# Analysis of microparticle penetration into human and porcine skin: non-invasive imaging with multiphoton excitation microscopy

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## ABSTRACT

At the University of Oxford and PowderJect Pharmaceuticals plc, a unique form of needle-free injection technology has been developed. Powdered vaccines and drugs in micro-particle form are accelerated in a high-speed gas flow to sufficient velocity to enter the skin, subsequently achieving a pharmaceutical effect. To optimize the delivery of vaccines and drugs with this method a detailed understanding of the interactive processes that occur between the micro-particles and the skin is necessary. Investigations to date of micro-particle delivery into excised human and animal tissue have involved image analyses of histology sections. In the present study, a series of investigations were conducted on excised human and porcine skin using the technique of Multi-Photon fluorescence excitation Microscopy (MPM) to image particles and skin structures post-penetration. Micro-particles of various size and composition were imaged with infrared laser excitation. Three-dimensional images of stratum corneum and epidermal cell deformation due to micro-particle penetration were obtained. Measurements of micro-particle penetration depth taken from z-scan image stacks were used to successfully quantify micro-particle distribution within the skin, without invasively disrupting the skin target. This study has shown that MPM has great potential for the non-invasive imaging of particle-skin interactive processes that occur with the transdermal delivery of powdered micro-particle vaccines and drugs.

Keywords: Multiphoton, micro-particle, drug delivery, impact, shock tube, DNA vaccine, skin, epidermis, biolistics.

## 1. INTRODUCTION

In the early 1990s a unique needle-free injection method was conceived at Oxford University [1, 2]. The concept behind this delivery method is to accelerate powdered vaccines and drugs in micro-particle form to sufficient velocity in a high-speed gas flow for penetration into the skin. Advances in research and design at the University and PowderJect Pharmaceuticals plc led to the development of hand-held, clinically effective, pain-free needle-less injection systems [3, 4]. In addition delivery systems based upon shock tube design have been shown to be effective in delivering micro-particles under narrow and controllable velocity ranges and uniform spatial distribution [5, 6]. This technology can deliver particles to specific depths within the skin, enabling the targeted delivery of vaccine and drug material.

The skin itself is a physically and biologically heterogeneous target with various cell populations residing at different depths. The stratum corneum – the skin's outer most layer, consists of flat, keratinized epidermal cells. This cell layer is a major physical barrier to penetration and of primary importance in the ballistic delivery of micro-particle vaccine and drugs. For optimized penetration, micro-particles are to impact the skin with sufficient momentum such that they penetrate the stratum corneum, enabling the active agent to be delivered to cells resident at greater depths. Understanding the mechanics of failure of this layer is fundamental in the design of optimal delivery systems and the development of valid engineering models of the impact processes. These models can identify the properties of the skin such as stratum corneum thickness, density and shear strength that have the greatest influence on the results of a powdered drug admission [7, 8, 9].

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The viable epidermis contains cells that eventually keratinize and move outwards to form the stratum corneum; it also contains specialized cells of the immune system. Langerhans Cells (LCs) represent approximately 2% of all epidermal cells and reside at suprabasal depths in the epidermis [10]. They are extremely effective antigen presenting cells (APCs), responsible for the uptake and processing of foreign materials in order to generate an effective immune response [11, 12]. The delivery of DNA material to LC nuclei using ballistic methods has been effective in providing immunity to a range of diseases in animal laboratory models including hepatitis B and influenza [13, 14]. The application of scientific methods to evaluate the micro-particle distribution within the skin will lead to improvements in the efficiency with which LCs are targetted and further an understanding of epidermal DNA vaccine delivery.

Published results to date report measurements of particle penetration depth and distribution routinely obtained from the analysis of histology biopsies of porcine and excised human skin injected with model drug micro-particles [8, 15]. In these studies model particles of various size and composition such as gold, polystyrene and glass beads are delivered to the skin, representing the physical behavior of typical drug and vaccine payloads during penetration. Models of the impact process derived from the results of these studies show that penetration depth is a function of the particle size, density and impact velocity. These models are key to understanding the mechanics of ballistic vaccine and drug delivery.

Other researchers have investigated the immunological and biochemical processes involved in the initiation of an immune response following micro-particle delivery [13, 14, 17-19]. Typically, neutralizing antibody titers are used to evaluate the strength of the immune response. Reporter genes such as GFP or luciferese plasmids are also used to evaluate the extent and detail of localized transfection within the target site immediately following delivery. The imaging of transfection and subsequent gene expression has, however, been limited to histology sections taken from the footprint.

There are drawbacks to the use of histology methods in the investigation of micro-particle impact. Primarily, histological studies of the skin are limited to excised animal and human samples- live tissue cannot be studied in-vivo [16]. The histology process is also labor intensive: slides are prepared from across the footprint, imaged and analyzed individually using a microscope. In addition, the results obtained are in two dimensions, inhibiting a thorough examination of the skin following micro-particle penetration. This disadvantage of histology is partially mitigated by skin surface mapping using SEM. It is preferable however, to employ a method of evaluating micro-particle distribution in the skin non-invasively.

In comparison to the histology, a non-invasive approach to the analysis of particle impact has a number of advantages. Firstly, disruption to the site such as tearing of the stratum corneum and movement of particles which can occur during the sectioning process, does not exist. When this uncertainty is removed, final particle location can be measured more reliably and more valid observations made of stratum corneum penetration. The freedom to resample an entire footprint using a different sampling pattern is also an advantage of a non-invasive imaging technique. With automation, such a technique would be superior to the image capturing of manually processed histology sections. The reference location of an image in the footprint and hence the particles within that image can be stored directly to digital format, making it an ideal component of an automated 3-dimensional target mapping methodology.

Knowledge of the biological interactions that occur locally between the micro-particles and the surrounding tissue are fundamental to furthering an understanding of this novel drug delivery method. The use of a non-invasive imaging technique to obtain images of the target footprint post-penetration lends itself to the application of imaging live subjects, both animal and human, in real time – a key prospect for the detailed analysis of biological interactions resulting from micro-particle penetration. The analysis of high resolution 3D generated pictures from a footprint can also provide an opportunity to observe structures and processes in the skin such as penetration tracks and skin deformation in much greater detail than two-dimensional images obtained from histology sections.

With the benefits of employing a non-invasive method to investigate the mechanisms of epidermal vaccine and drug delivery realized, an examination of current techniques was undertaken. Reports from the literature identify a number of possible candidates for the non-invasive imaging of micro-particles delivered to the skin. Sonography was successfully used by El. Gammal et. al. [20] to visualize structures in the upper layers of human skin in-vivo. An axial resolution of 8.5μm and a depth of field of 3.2mm were obtained. Stratum corneum and viable epidermal thickness as well as stratum corneum density (as measured by the echoic properties of the layer) were successfully measured.

Gladkova et. al. report the application of Optical Coherence Tomography (OCT) to the imaging of functional and structural processes within the skin in-vivo [21]. Imaging was conducted to a depth of 1.5 mm with a resolution of 15-30μm. Information from the images was used to assist diagnosis of skin diseases associated with structural changes in the epidermis and dermis.

Confocal microscopy (CM) has been successfully used in-vivo in research investigating the structure and morphology of human skin [22, 23]. In these studies the stratum corneum, epidermal and dermal cells were clearly imaged with the technique. Images obtained are comparable to those of conventional histology, with the added benefit of being non-invasive. CM does however suffer from the effects of photobleaching and photodamage due to the use of ultraviolet (UV) laser light [24]. These effects extend above and below the focal plane in which fluorescence is collected with CM.

Multiphoton microscopy (MPM), a technique first demonstrated in 1990 [25] has found application with many researchers for the non-invasive imaging of biological structures including skin [24, 26-29]. As compared to CM, MPM is less invasive since only the focal plane 'sees' the energy equivalent to short wavelengths, the rest of the tissue volume 'sees' single photons of near-infrared energy. Many advantages are provided with MPM, including reduced phototoxicity, increased penetration depth and the ability to excite fluorophores in the UV spectrum without using UV laser excitation [26, 27]. The technique has ideal characteristics for the non-invasive imaging of micro-particles and skin structures. Collagen and elastin fibers of the dermis possess broad single-photon excitation bands between 335-440nm, with epidermal structures excited at 270-295nm [30]. It is expected that microparticles containing organic molecules will also fluoresce with UV excitation.

In this paper we report on a feasibility study configured with the aim of assessing the applicability of non-invasive imaging with MPM to the biolistic study of epidermal vaccine and drug delivery. To meet this goal we will examine the application of the technique at two levels. The first objective of this paper is to assess the level of resolution, contrast, imaging depth and detail of micro-particles delivered to the skin. Micro-particles of various compositions are delivered to the skin and imaged in detail with the MPM system. The advantage of imaging skin structural detail in three-dimensions is also realized, with penetration tracks, skin structures and skin deformation imaged in 3 dimensions around micro-particles. Following this qualitative approach, the practical aspects of the MPM technique are examined in the context of the development of a high-throughput, routine target mapping procedure. Images taken from across the footprint are analyzed to obtain quantitative data of micro-particle position within the target. This information is used to characterize the performance of a delivery system in the skin target.

## 2. MATERIALS AND METHODS

### 2.1 SKIN TARGET SAMPLES

Micro-particles were delivered into porcine and excised human skin. The human skin was harvested by dermatome from cadaver, before cryo-preservation and storage at –80°C. The skin was then thawed, washed, rehydrated from beneath and reheated to 37°C. After micro-particle delivery the skin was washed with 0.1 mole Phosphate Buffered Solution (PBS) and fixed in 4% Paraformaldehyde for later imaging with the MPM system.

As part of two larger independent studies conducted by PowderJect plc, micro-particles were delivered to three anesthetized pigs aged between 12 and 16 weeks of age. Particles were delivered to the flank and inguinal regions, with each delivery site circumscribed by a 13mm circle outlined in ink. Following delivery the animals were euthanased. The delivery sites were then excised and punch biopsied. The skin's elasticity and subsequent shrinkage under relaxation made it necessary to place each biopsy between a glass slide and wire gauze with compression applied to return the biopsy to its original size (the 13mm diameter mark was used as a reference measurement). The samples were then washed in 0.1 mole PBS, before fixing in 4% Paraformaldehyde. After fixing, the samples were removed from the glass slide and placed in fresh Paraformaldehyde for storage and later imaging with the MPM system.

## 2.2 MICRO-PARTICLE DELIVERY SYSTEMS

Variants of the Contoured Shock Tube (CST) device family described in Kendall [5] were used to deliver model micro-particles. A schematic of such a device is shown in Figure I.

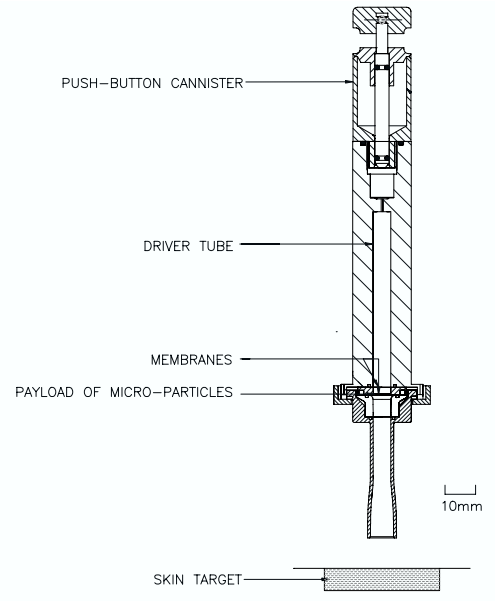


Figure I: A version of the contoured shock tube [5, 6].

Prior to actuation the micro-particles are filled in a cassette sealed between two polycarbonate diaphragms. The loaded cassette is then placed in the device. The gas reservoir is filled with helium to between 4 and 6 MPa. Actuation of the device results in the release of helium gas from the high-pressure reservoir, subsequently filling the driver volume and loading the diaphragms of the cassette to rupture. Rupturing of the diaphragms initiates a quasi-steady accelerating flow through the shock tube. The micro-particles are entrained in the gas flow and thus accelerated down the shock tube and out of the exit of the device, where they penetrate the skin target.

Model micro-particles of various size, density and composition were used in this study. Fluorescent polystyrene beads (Duke Scientific Corp., Palo Alto, C.A., U.S.A.) with an average size 48 μm and density 1.05 kg/m$^3$ were delivered with an impact velocity of 305 m/s. Agarose beads with an average size of 50 μm, density of 0.6 kg/m$^3$ were delivered with an impact velocity of approx. 500 m/s. Gold micro-particles with a mean size of 1.82 μm and density 18700 kg/m3 were delivered at approximately 600 m/s. 0.5 mg payloads of micro-particles were used in each condition.

## 2.3 IMAGE ACQUISITION AND ANALYSIS

All samples were imaged using a standard Radiance 2000MP multiphoton laser scanning system with 1 bi-alkaline and 2 multi-alkaline direct detectors (Bio-Rad Microscience Ltd, Hemel Hempstead, U.K.) attached to a TE300 Microscope (Nikon UK Ltd, Surrey, U.K.). A 60x 1.2 N.A. plan-apochromat water immersion lens (Nikon) and 10x 0.45 N.A. plan-apochromat air lens (Nikon) were used as focusing objectives. An 8W, Verdi pumped, Mira 900 Ti:Sapphire laser (Coherent Inc, Ely, U.K.) was tuned to provide laser excitation between 910 and 920nm, pulse width 100fs, repetition rate of 80MHz. The maximum laser power emmitted at the exit of the objective lens was approximately 120mW. Neutral density filter wheels limited the laser to between 10-100% of this value depending upon the depth of the focal plane in the tissue.

Blue (450/80), Green (515/30) and Red (620/100) emission filters (Bio-Rad) were used with three direct detectors to separate the fluorescence emitting from the samples into three component bands. A single bi-alkaline PMT was used with (UV670LP) filters for single channel detection in the UV to red wavelength range.

All skin samples were viewed through a No. 1.5 coverslip. A drop of water was placed between the surface of the skin and the coverslip. Objective focusing within the sample was controlled by a z-axis stepper motor drive (Bio-Rad). Images and image stacks were captured with LaserSharp software (Bio-Rad) as 512x512 pixel, 8-bit grayscale images. Image quality was improved by Kalman filtering over 2 to 30 frames.

All images were processed using MetaMorph analysis software (Universal Imaging Corp, Downingtown, P.A., U.S.A.).

### 2.4 EXPERIMENTAL DESIGN

Table 1 summarizes the experimental configurations under which each image set was captured. Configurations A and B were chosen to assess the resolution of the system and its ability to image detailed structures and conditions of the skin and particles beneath the surface. Cofigurations C and D were chosen to capture images within a reasonable time frame, in order to evaluate the system's applicability for high-throughput, routine target sampling.

| Configuration | Target tissue | Micro-particle | Excitation wavelength (nm) | Emmission filters | Objective lens | Scanning rate (l.p.s) | Kalman | z-axis image separation (μm) | maximum image depth (μm) |
|---|---|---|---|---|---|---|---|---|---|
| A | Porcine | Agarose | 910 | UV670LP | 60x | 500 | 30 | 0.4 | 64 |
| B | Porcine | Gold | 912 | B,G,R | 60x | 500 | 20 | 0.4 | 50 |
| C | Porcine | Agarose | 910 | UV670LP | 60x | 500 | 5 | 10 | 120 |
| D | Human | Fluor. Polysty. | 920 | B,G,R | 10x | 166 | 1 | 2 | 110 |

Table I: Experimental conditions for MPM investigation. Configurations A and B were selected for fast sampling

## 3. RESULTS AND DISCUSSION

### 3.1 HIGH RESOLUTION IMAGING

High-resolution images of agarose beads delivered to the skin were acquired with the MPM system. Figure II shows one of 162 images taken from a 200x200x64μm stack that extends from the surface through to the dermal layer at 0.4μm intervals (configuration A). In the center of the image is an agarose bead contained within the epidermis. The agarose bead is clearly distinguishable by its dark center and bright circumference. The images reveal distorted epidermal cells around the immediate perimeter of the particle, which appear to have deformed elastically to accommodate the micro-particle. Dermal papillae can be seen protruding through in certain areas of the image. The fibers of these papillae are relatively bright and readily visible in the image. In regions surrounding the papillae the epidermal cell density is greater. These observations reflect the natural non-uniformity of epidermal thickness within normal skin.

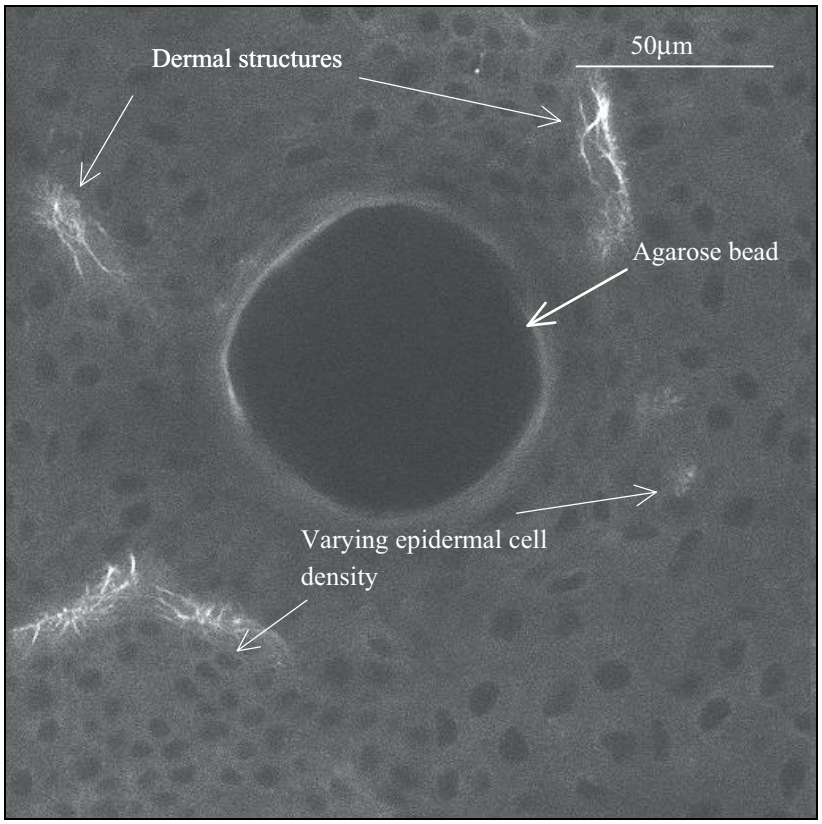


**Figure II:** 50 μm agarose bead delivered to the epidermis captured in configuration A. Image depth 42 μm.

Gold particles delivered to porcine skin were also imaged in three channel direct detectors under multiphoton excitation at 912nm in configuration B. Figure III shows blue (b), green (c), red (d) and color combined 3-channel (a) images from within the sample. The gold particles are brightest in the blue spectrum and can be seen in the red and green channels also. Dermal papillae show strong emission in the blue band only. A number of gold particles can be seen in the nuclei of epidermal cells.

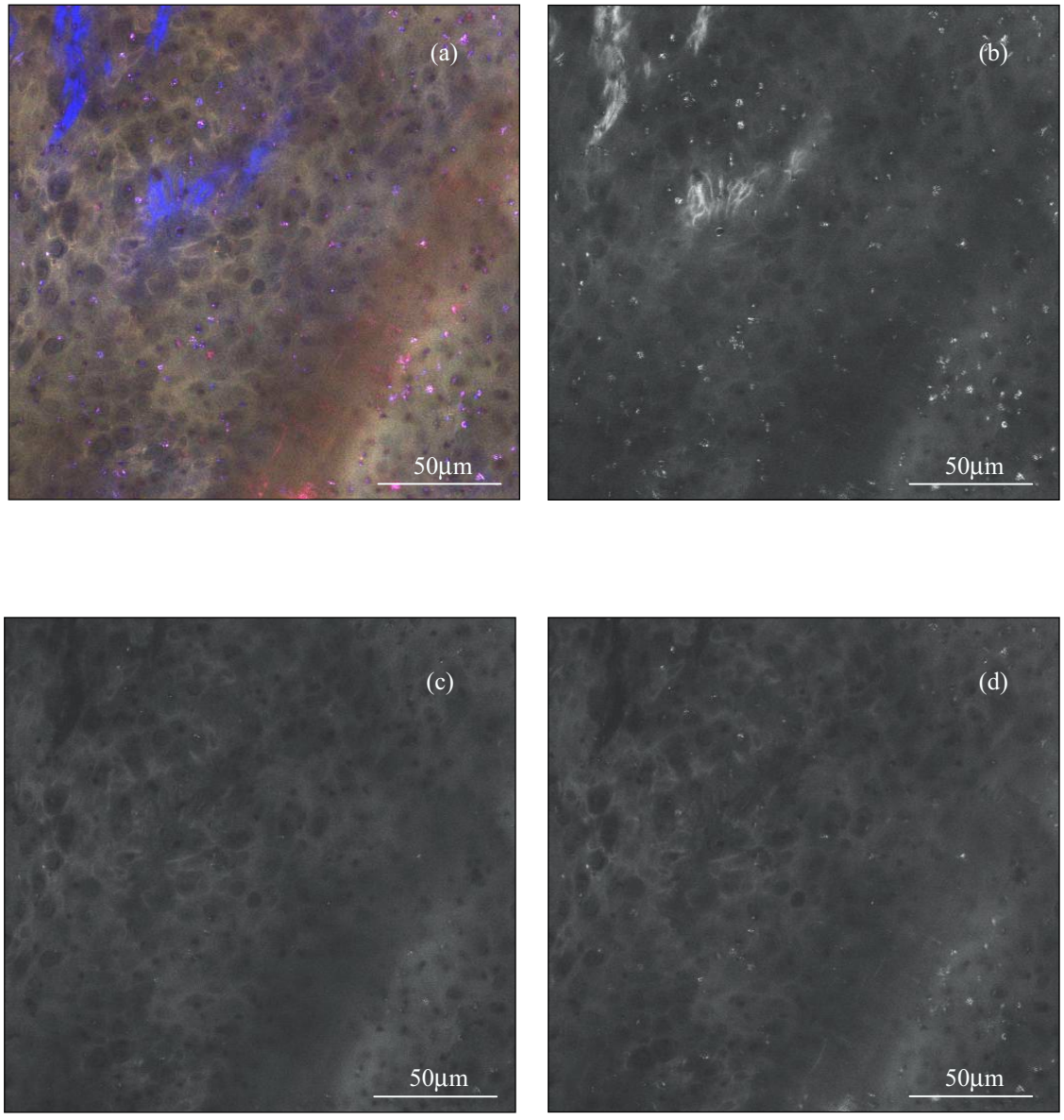


**Figure III**: Gold micro-particles delivered into the epidermis. Colour combined (a), blue (b), green (c) and red (d) channel images captured from 27µm beneath the surface. Configuration B.

Qualitative information was also obtained from the images across the footprint in configuration C. A series of slides taken from near the center of the footprint at depths of 0, 5, 10, 20 and 30μm from the surface are shown in figure IV (a to e). Images IV (a) and (b) show a hole in the stratum corneum through which two agarose beads have penetrated and come to rest adjacent to one another (IV (c), (d) and (e)). The hole appears to take the form of one or more hexagonally shaped squames of the stratum corneum.

The images show two important phenomena of the impact process. Firstly, the shape of the penetration track in the stratum corneum reflects intercellular as opposed to intra-cellular failure of stratum corneum cells under impact. This is expected since the intercellular matrix of the stratum corneum exhibits less strength than the cells of the stratum corneum themselves [7, 9]. Combined with the results of Figure II, this shows that during penetration agarose beads push apart cells of the epidermis as opposed to disrupting their internal structure. Secondly, the close proximity of the particles suggests that a particle-particle collision has occurred during impact. This acts to push the first of the two particles deeper into the skin.

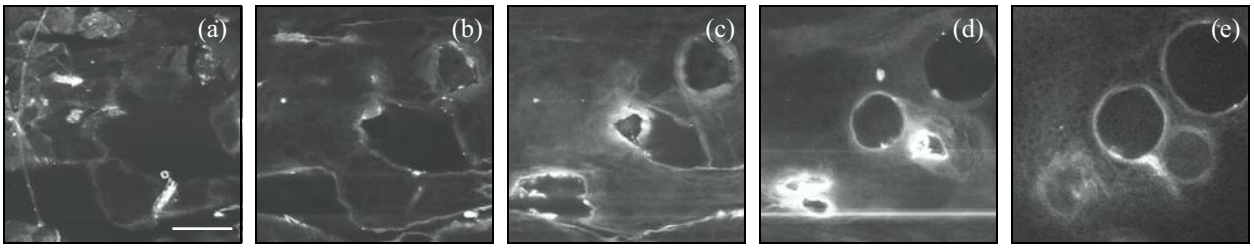


**Figure IV**: A series of images taken at the surface (a) and depths of 5μm (b), 10μm (c), 20μm (d) and 30μm (e) beneath the surface of the skin showing penetration tracks behind agarose beads delivered to the epidermis. Configuration C. Scale bar represents 50μm.

### 3.2 QUANTITATIVE TARGET MAPPING

MPM has been used to capture images of skin structures and micro-particles at high resolution. Following these results an evaluation of the system's ability to capture large amounts of data for the assessment of particle distribution was conducted. Figure V shows an image captured in the blue channel from a position 46μm beneath the surface of the skin of polystyrene spheres delivered to the epidermis. The capture time for each 1.24 x 1.24mm image frame was 3.1s when using the 10x lens. The deepest particles were seen at depths of approximately 85μm. In contrast to the level of fluorescence emitted by endogenous fluorophores of the epidermis, the fluorescent spheres were extremely bright. Very similar results were found in the green and red channels, indicating the polystyrene beads had a broad emission spectrum, along with a relatively high brightness as compared to the skin across all channels. Due to the order of magnitude difference in brightness between the beads and the skin, it was not possible to view skin detail in these images.

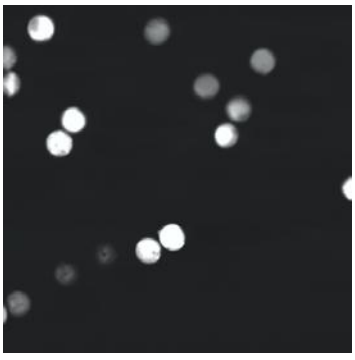

**Figure V**: Excitation with 920nm pulsed laser light of 48μm polystyrene spheres delivered to the epidermis (configuration D). Image depth: 46μm. Image size: 1.24mm

Beads that penetrated deeper into the skin appeared darker in the same image plane. This result suggests that the beads were more transparent than the skin tissue, although the exact absorption and scattering properties of the beads were not measured.

Figure VI outlines the sampling methodology used with a porcine skin target to analyze agarose micro-particle distribution across the footprint non-invasively. A total of 52 image stacks were captured with a 60x lens (image size 206x206µm) and montaged to form a collection of virtual transverse "slices" through the diameter of the footprint. The image stacks traversed from the surface to a maximum depth of 120µm within the skin at 10µm intervals.

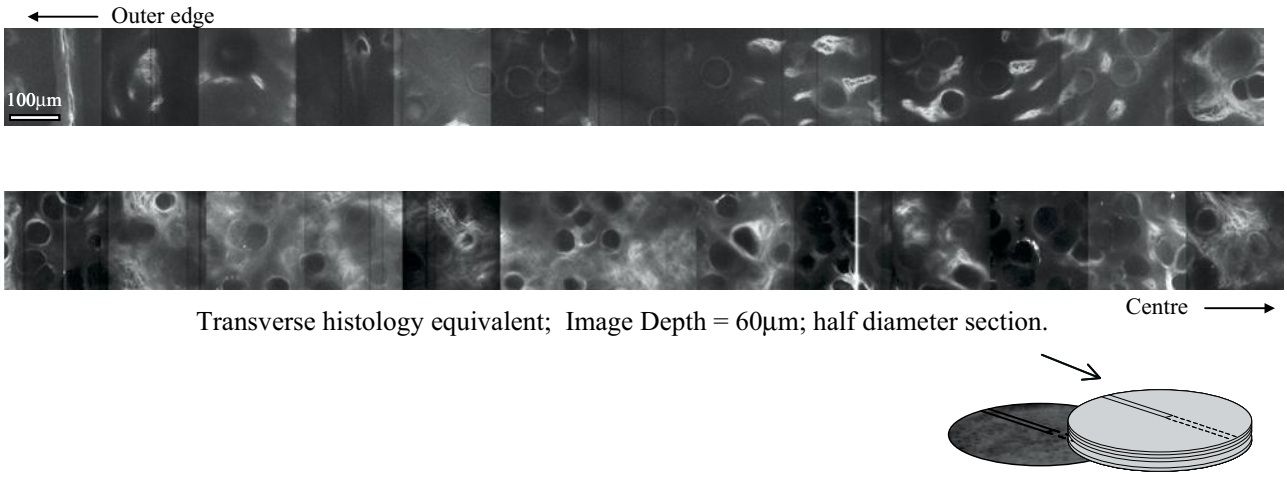


**Figure VI**: In configuration C, the MPM system captured a virtual slice through the footprint of a porcine skin sample targeted with agarose beads. Viewed from above, the half diameter section shown was captured at a depth of 60µm.

Individual agarose bead micro-particles were identified in the images as dark circles with light circumferences. Lateral bead position was measured to the nearest image frame, relative to the edge of the footprint. Depth positioning within the sample was measured by evaluating the frame in which the diameter of the bead was largest, providing an estimate of the bead's center to the nearest frame 10µm (frame separation in the z direction). A measurement of the bead radius was then taken and added to this value, giving the distance from the surface of the skin to the bottom face of the bead. In this way the depth position of each bead in the sample slice was measured, producing the penetration distribution profile within the sample slice as shown in figure IV below.

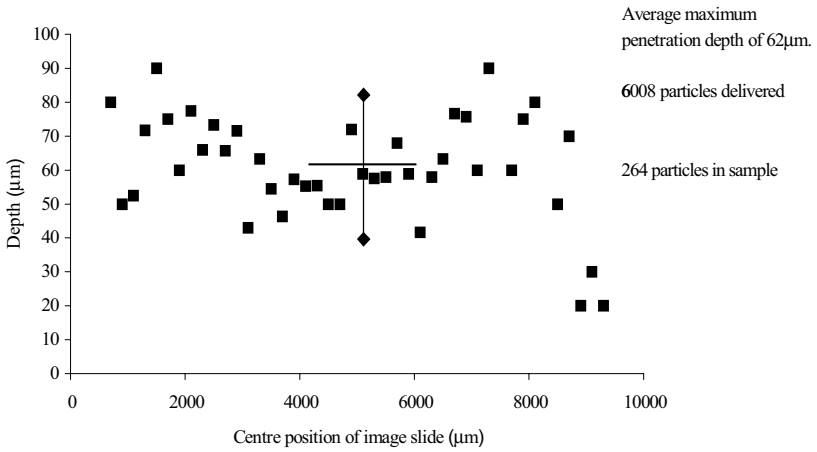


**Figure VII**: Penetration distribution profile of agarose beads delivered to a porcine skin sample. Configuration C.

A total of 264 particles were counted in the virtual slice through the footprint, providing estimates of the mean (62μm) and standard deviation (21μm) of the penetrated particles. Knowing the position of each particle within the slice also allowed the total number of particles in the footprint to be estimated at over 6000.

## 4. CONCLUSIONS

Micro-particles delivered to the skin with needle-less injection technology have been imaged non-invasively with multiphoton microscopy. This achievement represents a significant advancement in the investigation of ballistic epidermal vaccine and drug delivery. Endogenous fluorophores of micro-particles and skin structures were excited with infra-red light. Cell deformation and penetration tracks caused by micro-particle delivery were identified in images captured with the system in three dimensions. Images also provided data for the quantitative analysis of micro-particle distribution in a target footprint. The results presented in this paper identify further applications of the MPM technique in the investigation of micro-particle vaccine and drug delivery.

With automation it is expected that the MPM technique will enable the mapping of complete footprints of micro-particles delivered to the skin in a routine and non-invasive manner, furthering an understanding of the characteristics of particle distribution necessary for optimized vaccine and drug delivery. Further investigations will determine the ability of MPM to image the processes of cell transfection and drug dissolution and diffusion non-invasively, over time, in-vivo. Such studies will provide information fundamental to furthering an understanding of the vaccine and drug delivery process and will also realize the potential use of MPM as a key investigative tool in this exciting field of research.

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## REFERENCES

1. B. J. Bellhouse, D. F. Sarphie and C. J. Greenford, "Needle-less syringe using gas flow for particle delivery," *International patent WO94 24263*, 1994.
2. B. J. Bellhouse, N. J. Quinlan and R. W. Ainsworth, "Needle-less delivery of drugs, in dry powder form, using shock waves and supersonic gas flow," *Proc. 21st Int. Symp. Shock Waves*, 1997.
3. T. J. Burkoth, B. J. Bellhouse, G. Hewson, D. J. Longridge, A. G. Muddle and D. F. Sarphie, "Transdermal and transmucosal powdered drug delivery," *Crit, Rev. in Drug Carrier Syst.*, **16**, 331-384, 1999.
4. M. A. F. Kendall, N. J. Quinlan, S. J. Thorpe, R. W. Ainsworth and B. J. Bellhouse, "The Gas-Particle Dynamics of a High-Speed Needle-free Drug Delivery System," Proc. 22nd Int. Symp. Shock Waves, 605-610, 1999.
5. M. A. F. Kendall, "A shock tube-based system for the delivery of powdered vaccines and drugs: From concept to technology platform," *Plenary Lecture, 23rd Int. Symp. on Shock Waves*, 2001.
6. M. A. F. Kendall, G. L. Brown and B. J. Bellhouse, "Needle-free, dermal delivery of dry powdered drugs using contoured shock tubes," *Fluids 2000*, 2000.
7. P. Wrighton Smith, *D Phil Thesis, Uni. Oxford*, 2001.
8. M. A. F. Kendall, P. J. Wrighton Smith, B. J. Bellhouse, "Transdermal ballistic delivery of micro-particles: Investigation into skin penetration," *Proc. World Cong. Of Med. Phys. Biomed. Eng.*, 2000.
9. T. J. Mitchell, M. A. F. Kendall and B. J. Bellhouse, "Micro-Particle Penetration to the Oral Mucosa," *ASME 2001 Bioeng. Conf.*, 2001.
10. J. Bauer, F. A. Bahmer, J. Worl, W. Neuhuber, G. Schuler and M. Fartasch, "A strikingly constant ratio exists between langerhans cells and other epidermal cells in human skin. A stereologic study using the optical dissector methods and the confocal laser scanning microscope," *J. Invest. Dermatol.*, **116**, 313-318, 2001.

10. J. Bauer, F. A. Bahmer, J. Worl, W. Neuhuber, G. Schuler and M. Fartasch, "A strikingly constant ratio exists between langerhans cells and other epidermal cells in human skin. A stereologic study using the optical dissector methods and the confocal laser scanning microscope," *J. Invest. Dermatol.*, **116**, 313-318, 2001.
11. E. C. McKinney and J. W. Streilein, "On the extraordinary capacity of allogeneic epidermal Langerhans cells to prime cyto-toxic T-cells in vivo," *J. Immunol.*, **143**, 1560-1564, 1989.
12. L. Timares, A. Takashima and S. A. Johnston, "Quantitative analysis of the immunopotency of genetically transfected dendritic cells," *Proc. Natl. Acad. Sci.*, **95**, 13147-13152, 1998.
13. D. C. Chen, R. L. Endres, C. A. Erickson, K. F. Weis, M. W. McGregor, Y. Kawaoka and K. F. Payne, "Epidermal immunization by a needle-free powder delivery technology: Immunogenicity of influenza vaccine and protection in mice," *Nature Medicine* **6**, 1187-1190, 2000.
14. M. J. Roy et. al. "Induction of antigen-specific CD8+ T cells, T helper cells and protective levels of antibody in humans by particle-mediated administration of a hepatitis B virus DNA vaccine," *Vaccine* **19**, 764-778, 2001.
15. M. A. F. Kendall, T. J. Mitchell, M. P. Hardy and B. J. Bellhouse, "The ballistic delivery of high density, high velocity micro-particles into excised human skin," *Proc. ASME 2001 Bioeng. Conf.*, 2001.
16. M. A. F. Kendall, F. V. Carter, T. J. Mitchell and B. J. Bellhouse, "Comparison of the transdermal ballistic delivery of micro-particles into human and porcine skin," *Proc. 23rd Int. Conf. IEEE Med. Biol. Soc*, 2001.
17. J. K. Burkholder, J. Decker and N. Yang, "Rapid transgene expression in lymphocyte and macrophage primary cultures after particle bombardment-mediated gene transfer," *J. of Immunol. Methods* **165**, 149-156, 1993.
18. B.I. Loehr, P. Willson L. A. Babiuk and S. van Drunenlittel-van Denhurk, "Gene gun-mediated DNA immunization primes development of mucosal immunity against bovine herpesvirus 1 in cattle," *J. Virol.*, **74**, 6077-6086, 2000.
19. D. L. Lodmell, N. B. Ray, L. C. Ewalt, C. A. Hanlon, J. H. Shaddock, D. S. Sanderlin and C. E. Rupprecht, "DNA immunization protects nonhuman primates against rabies virus," *Nature Medicine* **4**, 949-952, 1998.
20. S. El Gammal, C. El Gammal, K. Kaspar, C. Pieck, P. Altmeyer, V. Vogt and H. Ermert, "Sonography of the skin at 100Mhz enables in vivo visualization of stratum corneum and viable epidermis in palmar skin and psoriatic plaques," *J. Invest. Dermatol.*, **113**, 821-829, 1999.
21. N. D. Gladkova et. al., "In vivo optical coherence tomography imaging of human skin: norm and pathology," *Skin Res. Tech.*, **6**, 6-16, 2000.
22. B. R. Masters and P. T. C. So, "Confocal microscopy and multiphoton excitation microscopy of human skin in vivo," *Optics Express* **8**, 2-10, 2001.
23. M. Rajadhyaksha et. al., "In vivo confocal scanning laser microscopy of human skin 2: Advances in instrumentation and comparison with histology," *J. Invest. Dermatol.,* **113**, 293-303.
24. K. Konig, "Multiphoton microscopy in life sciences," *J.Microsc.* **200 (2),** 83-104, 2000.
25. W. Denk, J. H. Strickler, W. W. Webb, "Two-photon laser scanning fluorescence microscopy," *Science,* **248**, 73-76, 1990.
26. B. R. Masters, P. T. C. So, K. H. Kim, C. Buehler and E. Gratton, "Multiphoton excitation microscopy, confocal microscopy and spectroscopy of living cells and tissues: Functional metabolic imaging of human skin in vivo," *Methods Enzym.* **307**, 513-536, 1999.
27. N. S. White and R. J. Errington, "Improved laser scanning fluorescence microscopy by multiphoton excitation," *Advances Imag. Elect. Phys.*, **113**, 249-277, 2000.
28. B. R. Masters, P. T. C. So and E. Gratton, "Optical biopsy of in vivo human skin: Multiphoton excitation microscopy," *Lasers Med. Sci.***13**, 196-203, 1998.
29. P. T. C. So, H. Kim and I. E. Kochevar, "Two-photon deep tissue ex vivo imaging of mouse dermal and subcutaneous structures," *Optics Express* **3**, 339-350, 1998.
30. R. Gillies, G. Zonios, R. R. Anderson and N. Kollias, "Fluorescence excitation spectroscopy provides information about human skin in vivo," *J. Invest. Dermatol.* **115**, 704-707, 2000.