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Cellular metabolism and pore lifetime of human skin following microprojection array mediation

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Keywords
Skin, 3D confocal microscopy; pore lifetime; metabolic lifetime; microneedles; drug delivery; histamine sensitisation, FLIM
Graphical Abstract
Abstract
Skin-targeting microscale medical devices are becoming popular for therapeutic delivery and diagnosis. We used cryo-SEM, fluorescence lifetime imaging microscopy (FLIM), autofluorescence imaging microscopy and inflammatory response to study the puncturing and recovery of human skin *ex vivo* and *in vivo* after discretised puncturing by a microneedle array (Nanopatch®). Pores induced by the microprojections were found to close by ~25% in diameter within the first 30 minutes, and almost completely close by ~6 hours. FLIM images of *ex vivo* viable epidermis showed a stable fluorescence lifetime for unpatched areas of ~1000 ps up to 24 hours. Only the cells in the immediate puncture zones (in direct contact with projections) showed a reduction in the observed fluorescence lifetimes to between ~518-583 ps. The ratio of free-bound NAD(P)H (α1/α2) in unaffected areas of the viable epidermis was ~2.5-3.0, whereas the ratio at puncture holes was almost double at ~4.2-4.6. An exploratory pilot *in vivo* study also suggested similar closure rate with histamine administration to the forearms of human volunteers after Nanopatch® treatment, although a prolonged inflammation was observed with Tissue Viability Imaging. Overall, this work shows that the pores created by the microneedle-type medical device, Nanopatch®, are transient, with the skin recovering rapidly within 1-2 days in the epidermis after application.
1. Introduction

Microscale medical devices are becoming popular for their ability to target various skin sites, for the delivery of vaccines and drugs, to monitor analytes through skin and to detect cancer cells in clinical settings. These miniaturised devices are designed to access the skin with minimal invasiveness and discomfort, and so promote faster recovery and healing of skin than is possible for more invasive and painful devices, like needles and syringes.

We have previously shown that the Nanopatch®, a high-density microneedle (10,000-20,000 projections per cm², ~110-250 µm in length, ~20-40 µm in base width) vaccine delivery device, caused cellular damage in a mouse model that was limited to the immediate and proximate surrounding cells that were in direct contact with the microneedle impact. The Nanopatch® is applied dynamically at high velocities of >3 m s⁻¹. In contrast, the Dermaroller microneedle device punctures facial skin by a hand driven quasistatic rolling motion. Recovery from the skin damage induced by this latter device, used mainly to promote anti-ageing, takes 3-4 weeks. It is hypothesised that discretised cell damage leads to programmed cell death (PCD) and, in turn, stimulates the immune response through alarmins released to neighbouring cells. In general, microneedle devices cause less trauma than larger, more invasive devices, like hypodermic needles.

In this study, we first investigated the skin viability and response to Nanopatch® application in an ex vivo human skin model. We used fluorescence lifetime imaging microscopy (FLIM), a method that quantifies the viability of cells through the decay of their endogenous fluorescence, to quantify the regions punctured by microprojections and surrounding areas. FLIM was performed from the surface down to 100 µm over the 24-hour period following Nanopatch® application. FLIM has been used to quantitate the effects of sunscreen and other formulations topically applied on skin, and the viability and suitability of ex vivo skin after storage, but not the effect of microneedles on the skin. These studies focused on monitoring the autofluorescence in the viable epidermis (VE) region of the skin as a measure of the epidermal redox state, whereas dermal collagen mainly provides morphological data.

We also tracked the pore lifetimes (hole closure rates) of microprojection-treated skin using multiphoton microscopy over 30 minutes. Pore lifetimes for conventional sized
microneedles are reported to range from hours\textsuperscript{15} to days\textsuperscript{16}, with the longer time frame being of concern in terms of skin infection susceptibility following drug delivery. For example, Enfield et al.\textsuperscript{17} used non-invasive optical coherence tomography (OCT) to quantify pore closure, by measuring a decreasing pore depth from an average of 160 µm to 80 µm over 90 minutes after microneedle (~280 µm long, ~150 µm wide at base) puncturing. Previous work on large microneedles showed that volunteer skin resealed 2 hours after microneedle (~500 - 1500 µm long, ~200 - 500 µm wide at base) application\textsuperscript{18}. The ability to extend pore lifetime, particularly important for “poke and patch” style microneedle devices, has also been demonstrated\textsuperscript{15,19,20}. In this work, we have also examined the change in pore width over time that is more readily quantified with the thinner Nanopatch\textsuperscript{®} microneedles and the use of multiphoton microscopy.

Finally, we investigated skin recovery \textit{in vivo}, in an exploratory pilot clinical case study that is close to real-life scenarios, by measuring skin recovery and erythema reduction over 48 hours after deliberate histamine provocation at the Nanopatch\textsuperscript{®}-mediated site. In this case study, we used tissue viability imaging (TiVi), which quantifies the erythema induced in superficial capillaries of skin through subsurface polarised light spectroscopy\textsuperscript{21}. TiVi has been used to examine cutaneous microcirculation after cryotherapy\textsuperscript{22}, heat therapy\textsuperscript{21}, drug delivery through iontophoresis\textsuperscript{23}, hyaluronic acid and histamine provocations\textsuperscript{24}, but not in relation to microneedle mediation. It is hypothesised that this method may provide valuable data because of the potential of pro-inflammatory stimulation induced by high-density microneedles\textsuperscript{25}. We therefore combined TiVi imaging with histamine provocation. The use of histamine in a skin prick test is a conventional method to evaluate allergic reaction on the subject\textsuperscript{26}. In this case, the ‘prick’ is carried out using the Nanopatch\textsuperscript{®}, and it is hypothesised that the micropores created will allow exposure to the allergen. It is further hypothesised that due to the microscopic pores and the limited cellular damage induced by the Nanopatch\textsuperscript{®}, pores should close and seal quickly within hours, which can be explored using a microneedle-mediated TiVi-histamine erythema case study.
2. Results

2.1 Cryo-SEM assessment of puncture hole aperture

Cryo-SEM images of poration of skin surfaces as a result of microprojection penetration are shown qualitatively at different magnifications in Figure 1 (a-c). Larger projection holes were found towards the edges of the patched area and all projection holes were circular in shape.

![Figure 1. (a-b) Representative SE Cryo-SEM images of ex vivo human skin with a top-down view of puncture holes created by the Nanopatch® application, at 25x and 75x (cropped), respectively. (c) Representative SE Cryo-SEM image of a top-down view of a single puncture hole created by Nanopatch® application at 1000x.](image)

2.2 Cellular viability of skin specimens post Nanopatch® application

Representative FLIM images of the stratum corneum (SC), stratum granulosum (SG), stratum spinosum (SS), and stratum basale (SB) acquired prior to and at four time points after application of Nanopatch® are displayed in Figure 2 (a). The SG, SS and SB are clearly identified by the morphology and density of the keratinocytes in each of these layers. Patching of skin with the Nanopatch® created puncture spots or holes in the skin as observed in the FLIM images. The SC was torn where the microprojections pierced the skin and puncture holes were created where the microprojections pierced the VE. These puncture holes exhibited a blue pseudocolouring, which indicated lower lifetime values. At 24 hours post patching, puncture holes appeared to be obscured or covered up. Cells surrounding the puncture holes appeared to have retained their morphology at all time points.

2.3 Assessing changes in induced pores over time using fluorescence lifetimes

The fluorescence lifetime images of the VE before and after poration over time, as shown in Figure 2 (a), can also be used to assess changes in the induced pores over time. It is apparent that the lower lifetime associated with a pore, as depicted by closed blue circular regions in Figure 2 (a), began disappearing after 24 hours in the SG and SS. The lifetime
values of each pixel along the white line drawn on the representative FLIM images of untreated skin and patched skin (0.5 hour post Nanopatch® application) are presented in Figure 2 (b). The lifetime profiles of untreated SG, SS and SB are indicated in red and were stable between 1000 and 1500 ps. Average lifetime values of untreated SG, SS and SB were measured to be around 1000 ps. Figure 2 (a) shows a narrowing in the spread of the low lifetimes around a pore, which was more rapid in the SG and SS than in the SB. These changes are consistent with a faster closure rate in the more superficial VE than the deeper VE and papillary dermis (PD) with time. Figure 2 (c) shows that lifetime values of puncture holes are invariant with VE depth and are 553.99 ± 122.49 ps, 583.30 ± 111.17 ps, and 519.77 ± 103.28 ps in the SG, SS and SB respectively immediately after (t = 0 hour) application of the Nanopatch®. In the representative image, one of the microprojection holes can be seen obscured in the SG after 4 hours (Figure 2 (a), second row, fourth column) and after 24 hours; the puncture holes in the SG and SS were obscured, whereas one of the holes in the SB was less covered (Figure 2 (a), fifth column). The ratio of the coefficient amplitudes of free and bound NAD(P)H (α1/α2) of the puncture hole, viable epidermis and total sample area are presented in Figure 2 (d). The α1/α2 ratios detected in untreated skin are in the range of 2.5 – 3.0. Puncturing of the skin by the Nanopatch® is characterised by a significant increase in α1/α2 ratio localised to the puncture hole when compared to untreated skin, with an average of 4.60 ± 0.78, 4.59 ± 1.18, and 4.18 ± 0.48 (n=100 px) detected in the SG, SS and SB respectively. The α1/α2 values of the rest of the viable epidermis and the total sample area are comparable to untreated skin.
Figure 2. (a) Representative pseudocoloured FLIM images of untreated human VE and for patched human VE over time for lifetimes ranging from 0 to 2000 ps (see legend). The closed blue circles are the sites of needle projections whereas the wavy blue lines correspond to the furrows. More detailed examination of changes in VE lifetime adjacent to an induced pore as defined by white lines drawn as shown in the following subfigures: (b) Lifetime profiles of untreated and patched SG, SS and SB, obtained 0.5 hours post patching. (c) Average NAD(P)H lifetime values ($\tau_m$) of different ROIs 0, 0.5, 4, and 24 hours post application of Nanopatch®. (d) Ratios of amplitude coefficients of free and bound NAD(P)H ($\alpha_1/\alpha_2$) of ROIs 0, 0.5, 4, and 24 hours post patching. Values are expressed as mean ± SEM (n = 3).

2.4 Assessing changes in induced pores over time using autofluorescence

Autofluorescence was used as an alternative method to quantitatively assess induced hole closure rates. A representative example of a series of spaced cross sections of human
skin at various times using the autofluorescence channel is shown in Figure 3. Each replicate was analysed from immediately after patching to 30 minutes. Averaged replicates are shown in Figure 4 (a). Between all replicates and through the entire z-stack, the aperture sizes appeared similar, with initial radii between 20-23 µm, falling to 18-20 µm (n=12) after 30 minutes. Puncture spots also appeared relatively uniform and cylindrical in width down the entire 100 µm imaging stack from the surface, except for a prominent contraction towards the SC-VE junction. A representative stack was compiled into a 3D space-time reconstruction and is included in Supplementary Error! Reference source not found..

![Figure 3](image.png)

**Figure 3.** Representative multiphoton fluorescence intensity images (autofluorescence emission 410-485 nm emission filter) at different depths from the surface of the skin and at 2, 10, 20 and 30 minutes post application of Nanopatch® onto ex vivo human skin.

Hole closure rate appeared slow but steady, with the radius of the puncture hole decreasing by approximately 3-5 µm (n=12) over 30 minutes, corresponding to approximately a 25% reduction in area. In Figure 4 (a), mean radius is shown to reach a minimum in the region of the SC-VE junction, between 10 and 30 µm from the surface. Assuming an average closure speed of 3.3 µm h⁻¹ obtained from Figure 4 (b) and unchanged metabolic conditions, we estimate that it would take about ~6 hours for complete closure, but not necessarily healing, of a puncture hole with a diameter of approximately 40 µm corresponding to that of a Nanopatch® projection.
Figure 4. (a) Mean microprojection hole radius (µm) at different depths over time. Each line indicates average radius per time point. The first time point (2 mins) and last time point (30 mins) are indicated with filled and open circles respectively. SD is shaded in black (n = 12). (b) Mean aperture closure rate (µm.h⁻¹) by depth over 30 mins. Data is the average of 12 replicates ± SEM.

2.5 Inflammatory response pilot study: in vivo histamine application

Results of the exploratory study after Nanopatch® and histamine application are shown in Figure 5 for the erythema scores and the itching scores in Table 1. Erythema score appeared to increase over time, in contrast to the cessation of the itching response. A small bump (wheal) was observed on the patched site with itchiness reported from participants. The bump and itching faded away after a few minutes post-application, and no complaints from participants were reported. One participant (subject 6) withdrew from the study after patch application due to higher than expected erythema. Consequently, no histamine was applied, but TiVi recording was continued, that showed continued reduction to 62 % of the original score at the end of the observation period, whereas the mean score for the other five participants increased to 72 % higher than the immediate reading by day 3. Two individual photographs of different time points that were not saved successfully were excluded from the analysis. One participant was unavailable for the 48-hour time point. A representative, cropped series of images used in the Tissue Viability Imager after patch application is shown in Error! Reference source not found.. The extent of pronounced erythema was limited to the immediate surroundings of the patched region, although faint redness was observed over the skin, comparable to a mosquito bite,
and reduced after 24 hours. Erythema induced by histamine provocation appeared minor, and the redness was more uniform and was spread across larger areas of the forearm.

![Graph showing erythema intensity over time](image)

**Figure 5.** Erythema intensity score from Nanopatch® application followed by histamine intervention. Subject #6 was excluded from histamine provocation, and only images were taken, due to high erythema after patching.

**Table 1.** The presence of itching and response intensity from participants after histamine application. All participants reported near-instantaneous itchiness and wheal was observed on Day 1. (Yes/No indicates presence of itching and comments for Day 2 indicate response intensity)

<table>
<thead>
<tr>
<th>Subject</th>
<th>Time point</th>
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<tbody>
<tr>
<td></td>
<td>Day 1</td>
</tr>
<tr>
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<td>Yes</td>
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<tr>
<td>2</td>
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<td>5</td>
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3. Discussion
We assessed changes in pores induced by Nanopatch® projections after application to ex vivo and in vivo skin using a range of techniques. We first quantified the punctures caused by Nanopatch® microprojections applied to human skin by cryoSEM and then studied changes in the viable epidermal redox state using FLIM. Only the viable epidermis in the
immediate vicinity of the puncture was affected and the punctures were shown to disappear over time. Fluorescence imaging showed a reduction in the puncture hole size of ~25% over the first 30 mins. These results were then validated in vivo, in an exploratory pilot clinical case study, in which we measured epidermal response to histamine provocation after patch application. Wheals induced by histamine disappeared quickly after application and did not reappear after repeat topical histamine provocations between 24 to 48 hours. These responses were supported by participant feedback reporting the lack of itching sensation by day three, agreeing with an earlier report. Topically-applied histamine induced further superficial erythema, which was sustained throughout the observation period. This work has demonstrated short pore closure duration after Nanopatch® application, important in real-life situations and emphasising the significant advantage microscale medical devices targeting the skin have over devices with larger interfacing areas. A fast closure time promotes skin healing and reduces the chance of infections and entry of exogenous matter to the skin after micro-device mediated vaccine delivery or the completion of diagnosis through biomarker or analyte detection.

FLIM was used to quantify the redox state of ex vivo viable epidermis after application of the Nanopatch®. A decrease in average NAD(P)H fluorescence lifetime and an increase in redox ($\alpha_1/\alpha_2$) ratio was observed in all strata, in the areas where the microprojections pierced the skin, which is indicative of cell death due to metabolic stress. The mechanical stress exerted by the microprojections created holes in the viable epidermis by puncturing through keratinocytes. In earlier studies performed on mouse skin, cell death (through decreased lifetime) was observed around microprojection holes and attributed to stress exerted by Nanopatch® application. Here, in human skin, the reduction in metabolic activity was also localised to puncture spots with adjacent cells unaffected metabolically over a period of 24 hours (Figure 2). Cell death associated with a Nanopatch® application was reported to have an adjuvant effect on adjacent viable cells, enhancing vaccine activity.

In this work, we found that hole closure rates varied between different skin strata, with the lowest rate occurring at approximately the dermal-epidermal junction (DEJ: Figure 4 (a)), although the closure of microscale puncture holes is projected to occur ~6 hours after Nanopatch® application, in line with previous work in mice. This reduction in the rate of hole closure surrounding the DEJ may suggest that this boundary region had experienced more plastic deformation than the other constituent layers. Nevertheless, a ~6 h estimate
of pore lifetime is also in agreement with observations that transepidermal water loss (TEWL) from ex vivo dermatomed skin returned to pre-puncture levels by 4-6 hours. Longer microneedle lengths than used in the Nanopatch® have been reported to be associated with longer recovery rates; for example: 8-24 hours (180-280 µm), 3-40 hours (500-1500 µm), and 48-72 hours (620 µm). Clearly, rapid skin closure is desirable after a single vaccination. However in contrast, it has been argued that sustained pore lifetimes of up to 7 days, that could be induced by enzyme inhibition to delay wound healing, may be beneficial for applications requiring extending transdermal patch wear time. We propose that the quantification of pore lifetime done in this work using ex vivo viable human skin is indicative of in vivo pore lifetimes. Indeed, in vivo provocation of histamine also showed a reduction in itching from day 2. Closure rate is an important parameter to consider for microscale devices, as it is advantageous to minimise skin damage and to promote rapid healing of micro-wounds, while at the same time inducing a mechanical adjuvant pro-immune effect for vaccine delivery. Closure rates are particularly relevant to poke and patch microneedles where the extension of pore lifetime allows for the drug diffusion at the site of application. Pore lifetime can be extended with the application of Diclofenac, Fluvastatin and prolonged occlusion of the application site. Hole closure rate is likely affected by the mechanical properties of skin (as well as the freshness of the skin model if not in vivo), especially the viscoelastic component. From previous work on the mechanical characterisation of the skin, we suggest hole closure occurs in two stages: initial, fast reduction in hole diameter within seconds of the Nanopatch® being removed prior to image acquisition, followed by a steady and gradual return to its original state. The results presented in Figure 4(a) are therefore most likely describing the steady state phase (see Figure S3).

In the exploratory pilot case study, the 24 hour increase in erythema after patching we observed was consistent with other studies, for instance that of Nyman, using the TiVi system after hyaluronic acid or sodium chloride provocation. Nyman proposed that cells at 24 hours were responding to wound trauma in a pro-inflammatory manner, which supports the increasing erythema score (Figure 5) over that time period returning to baseline after 14 days. We note that in this study, the wounds were created by a single puncture with a 1.6x1.8 mm lancet, and were significantly larger and deeper than those made from the ~4000 smaller projection on a 4x4 mm Nanopatch®. Another Nanopatch® study reported erythema faded between days 3-7. Using skin impedance as measure of skin permeability, Gupta et al. suggested that the skin barrier was re-established within 2
hours after large microneedle application. They also found that a 50% increase in microneedle length resulted in a 35% increase in barrier reopening time. A previous TEWL experiment\textsuperscript{35} showed that the rate of water loss returned close to baseline by 8 hours after Nanopatch\textsuperscript{®} application, but was dependent on projection geometry. Here, we suggest that using microneedles significantly larger than Nanopatch\textsuperscript{®} microprojections may induce more plastic deformation within skin layers, which leads to high cell death, erythema, longer pore lifetime and reduced fluorescence lifetime. By extension, it is likely that the complete healing of Nanopatch\textsuperscript{®} treated skin, with its microscopic and shallow puncture holes, will also be significantly faster than hypodermic needles.

We further hypothesised that the DEJ boundary puncture spots may have healed around day 2-3 after patching, and that histamine may have thus mainly reacted with the epidermal layer only. This may explain the increasing mean erythema score after each histamine provocation, despite reportedly reduced itching sensation. Histamine may have been out of reach of receptors within the dermis. This is supported by Enfield et al. who reported an 80 µm decrease in pore depth over 90 mins (with microneedles larger than the Nanopatch\textsuperscript{®})\textsuperscript{17}. The keratinised, dead layer of SC may have closed more slowly due to the non-vascularised nature of the epidermis, allowing histamine to continue to permeate and to generate a pro-inflammatory response to promote wound healing\textsuperscript{17}, over 2-5 days\textsuperscript{36}, depending on wound size and depth. This window of opportunity could therefore potentially be exploited for topical delivery of drugs into skin specifically targeting the epidermis, such as allergen-specific immunotherapy (SIT)\textsuperscript{37}, cancer\textsuperscript{38} and vaccine delivery for enhanced antigen presentation.

Finally, we envisage puncture hole closure rates explored in this study will be useful beyond microneedle and topical pharmaceutical delivery – at this scale, it is applicable to micro-surgery interventions, wound healing, skin-based patches and sensors.

4. Conclusions
In this work, we demonstrated the discretised, microscopic puncturing of skin using the Nanopatch\textsuperscript{®}. The amount of damage due to microprojections was examined using FLIM, which showed that only the immediate cells at the puncture holes were affected in an \textit{ex vivo} human skin model. The metabolic lifetimes of surrounding cells remained unaffected over the course of 24 hours. Furthermore, the pore lifetimes calculated from the
autofluorescence of skin viewed under multiphoton microscopy suggest a reduction of ~25 % in area over the first 30 minutes with an estimated full closure of ~6 hours. Additionally, a clinical case study on six human subjects with Nanopatch® mediated wound showed no reaction to deliberate histamine provocation after 24-48 hours, despite a continued strengthening in inflammation examined with as reflected by erythema score. Overall, data from this paper showed the skin’s ability to recover rapidly after Nanopatch® application within hours below the DEJ, and 1-2 days more above it. This relatively short pore lifetime makes skin-interfacing microneedle-type devices, in the Nanopatch® projection length scales of ~110-250 µm, an attractive platform for a range of therapeutic treatments, interventions and diagnoses. We believe that this work also adds to the information required by regulatory bodies as they assess the safety and efficacy of microdevices.

5. Materials and methods

5.1 Ethics statement
All work carried out has been approved by The University of Queensland Medical Research Ethics Committee (ethics approval number 2008001342) and the Regional Ethics Examination Board in Linköping (ethics approval number 2015/291-31). Written informed consent was obtained from all participants. All experiments were carried out as per The University of Queensland guidelines and adhered to the Declaration of Helsinki guidelines.

5.2 Nanopatch® manufacture
Nanopatch® wafers, diced into 4x4 mm squares shown in Figure 6 (a), were manufactured at the Australian National Fabrication Facility – Queensland Node (ANFF-Q) in Brisbane, Australia. Wafers were obtained as 1 mm thick, 6” silicon wafer and etched using the deep reactive ion etching method, as described by Jenkins et al. 39, to a specification of 10,000 projections per cm², tapered to approximately 250 µm tall and 40 µm wide at the base, illustrated in Figure 6 (b). SU-8 (MicroChem, Westborough MA, USA) was used as photoresist during patterning.
5.3 *Ex vivo* skin tissue collection, preparation and characterisation

The skin used for this study was collected from female patients undergoing abdominoplasty surgery at the Princess Alexandra Hospital (Brisbane, Australia). Subcutaneous fat was discarded after collection. Skin was wrapped in aluminium foil and stored in 4°C refrigeration until usage within 48 hours. The skin was previously measured for its layer thicknesses in Wei et al. 34. Previous studies demonstrated the suitability of storing skin up to seven days without compromising skin’s cellular viability 12. Excised samples were kept in covered 12-well plates above a layer of 0.9 % w/v saline moistened (Baxter, Old Toongabbie NSW, Australia) folded paper towel (Kimberley-Clark Australia, Milsons Point NSW, Australia) between time points at 32 °C.

5.4 Fluorescence lifetime and pore lifetime experimental design

The experiments were carried out on a total donor skin count of n=15, mean age 36±7.8 years old. Fluorescence lifetime was carried out on n=12 replicates, with n=3 on FLIM. FLIM images were then acquired at 0, 0.5, 4 and 24 hours after Nanopatch® application and removal from the skin. Pore lifetime auto-fluorescence was carried out on 12 replicates, imaged continuously from immediately after patching to 30 minutes post patching. Each scan from 0-100 µm from the surface with 5 µm spacing took approximately 2 minutes.

5.5 Nanopatch® application onto *ex vivo* skin

Nanopatch® patches were uncoated upon application. Each specimen was patched once using a custom-built, handheld, spring-loaded applicator (*Figure 7 (a)*), applied at a velocity of ~15 m s⁻¹. The skin was placed on a polystyrene tray and held down by hand to prevent collapsing due to patch application. The patch was removed directly after, and the

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**Figure 6.** (a) Photograph of several 4 x 4 mm Nanopatch® patches in a well plate. (b) Representative image of SE detector SEM of a coated Nanopatch®. (c) Representative BSE detector SEM of a Nanopatch® applied on full thickness *ex vivo* human skin and removed at 500x.
patched area was cut out and imaged immediately. First time point captured was approximately two minutes after biopsy, factoring in excision and microscope adjustment.

![Figure 7](image-url)

**Figure 7.** Method of applying the Nanopatch® onto the skin, *in vivo* and *ex vivo*. (a) In-house designed and manufactured applicator with adjustment for application velocity, (b) *In vivo* post application forearm, (c) The setup where the camera is placed directly above the forearm for the TiVi system. A lens cover and a ring-shaped LED light are attached to the lens.

5.6 Selection of participants for *in vivo* histamine case study

Six healthy volunteers (three males, three females, ages ranging from 25-65, median age 40 years) with no known skin conditions at the time of the study, consisting of university/hospital staff and students were recruited without remuneration (including one participant withdrawn and replaced). Participants may withdraw at any time without reason. Testing was performed at the Department of Dermatology at Linköping University Hospital under dermatologist supervision (co-author CDA). The spring-loaded applicator mechanism and the patch were first demonstrated to the participants before applying the patch to their skin. After Nanopatch® application at the volar surface of the proximal forearm (*Figure 7 (b)*), the skin was not further treated or covered, except for histamine provocation after patch application, and at 24 and 48 hours. Participants were allowed to return to their usual daily routine, including showering and cleaning of the patched site. The experiment was carried out during weekdays while participants were working indoors during office hours, to mitigate potential effects of sun-tan and sunburn.

5.7 Scanning electron microscopy (SEM)

All SEM images were only processed for brightness and contrast adjustment and labelling with Adobe Photoshop (San Jose CA, USA), except otherwise stated.
5.7.1 SEM
Scanning electron microscopy (SEM) examinations of the projection profiles shown in Figure 6 (b-c) was taken with the Hitachi SU3500 (Tokyo, Japan) and the Jeol Neoscope (Tokyo, Japan) using secondary electron detector with the accelerating voltage set at 10-15 kV.

5.7.2 Cryo-SEM
Cryo-SEM images illustrating top-down penetration of projections in skin were taken using the Jeol 7100 (Tokyo, Japan). The method was similar to previous experiments in Fernando et al.40. Briefly, skin specimen was patched, excised, patch removed and submerged under liquid nitrogen for freezing. To further remove ice crystal build-up, the sample was sublimated at -105ºC. Imaging temperatures were set to -145ºC (chamber/pre-chamber) -195ºC in the anti-contaminator. Images were taken with secondary electron detector and its accelerating voltage at 2-5 kV.

5.8 Fluorescence lifetime imaging microscopy (FLIM)
FLIM was used to evaluate the metabolic state of the viable epidermis after application of the Nanopatch® on fresh full thickness human skin. Images were acquired using the DermalInspect multiphoton microscope (JenLab GmbH, Jena, Germany) equipped with a Titanium:Sapphire femtosecond-pulse Mai-Tai laser (Spectra Physics, CA, USA) and a TCSPC830 detection module (Becker & Hickl GmbH, Berlin, Germany). FLIM images were then acquired at 0, 0.5, 4 and 24 hours after Nanopatch® application and removal from the skin. The skin samples were excited at a wavelength of 760 nm with a laser power of 21 mW and acquisition time was set at 13.4 s. 256 x 256 µm images of the epidermis at four depths corresponding to the SC surface (but was excluded from the analysis, as it is not a living layer part of the viable epidermis), SG, SS and SB were acquired. The depths were determined by the morphology of the cells, however, SG is generally ~5-6 µm below the SC; SS ~7-8 µm and SB ~12 µm. Fluorescence emitted at a wavelength range of 350 – 450nm, corresponding to the emission range of NAD(P)H was detected using an in-built band-pass filter.

FLIM images were analysed using SPCImage 5.2 (Becker & Hickl GmbH, Berlin, Germany) as previously described12,40,41. The masking function on this software was used to define regions of interest, such as the VE, the puncture spot, and total sample area. The average weighted (to $\alpha_1$ and $\alpha_2$) fluorescence lifetime of NAD(P)H ($\tau_m$), and the ratio of amplitudes
of free and bound NAD(P)H (α₁/α₂) of these regions of interest were exported from the software.

5.9 Multiphoton microscopy (MPM)
Pore lifetime was analysed using data from MPM images. Auto-fluorescence from skin was captured using the 410-485 nm bandpass filter on the LaVision Biotec Nikon Ti-U inverted microscope (Bielefeld, Germany) with the Olympus 20x 0.95 NA water immersion lens (Tokyo, Japan) and the Spectra Physics Ti-sapphire Mai-Tai laser (Santa Clara CA, USA) set at 760 nm. Imaging power was set at 14.5 mW. Images were taken at 1047 x 1047 pixels over an area of 500 x 500 µm over 100 µm from the skin surface, at 5 µm spacing to form a stack. Each stack was repeatedly imaged every two minutes for 30 minutes. Exported images were processed using Fiji and pseudocoloured green and saved as .jpg images. A 3D space-time reconstruction of the stack was created using Imaris 6.3.1 (Bitplane, Belfast, United Kingdom).

5.10 Pore lifetime analysis
Exported auto-fluorescence images were analysed in Matlab 2016b/2017a (MathWorks, Natick MA, USA). Each layer in each stack was analysed independently of one another. A Matlab code was written for manual selection of approximately 4-5 projection puncture hole (pore) centres. The coordinates of these holes were used for each complete stack over all time points. By selecting a hole, a circular area of diameter 50 µm (slightly larger than a projection’s diameter) was created around the centre.

The selected circular areas were first converted to greyscale using the Matlab function rgb2gray. Intensity was normalised with the function imadjust. Images were further converted to black and white using the function imbinarize with a sensitivity of 0.2 after testing values between 0.1-0.5, where 0.2 returned the most consistent values for non-puncture areas throughout complete stacks. The ‘adaptive’ function accommodated for decreased signal in deeper layers. The white area in the selected circular region was used to determine a mean puncture hole size. This was repeated for all holes chosen in the
same image, then through the entire z-stack, and for all samples. An example is shown in

![Figure 8](image.png)

**Figure 8.**

(a) Representative pseudocoloured multiphoton image (410-485 nm emission filter) of patched skin. The purple crosshairs indicate the centre of the puncture hole. (b) Threshold area used to estimate puncture hole size.

5.11 *In vivo* histamine exploratory pilot study experimental design

Histamine was used as an agent to determine whether skin pores induced by microprojections could close and recover rapidly. First, a suitable, scar-free, uniform region of the volunteer forearm was selected. One drop of histamine hydrochloride 10 mg ml$^{-1}$
(Soluprick SQ positive control, ALK-Abelló, Hørsholm, Denmark) was dispensed onto skin and wiped off with tissue paper after 30 s as a negative control. The skin was observed for any reaction (positive reaction was considered as >3 mm diameter 43 (intact skin should not react). A 5 mm wide colour-checker chart 44 for black-white point balance correction was placed on the skin (Error! Reference source not found. (a)) and imaged using the TiVi 700 system (WheelsBridge, Linköping, Sweden) for reference. Nanopatch® patches were uncoated, and no vaccine was delivered. Patches were disinfected by soaking in 70 % w/w ethanol and let dry completely before application. The same Nanopatch® geometry and application conditions used in Section 5.5 were used in the in vivo test. Immediately after application (approximately five mins), the patched area was imaged again, with a colour-checker chart alongside. Histamine drop was applied to the patched area and again wiped off after 30 s. Imaging was repeated at 10 mins and 90 mins (when the strongest reaction should occur) 24. Histamine was reapplied after 24 and 48 hours with images taken before and ten minutes after each application to assess the degree of reaction. Histamine reaction was evaluated with wheal diameter, inflammation (TiVi) and volunteer oral response to itchy sensation.

5.12 Erythema analysis
Images captured using the TiVi700 system were first imported into Photoshop because slight differences in the tone was observed between images. The Auto Tone adjustment feature was used to ensure all images taken prior to analysing are consistent. This feature darkens the darkest pixel to black, and vice versa to white, and redistributes all tonal values in between for each RGB channel 45. Images were then straightened and cropped into a circular Region of Interest (ROI) so only the patched area, excluding pronounced edge effects that may bias the results 46 due to high-stress regions at the square corners, was used for analysis. Images were imported back into the TiVi software for analysis, normalised with untreated control (unprovoked skin) by subtracting from the baseline as the reference. Values (au) were recorded as an intensity indicator for erythema.

5.13 Measurement of microprojection penetration depth
Projection penetration depth through the full imaging stack of at least 100 µm was confirmed using the same application procedure, conditions, and skin specimens, reported in a previous study 47. In brief, patches were coated as per 35 with FluoSpheres fluorescent labelling dye. After application onto skin, specimens were fixed and sectioned using histology with penetration tracks viewed with multiphoton microscopy.
Data availability
The datasets generated and/or analysed during the current study are available from the corresponding author on reasonable request.

Competing Interests
JCJW and MLC were former Vaxxas (a company developing the Nanopatch® for clinical trials) staff employed through UQ. MAFK was a former board member of Vaxxas. CDA is a consultant for Vaxxas. The Nanopatch® technology has been licensed to Vaxxas for use in clinical development. There has been no financial support for this work that could have influenced its outcome. The remaining authors declare no conflicts of interest.

Contributions
(alphabetically listed) CDA, MLC, INH, JH, SCM, YHM, WYS, JCJW performed the experiments. MLC, INH, YHM, JCJW prepared the figures and wrote the manuscript. HAEB, MLC, JEG, MAFK, YHM, MSR provided feedback on the manuscript, supervision, scientific oversight on the experimental design, data analysis and interpretation.

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References


