Supplementary Materials

Real-time *in vivo* structure-function study of scalp hair cycles: an experimental approach for monitoring living hair roots with a 20-year follow-up

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Supplementary File 1

Real-time *in vivo* structure-function study of scalp hair cycles: an experimental approach for monitoring living hair roots with a 20-year follow-up.

INTRODUCTION

As originally reported in 1989[1,2], the use of immersion fluids improved light penetration into the skin, allowing for better visualization of the intra-epidermal (acro-infundibulum) down to the most superficial intra-dermal aspects of the hair follicle. This presented an interesting opportunity for further study.

For the experimental approach presented herein, we built upon these early observations and focused on identifying methods for most superficial hair follicle implantation.

METHODS

Procedures for superficial implantation were selected (as reported in the main manuscript) after several unsuccessful attempts, including a failed transfection experiment. Naturally non-hairy sites on the flexural aspect of the forearm were carefully selected for two different implantation techniques.

To justify this experimental approach, we examined images of patients with hair loss (including those with or without scalp scarring). We observed pigmented hair shafts located within or just below the epidermis. Together with these clinical observations, the schematic structure of scalp hair follicles and the implantation plan are shown in Figure 1, stressing the importance of altering the angle in

1. Intra-epidermal implantation
2. Superficial dermal implantation

1. Intra-epidermal implantation

Suction blisters were induced using negative pressure (-250 mm Hg for 1.5 to 2 h[3]). After perforating the roof of the blister, marker hair fibres and five isolated follicle roots (one per blister) were implanted.

2. Superficial dermal implantation

The flexural aspect of the forearm was topically anesthetized (EMLA with 2h of occlusion under plastic), and three parallel channels were created in the most superficial dermal layers.

A small needle (BD microlance™ 3; 1.2 × 40 mm) was initially inserted at one of three entry points, advancing until the bevel exited the skin surface (Figure 1, lower panels; insertions at B1*, B2*, and B3* and progressing toward B1, B2, and B3).

The intradermal needle served as a guide for inserting a larger needle in the opposite direction (from B1, B2, and B3 back to B1*, B2*, and B3*). Once the bevel of the larger needle emerged from the skin (at B1*, B2*, or B3*), the smaller needle was discarded. Individual follicles were then gently placed inside the larger needle, which now served as a graft container. At the end of the backward movement, aimed at extracting the larger needle (bevel with the follicle inside returns to positions B1, B2, and B3), the operator carefully positioned the hair fibre at the skin surface, ensuring the root end smoothly resided in the superficial dermal channel. The single-use needles were discarded.

As planned, immediately following the micro-dissection of three individual hair follicles, one follicle was treated *ex vivo* in an attempt at LASER-assisted transfection. However, the LASER procedure (which involved excessive energy delivery and/or too many impacts) irreversibly damaged key components of the follicle before implantation at the most proximal site, preventing any follow-up on hair growth.

RESULTS: FROM FAILURES TO SUCCESS

In Figure 1, the upper panel (1) shows a simplified schematic illustrating the natural angle and depth of scalp hair implantation (top left; 45° - 60°), along with the depth of light penetration (represented by the vertical magenta bar, 100 µm), which allows visibility of superficial segments of the follicle (short green oblique segment; length between 100-200 µm).

Deeper segments of the scalp follicle remain inaccessible (blue vertical bar, approximately 4 mm deep) due to limited light penetration and the low resolution associated with backscattered light once it passes through the epidermis. This natural

anatomical arrangement restricts optical methods from generating high-resolution images unless the follicle is tilted between - 45° and - 55° (green line; deeper intra-follicular depths). Scalp immersion proxigraphy – more recently renamed trichoscopy - can only capture the minimal depth of the scalp hair (top right panels 1a to 1d; each magenta dot represents 100 µm).

The lower panel (2) highlights on the left the rationale for the very superficial implantation of single scalp hair follicles with access from surface to matrix or bulb.

Two modalities were tested by design:

- First (2-A): Intra-epidermal grafts (the single hair follicle) were engrafted between the base and roof of the suction blister, with the grafts positioned at the center of a geometrical reference field [a triangle with two dark beard hairs (A1 and A2) and one un-pigmented hair (A3)].
- Following intra-epidermal implantation, surface observation produced very sharp, clear images. However, all five grafts were rapidly eliminated as the epidermis healed and underwent desquamation. Consequently, this engrafting procedure was abandoned.
- Second (2-B): Channels were created at the pre-selected site on the flexural aspect of the forearm, where a single micro-dissected follicle was inserted into each channel (1 per channel; hair exits near B1*, B2*, and B3* (higher magnification). Follow-up imaging of hair growth, rest, shedding, and regrowth is reported in the main manuscript.

CONCLUSION

The only successful approach was via superficial dermal implantation, as demonstrated by long-term monitoring of hair growth, rest phases, shedding, and regrowth, followed by dormancy. This dormancy is reversible through reactivation of the anagen phase via topical minoxidil application.

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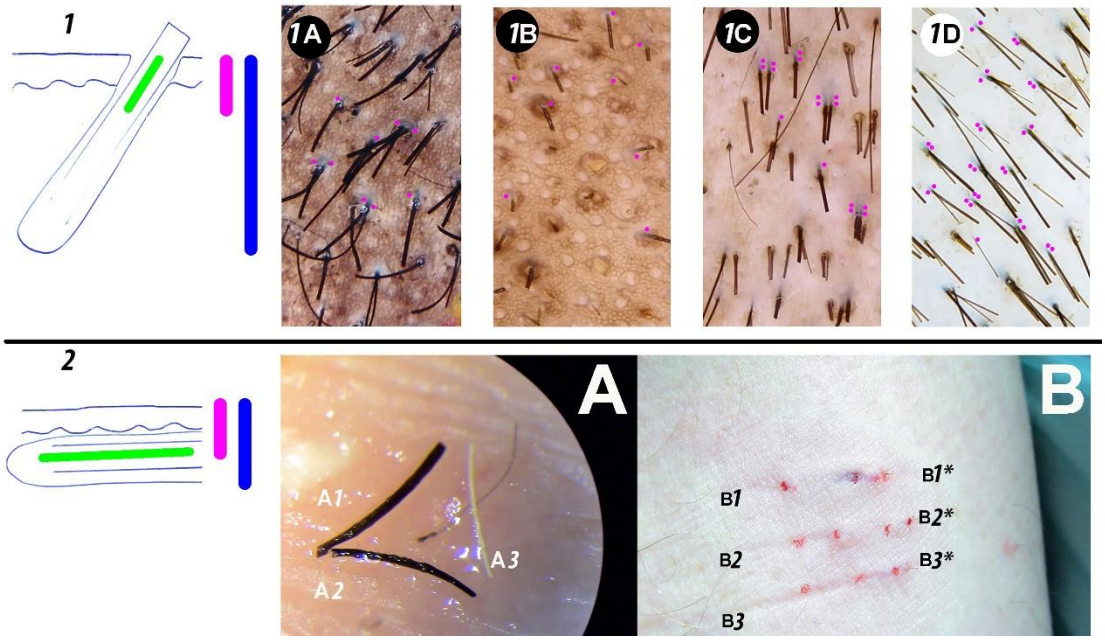

Figure 1. Rationale (1, 2) based on clinical observations (1A-1D) and view of failure (2A) and success (2B) of the superficial implantation of scalp hair follicles.

Supplementary File 2

Real-time *in vivo* structure-function study of scalp hair cycles: an experimental approach for monitoring living hair roots with a 20-year follow-up.

INTRODUCTION

In the present paper, superficial implantation was used to position the scalp hair grafts at a depth where confocal microscopy could capture biologically relevant images. This innovative approach enables the detailed observation of a living and functionally active structure - specifically, the hair follicle - from top to bottom. The success of live confocal microscopy observations depends heavily on the precision of the surgical implantation, ensuring the grafts are placed at the appropriate depth within appropriate recipient sites.

BACKGROUND AND BASICS

Theoretical aspects of imaging with confocal microscopy

The scanning process, dependent on the tissue depth, was performed with single scans of 4 s to 16 s, using a laser mean power of 5 mW to 25 mW at the target. The auto-fluorescence of various tissue structures was detected using femtosecond laser pulses at 760 nm (for NAD(P)H, elastin, pigmented cells) and 840 nm (for pigmented cells, collagen, Second Harmonic Generation or SHG).

Multiphoton laser microscopy uses the low out-of-focus absorption of tissue structures and endogenous fluorophores in the near-infrared (NIR) spectrum, allowing for deeper tissue penetration. Multiphoton excitation of fluorescence occurs through the simultaneous absorption of at least two NIR photons. To achieve this, high transient intensities (in the GW / cm^2 range) are required, which result from tightly focusing ultra-short laser pulses into a sub-femtoliter volume using objectives with high numerical apertures. Because of this very small reaction focal volume, out-of-focus photobleaching and tissue damage do not occur (at appropriate laser powers) and no pinhole is needed. The weak natural auto-fluorescence of cells and tissues such as the skin - due to NAD(P)H, flavones, co-enzymes, metal-free porphyrins, lipofuscin, keratin, melanin, collagen and elastin - can be excited simultaneously.

Practical aspects of imaging with confocal microscopy

The practical aspects of image acquisition with confocal microscopy are illustrated in Figure S2-1.

Before initiating the imaging session, three laboratory assistants involved in the placement and monitoring of hair growth from the grafted follicles helped spot the opening of hair follicles using optical microscopy (A).

Once the region of interest was spotted, with the flexural aspect of the forearm positioned on the microscope (B), the volunteer (with the help of assistants) remained still throughout the entire examination. Dual-confocal microscopy examination then began, with monitoring displayed on computer screens (C)

Expert assistants (not shown) guided the LSM 510 META system from Zeiss under the direction of the laboratory engineer (D: Iris Riemann). The images captured during the examination are presented in Figure 11 of the main manuscript, along with the detailed results.

DISCUSSION AND FUTURE PERSPECTIVES

During the normal hair cycle and when the cycle terminates ‘naturally’, cellular events directly related to anagen termination can be observed. These include the programmed cell death process during catagen, the involvement of elastic fibers during the shift of the hair bulb toward the upper part of the hair follicle, and the probable occurrence of Arao-Perkins bodies[1]. Additionally, the role of intermediate companion cell layers, which enable the parallel movement of adjacent cellular structures in opposite directions, could be examined. Also, visualisation of most early stages of follicular regrowth (before anagen VI when a hair becomes visible at the acro-infundibulum, i.e., anagen stages III-V), and activation blood vessels could provide further insight into hair follicle dynamics. This approach might also contribute to precise quantification and assessment of the structural volumes related to hair productivity[2], as well as the duration of the catagen and telogen phases. Classical estimates of these phases might be limited by the precision of optical methods, such as stereomicroscopy[3,4], scalp immersion proxigraphy, and skin replicas, along with the confirmation of exogen hair release at the end of the telogen phase using scanning electron microscopy[5]. Ultimately, current imaging techniques

and computer-assisted analysis have significant clinical relevance for understanding hair dynamics and productivity, as demonstrated by recent correlational studies[6-8].

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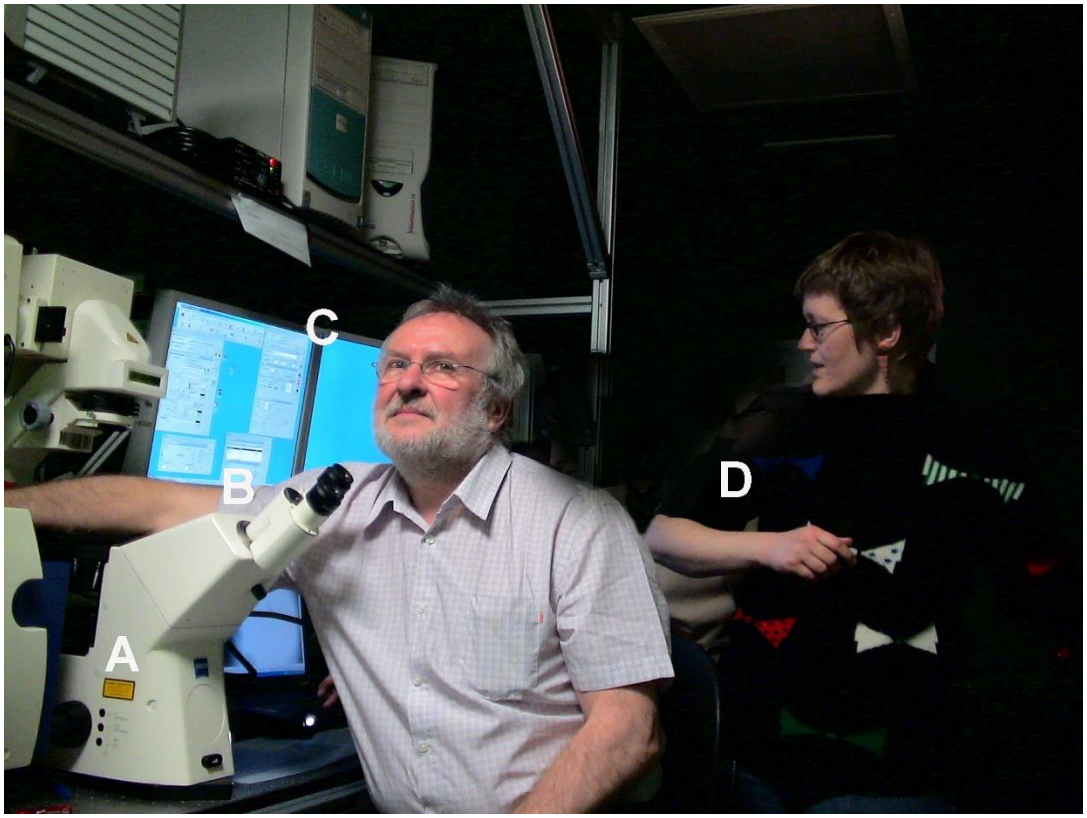

Figure 1. General view during the examination of grafts using dual confocal microscopy. Each of the two grafted follicles was first spotted using optical microscopy [(A); visual confirmation of the follicular opening and hair fiber. This was followed by dual-confocal microscopy examination (LSM 510 META; Zeiss)]. After confirming the follicular opening and hair fiber [(A); flexural aspect of the forearm on the microscope (B)], the volunteer remained still while the computer processing was managed on the screens (C) by the expert engineer and her assistants [(D): Iris Riemann, with permission)], capturing images of the grafted follicles.