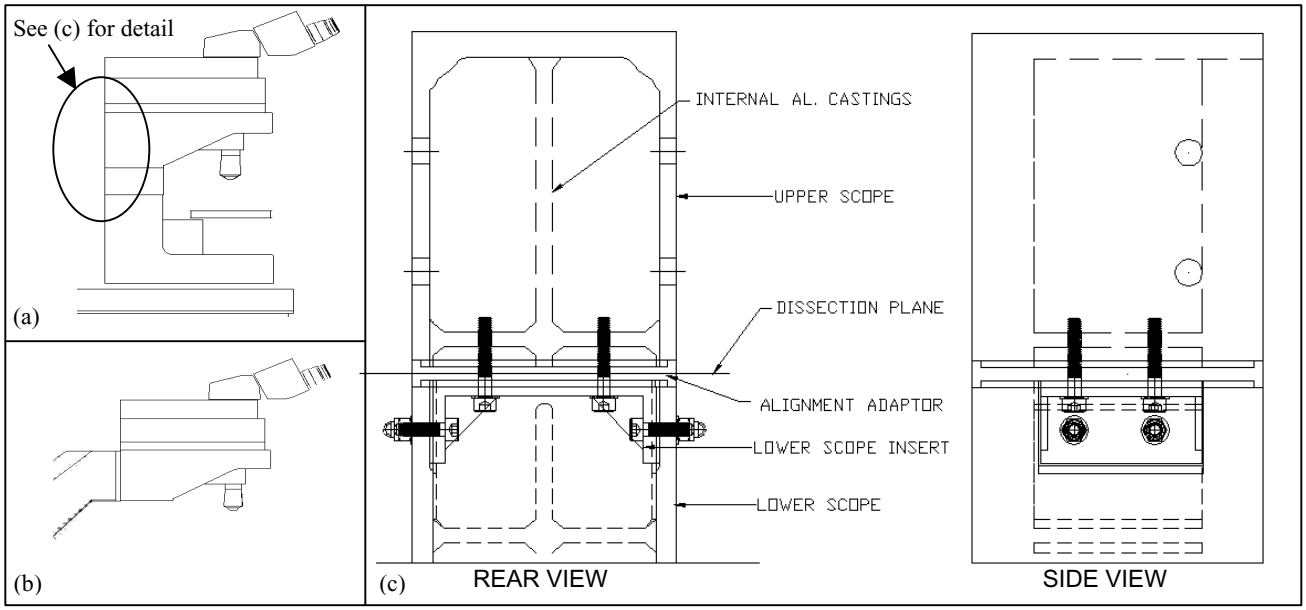

Figure 2: Modular attachment of the transmission half of a Nikon E600FN microscope required modifications to the microscope main body (c-detail). Alignment surfaces were machined into the sidewalls prior to complete separation of the two microscope halves. A precisely machined alignment adaptor (tolerance ±-0.05 mm) relocated the halves against the alignment surfaces for clamping. Webbing in the lower half was removed and a bracket was inserted to enable clamping forces to be transmitted via four M5 screws. The upper and lower halves can be readily separated by removal of these four screws.

2.2.3 Scope support arms

Additional modifications were required to provide lateral space between the objective lens and equipment mounted on the optical table to allow in-vivo models (e.g. volunteer laying on a trolley) to be positioned under the objective lens for imaging. To achieve this goal, the upper half of the microscope, scanning hardware and associated attachments to the microscope head were cantilevered off of the side of the optical table and supported by rigid stainless steel support arms fixed to the table's outer edge (Fig. 3 a, c). The arms provided in excess of 480 mm of lateral clearance between the optical axis of the objective lens and the laser table. Choice of arm material and physical design were governed by material properties including modulus of elasticity and co-efficient of heat transfer.

Under *in-vivo* imaging conditions, body heat transferred into the microscope head via the objective lens would be conducted through the microscope support arms to the optical table, with resulting thermal variations producing instabilities in optical system alignment. A comparison of aluminium alloys and stainless steels revealed the latter material to have a far lower co-efficient of heat transfer through conduction. Carbon composites are also excellent insulators, however the cost of manufacture of an arm made with such material was considered prohibitively expensive.

In normal operating conditions, the support arms would have users and/or patients positioned beneath them, thus they required sufficient strength to ensure zero risk of failure under excessive operational loads. Deflections under normal microscope operation were also to be kept to a minimum. A two-dimensional finite element method analysis showed that support arms manufactured from 2mm stainless steel sheet met deflection and strength criteria for safe and reliable operation. For example, a worse case analysis a design load of 150kg at the outer tip of the support arms (in the event of unintentional user loading against the microscope head) resulted in stresses on the arm of an order of magnitude lower than the yield strength of the material. Under normal operation, a 20 kg design load due to system hardware components produced a 16 μm deflection at the arm's outer tip. This steady-state deflection was easily corrected during initial beam alignment.

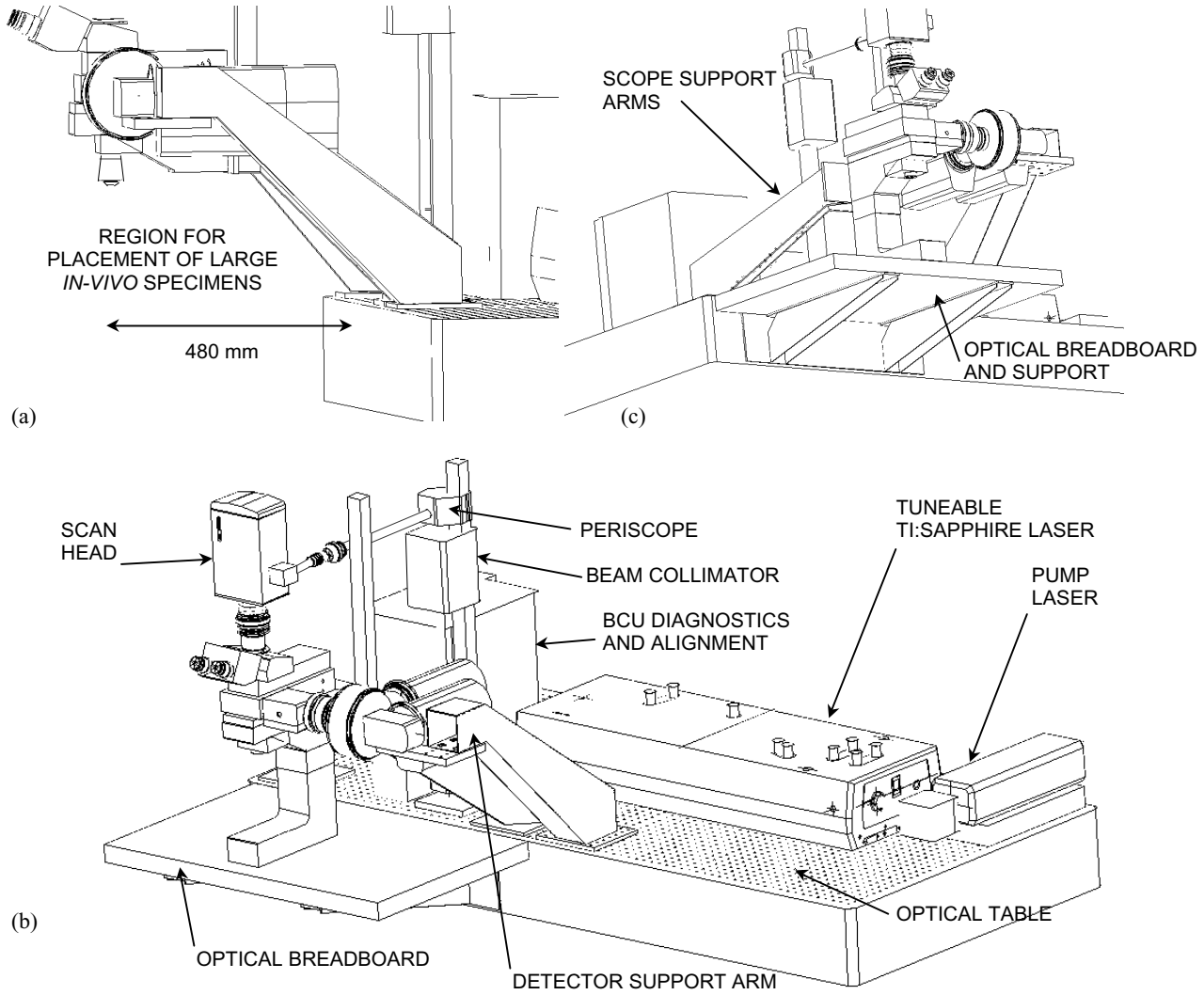

Figure 3: The modified multiphoton microscope. The upper half of a sectioned upright Nikon E600FN microscope is supported off the edge of an optical table by two stainless steel support arms fixed to the table (a). A third arm supports the four channel fluorescence detection system (b). Femtosecond pulses were provided by a tunable Ti:Sapphire laser positioned at the back of the optical table between a pump laser and beam conditioning unit (BCU). The laser beam is directed to the scan head via a beam collimator and periscope. The beam is focused through a high N. A. objective lens into the sample. A modular microscope transmission half can be selectively attached for imaging of small biological specimens to be mounted on a motorized x-y stage (b, c). The transmission half is supported by the upper scope and scope support arms, or via an optical breadboard cantilevered off the side of the table (c).

2.2.4 Detector support arm

An additional support arm provided mechanical support to fluorescence detectors mounted adjacent to the microscope head (Fig. 3 b, c). These arms were also manufactured from welded 2 mm thick stainless steel sheet. Four M10 bolts coupled the direct detectors to the support arm via a small aluminium mount. Alignment in the vertical plane was achieved by the insertion of shims at the coupling surface between the detector mount and support arms. Up to 10 mm of lateral adjustment in the x-y direction was also provided.

2.2.5 Transmission support stage

All components of the imaging system that are mounted to the microscope can be suspended from the upper scope and detector support arms, with forces transmitted to the modular transmission half via the bracket insert described in section 2.2.2. In addition, a small optical breadboard cantilevered from the side of the vibration isolation table can provide support to the microscope as required (Fig. 3 b, c). The inclusion of this redundant support is advantageous should the system need to be kept aligned whilst the main arms are removed for any necessary modifications. The transmission support stage also offers system users a readily accessible area for placement of tools, samples or imaging equipment.

3. RESULTS

3.1 Optical performance:

Figure 4 presents the average power levels (P_{AV}) measured at the exit of the laser and again at the back aperture of the objective lens. These measurements revealed a transmission efficiency through the MPLSM system of approximately 25%, which was approximately constant across the wavelength range from 750-950nm. Measurements of lateral and axial point spread functions (PSF) were also made for a plan- apochromat Nikon 100x, 1.4 N.A. immersion oil objective with enhanced transmission in the infrared region, as per a technique previously described [30]. Sub-resolution (210 nm) fluorescent beads (Polysciences, Warrington, P. A., USA) attached to a coverslip were imaged under NIR multiphoton excitation at 800 nm. The measured full width at half maximum (FWHM) lateral and axial resolutions were 280 ± 65 nm and 800 ± 95 nm respectively.

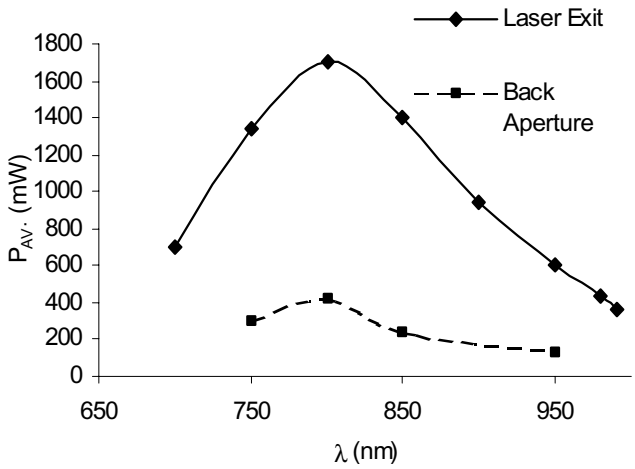

Figure 4: Measurements of average power levels through the system revealed IR transmission efficiency of approximately 25%.

3.2 Imaging with the system

The three-dimensional morphology of epidermal Langerhans cells in a pre-clinical biological model was investigated. Epidermal sheets were acquired from the dorsal surface of the ear of a euthanased mouse, aged six weeks. Prior to mounting on a glass slide with coverslip, each sheet was labeled with an anti-MHC Class II-FITC conjugate and DAPI stain [31]. All images were captured using a 100x oil immersion lens. Simultaneous excitation of both fluorophore labels was achieved by tuning the Ti:Sapphire laser to 800 nm. The average laser power level at the back aperture of the objective lens was attenuated to approx. 20 mW. The skin was imaged from a depth of 7 μm to 40 μm beneath the surface. Fluorescence emission was captured in the blue (410-490 nm) and green (500-530 nm) wavelength ranges using direct detectors in combination with emission filters. Montaged image stacks are presented in Figure 5. Fluorescence

emission in the green channel corresponds to immunolocalized MHC Class II molecules expressed on the surface of epidermal Langerhans cells. The dendrites of these cells are clearly discernable from Figure 5 (a). Fluorescence in the blue channel identifies epidermal cell nuclei labeled with DAPI (Fig. 5 b).

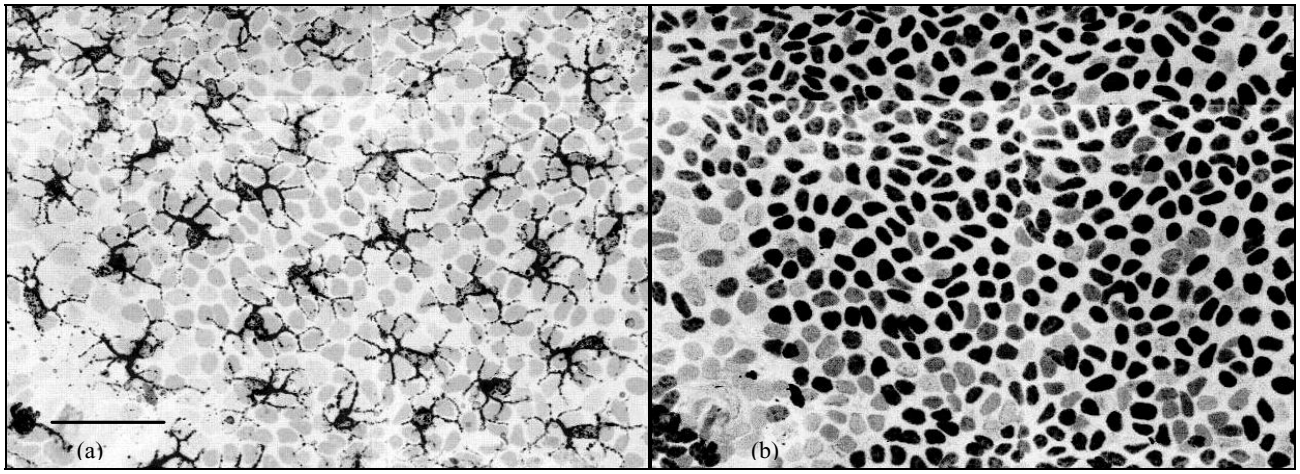

Figure 5: Fluorescence emission acquired in the blue and green channels under multiphoton excitation at 800 nm. Following excision of a murine skin sample, the epidermis was separated and labeled using immunohistochemical fluorescence staining methods. Fluorescence emission from FITC labeled MHC Class II surface molecules specific to Langerhans cells was captured in the green channel (a). Here the dendritic structure of the sub-population of LC resident in the surrounding epidermis can be clearly identified. Images in the blue channel (b) show epidermal cell nuclei labeled with DAPI. Scale bar represents 30 μm.

4. CONCLUSIONS

We have developed a directly coupled multiphoton microscope for *in-vivo* imaging of large animals and humans. A standard Nikon E600FN microscope, dissected above the optical plane was cantilevered from a vibration isolated table using rigid support arms. The modified microscope was coupled to an infrared optimised Bio-Rad Radiance 2100MPD, multiphoton dedicated laser scanning control and image acquisition system. Femtosecond laser pulses were emitted from a 10W Verdi pumped Mira Ti:Sapphire laser, supplied by Coherent Inc. The microscope was modified such that the transmission half could be selectively attached for conventional imaging with *ex-vivo* samples, cell culture samples and small animal models, or decoupled for *in-vivo* imaging of skin sites on the body of humans or large animal models. Preliminary measurements of the optical performance showed overall transmission efficiency through the system of approximately 25% in the wavelength range from 750 nm to 950 nm. The measured full width at half maximum resolution of the system at 800 nm excitation was 280 nm and 800 nm in the lateral and axial planes, respectively. The unique features of this versatile imaging system make it an ideal experimental tool for the investigation of a wide range of biological systems *ex-vivo* and *in-vivo*. Relevant to the study of biolistics and powdered epidermal vaccines, applications include mapping vaccine particles and target cells of the epidermis in three dimensions, tracking of migratory cells to and from the delivery site following vaccination and the study of sub-cellular processes such as cell transfection and gene expression.

Further engineering is required to fully utilize the unique potential of this microscope for *in-vivo* imaging of sites across the body. This work involves the development of a coupling mechanism to prevent inadvertent movement between the skin site of interest and the microscope head during image acquisition. A more detailed characterisation of excitation laser pulse shape and power throughout the optical system will lead to an improved understanding of the relative efficiency of transmission of individual optical components in the beam path. This work will also provide a better understanding of the localised photon fluxes within the femtoliter focal volume of the objective lens that a biological sample may be exposed to. These investigations are crucial to the development of a safe *in-vivo* multiphoton imaging system.

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