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TITLE:

Isolation of Papillary and Reticular Fibroblasts from Human Skin by Fluorescence-activated Cell Sorting

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KEYWORDS:

FACS, cell surface markers, fibroblast heterogeneity, fibroblast lineages, human skin, papillary and reticular fibroblasts

SUMMARY:

This manuscript describes a FACS-based protocol for isolation of papillary and reticular fibroblasts from human skin. It circumvents in vitro culture which was inevitable with the commonly used isolation protocol via explant cultures. The emanating fibroblast subsets are functionally distinct and display differential gene expression and localization within the dermis.

ABSTRACT:

Fibroblasts are a highly heterogeneous cell population implicated in the pathogenesis of many human diseases. In human skin dermis, fibroblasts have traditionally been attributed to the superficial papillary or lower reticular dermis according to their histological localization. In mouse dermis, papillary and reticular fibroblasts originate from two different lineages with diverging functions regarding physiological and pathological processes and a distinct cell surface marker expression profile by which they can be distinguished.

Importantly, evidence from explant cultures from superficial and lower dermal layers suggest that at least two functionally distinct dermal fibroblasts lineages exist in human skin dermis as well. However, unlike for mouse skin, cell surface markers enabling the discrimination of different fibroblast subsets have not yet been established for human skin. We developed a novel protocol for the isolation of human papillary and reticular fibroblast populations via fluorescence-activated cell sorting (FACS) using the two cell surface markers Fibroblast Activation Protein (FAP) and Thymocyte antigen 1 (Thy1)/CD90. This method enables the isolation of pure fibroblast

subsets without in vitro manipulation, which was shown to affect gene expression, thus permitting accurate functional analysis of human dermal fibroblast subsets in regard to tissue homeostasis or disease pathology.

INTRODUCTION:

As the main cellular component of connective tissue, fibroblasts are primarily responsible for the deposition of collagen and elastic fibers that ultimately form the extracellular matrix[1], and thus, their role in tissue homeostasis, regeneration and disease has been underestimated for a long time. However, fibroblasts have recently come under the spotlight of researchers, not only because they represent a prominent cellular source for induced pluripotent stem cells[2] but also because of their high plasticity and implication in the pathogenesis of a wide number of diseases such as organ fibrosis[3-5] or cancer[6,7].

Human skin is composed of a multi-layered epithelium, the epidermis, and its underlying connective tissue, the dermis, which can be histologically subdivided into the upper papillary and the lower reticular dermis and is mainly composed of fibroblasts and extracellular matrix[8] and the hypodermis. According to their location within the tissue, dermal fibroblasts have roughly been classified into papillary and reticular fibroblasts[1].

Importantly, recent data indicate that these dermal fibroblast subpopulations are not only histologically distinguishable, but also that their function is considerably diverse. In mouse skin, papillary and reticular fibroblasts arise from two distinct lineages during embryogenesis[9]. Several lines of evidence suggest that the two lineages exert different roles not only in tissue homeostasis, hair follicle morphogenesis, wound repair and fibrosis[7,9,10], but they also respond to different signals from neoplastic epidermal stem cells[11], suggesting diverging roles in cancer pathogenesis. Conveniently, both lineages express a distinct set of mutually exclusive cellular markers in adult mouse skin, thus enabling the isolation of pure dermal fibroblast populations and subsequent extensive analysis of their specific functions in vitro[9,11].

Correspondingly, at least two distinct fibroblast subsets with distinct morphology and functions, including diverging proliferation rates, tissue remodeling capacities[12,13], as well as the ability to support the growth of epidermal stem cells in vitro, have been described for human skin dermis[14,15]. However, most of the published studies on human dermal fibroblasts have been conducted using mixed fibroblast populations isolated from explant cultures from dermatomed skin, since specific cell surface marker sets enabling the isolation of pure human papillary or reticular fibroblast subpopulations in analogy to mouse dermis were yet to be established.

We have recently demonstrated, that human skin papillary and reticular fibroblasts are characterized by specific cell surface markers that enable the isolation of the respective subpopulations via fluorescence-activated cell sorting (FACS)[16]: FAP+CD90- fibroblasts represent papillary fibroblasts primarily located in the upper dermis, presenting higher proliferation rates, a distinct gene signature but no adipogenic potential. FAP+CD90+ and FAP-CD90+ fibroblasts belong to the reticular lineage of the lower dermal compartments, which are less proliferative but readily undergo adipogenesis—a hallmark for reticular fibroblasts. This method enables to

extensively study these distinct fibroblast subpopulations not only in regard to their specific functions under physiological conditions but also in the context of the pathogenesis of cutaneous diseases including skin cancer.

However, since fibroblasts alter their cell surface marker expression in two-dimensional in vitro culture[16-19], the application of our protocol is limited to the isolation of primary fibroblasts from human dermis and does not permit the identification of papillary or reticular fibroblasts in mixed cell culture populations. Importantly, although the expression of cell surface markers changes in vitro, we have demonstrated that the fibroblast subsets isolated according to the protocol described below retain their specific functionality when cultivated[16], thus enabling in vitro studies of subset-specific properties under physiological or pathological conditions.

In conclusion, we developed a protocol for the isolation of distinct fibroblast subsets via FACS that for the first time permits the isolation of pure fibroblast populations from human skin dermis in a naïve state.

PROTOCOL:

Human skin was obtained from Caucasian men and women aged 26 to 61 years (sun-protected skin; abdominal corrections, breast reductions). Written informed patient consent was obtained before tissue collection in accordance with the Declaration of Helsinki, and with approval from the Vienna Medical University Institutional Review Board.

NOTE: We provide a protocol for the isolation of functionally distinct fibroblast subpopulations either from full thickness dermis (please refer to section 1) or after sectioning human skin dermis into its different layers (skip section 1 and directly refer to section 2).

1. Preparation of full thickness dermis

1.1. Place a 10 cm x 10 cm human skin piece (e.g., from abdominal corrections or breast reductions) with the epidermis facing downwards on thick filter paper.

1.2. Hold the epidermis tightly on its edges with forceps and scrape off the subcutaneous fat layer with a scalpel. Then slice the tissue into 5 mm wide strips before putting them into a Petri dish.

NOTE: This facilitates penetration of Dispase II (referred to as dissociating enzyme henceforth, see section 3) and is used if the epidermis needs to be separated from the entire dermis. For this skip section 2 and directly refer to section 3. To avoid contamination, keep the tissue sterile after surgical removal or clean it thoroughly with ethanol or iodine solution. If at all, ethanol treatment of the skin surface causes only minor cell death. Work under sterile conditions in a tissue culture flow hood.

2. Sectioning of human skin dermis into papillary and reticular layers with a dermatome

2.1. Place a 10 cm x 10 cm skin piece (e.g., from abdominal corrections or breast reductions) on thick filter paper, with the epidermis facing upwards. Hold the skin tightly on its edges with forceps.

2.2. Slice the skin with an electric dermatome, adjusted to a cutting thickness/depth of 300 µm, by sliding the head of the dermatome away from oneself, with the blade being at a 90° angle in relation to the surface of the skin.

2.3. Take the first layer consisting of epidermis and papillary dermis (300 µm thick, "Dermal layer 1", **Figure 1** and **Figure 2**) and put it in a Petri dish. Proceed immediately to section 3 or add 1x PBS to keep the tissue from drying out.

NOTE: To avoid contamination, keep tissue sterile after surgical removal or clean it thoroughly with ethanol or iodine solution. Work under sterile conditions in a tissue culture flow hood.

CAUTION: Handle dermatome carefully since the blades are very sharp.

2.2. Repeat step 2.2 adjusting the dermatome to a cutting thickness of 700 µm and slice the remaining dermis. Place the upper slice which is defined as the upper reticular dermis ("Dermal layer 2", **Figure 2**) into a separate Petri dish. Proceed immediately to section 4 or add 1x PBS to keep the tissue from drying out.

2.3. Scrape away the subcutaneous fat layer with a scalpel from the residual lower reticular dermal layer (>1000 µm) and discard it. Collect the lower reticular dermis ("Dermal layer 3", **Figure 2**) in another Petri dish. Proceed immediately to section 4 or add 1x PBS to keep the tissue from drying out.

NOTE: Alternatively, instead of scraping the fat off with a scalpel, remove the fat layer with scissors. It may help to put the reticular dermis on ice prior to scraping the fat away.

3. Separation of epidermis and dermis

3.1. Prepare a 3 U/mL dissociating enzyme (i.e., Dispase II) solution in sterile 1x PBS. Place the 5 mm skin stripes (from section 1), or the epidermis/papillary dermis (from step 2.2), with the epidermis facing upward in a 10 cm Petri dish with 10 mL of 3 U/mL dissociating enzyme solution. Incubate the Petri dish at 37 °C for 1 h.

3.1.1. The protocol may be paused here. Incubate epidermis/papillary dermis in protease in the refrigerator at 4 °C overnight instead. As needed store the other layers (Dermal layer 1, 2 and 3) overnight immersed in 1x PBS in 50 mL tubes at 4 °C.

CAUTION: Work carefully with protease, it may irritate skin and eyes upon contact.

3.2. After incubation, transfer the epidermis/papillary dermis to the dry lid of the Petri dish and separate the epidermis from the upper dermis (Dermal layer 1) with two forceps each holding the edge of either the epidermis or dermis.

4. Enzymatic digestion of dermal tissue

4.1. Mince each dermal layer (Dermal layer 1, 2 and 3) separately with scissors/scalpel as thoroughly as possible; the smaller the pieces, the better the tissue digestion.

NOTE: The toughness of the dermis can vary depending on the body part it originates from (e.g., abdominal or upper arm skin).

4.2. Prepare the digestion mix by combining 1.25 mg/mL collagenase I, 0.5 mg/mL collagenase II, 0.5 mg/mL collagenase IV and 0.1 mg/mL hyaluronidase in Dulbecco's modified Eagle's medium (DMEM) with L-glutamine (see **Table of Materials**), 10% fetal calf serum (FCS) and 1% penicillin/streptomycin (P/S).

CAUTION: Work carefully with collagenases as these may irritate skin and eyes upon contact.

4.3. Put each of the minced tissues with 10 mL of the prepared digestion mix into a 50 mL tube and incubate in a 37 °C warm water bath for 1 h under permanent agitation. During digestion invert the tubes several times.

NOTE: One should consider adjusting the digestion volume according to the size of the minced skin piece. Do not overload 10 mL digestion mix with too much tissue otherwise the cell yield will be minimal. Less than one third of tissue within the total volume is recommended (1:2 w/v).

5. Preparation of single cell suspension and erythrocyte lysis

5.1. Stop enzymatic tissue digestion by adding 10 mL fibroblast medium (DMEM with L-glutamine, 10 % FCS and 1 % P/S) to the digested tissue. Work on ice from here on.

5.2. Pour the contents of each tube through a regular sterile stainless tea strainer and collect cell suspension in a clean Petri dish. Wash the strainer with medium and mash undigested tissue pieces with the edge of a syringe-plunger.

5.3. Afterwards, pipette collected cell suspension through a 70 µm cell strainer into a 50 mL tube. Rinse the cell strainer with medium and collect flow through into the same tube.

5.4. Centrifuge tubes at 500 x g at 4 °C for 10 min. Remove and discard supernatant and wash cell pellet with 5 mL fibroblast medium. Repeat the centrifugation step.

5.5. Remove and discard the supernatant and resuspend the pellet in 1 mL self-made ACK erythrocyte lysis buffer (0.15 M NH_4Cl, 10 mM $KHCO_3$, 0.1 mM Na_2EDTA; pH 7.2–7.4). Leave cells in lysis buffer for approximately 1 min at ambient temperature (20–22 °C).

5.6. Stop lysis by adding 9 mL of 1x PBS with 10% FCS and centrifuge the tubes again at 500 x g at 4 °C for 5 min. Discard the supernatant.

CAUTION: The incubation time in erythrocyte lysis may be adjusted if the lysis seems incomplete (red pellet). However, do not leave the cells in erythrocyte lysis buffer for too long to avoid lysis of fibroblasts and immune cells.

6. Blocking and staining fibroblasts for FACS

6.1. Prepare 5 µL of human Fc-receptor blocking solution in 100 µL of 1x PBS with 10% FCS and resuspend cells in 100 µL of human Fc blocker. Incubate on ice for 10 min.

6.2. Design a FACS staining panel to stain human dermal fibroblasts. Mix anti-human FAP APC (1:20), anti-human CD90 AF700 (1:30), anti-human E-cadherin PE (1:20), anti-human CD31 FITC (1:30), anti-human CD45 Pacific blue (1:20), anti-human ITGA6 PE (1:20), anti-human CD235ab Pacific blue (1:1000) and anti-human CD106 Pacific blue (1:100) in 1x PBS with 10% FCS.

6.3. After Fc receptor blocking, spin cells down at 500 x g at 4 °C for 3 min. Remove and discard the supernatant and resuspend cells in 100 µL of antibody mix. Incubate cells in the dark at 4 °C for 20 min.

6.3.1. Prior to staining, remove 20 µL of a sample with a high cell number for unstained and single stain FACS controls.

6.4. Wash the stained cells and FACS controls twice with 500 µL 1x PBS with 10% FCS and centrifuge at 500 x g at 4 °C for 3 min. In the meantime, add 4',6-diamidino-2-phenylindole (DAPI) to 1x PBS with 10% FCS to make a final concentration of 1 µg/mL.

6.5. Remove and discard supernatant, resuspend cells in 200 µL DAPI solution and filter each cell suspension through a 70 µm cell strainer into a 5 mL FACS tube.

NOTE: If FACS is going to be performed with delay, add DAPI and filter shortly before recording.

7. Gating strategy for human dermal fibroblast subset isolation and FACS

7.1. Record flow cytometry controls, set correct voltage settings and perform the appropriate compensation. Gate for single cells and live (DAPI-), Pacific blue, PE and FITC negative cells (CD45-, CD31-, CD235ab-, CD106-, ITGA6- and E-cadherin-) to get three fibroblast populations: FAP+CD90-, FAP+CD90+ and FAP-CD90+ cells. See **Figure 3**.

7.2. Sort three fibroblast subpopulations into separate 1.5 mL screw cap microcentrifuge tubes filled either with 350 µL lysis buffer for RNA isolation or filled with 350 µL fibroblast growth medium (see the **Table of Materials**) for cell culture. Invert tubes immediately after the sort and either put lysis buffer tubes into liquid nitrogen for snap freezing or put medium tubes on ice.

CAUTION: Prepare lysis buffer with 0.1 % β-Mercaptoethanol in fume hood and avoid inhalation.

8. Culturing of fibroblasts and adipogenesis assay

8.1. Following FACS, spin cells down at 500 x g for 3 min at 4 °C and plate equal cell numbers (5,000–10,000 cells/well) in fibroblast growth medium (see the **Table of Materials**) in 48-well cell culture dishes. Leave cells to grow until they reach 70% confluency.

8.2. Add 5 µg/mL insulin, 5 µM troglitazone, 0.5 mM 3-isobutyl-1-methylxanthine (IBMX) and 1 µM dexametasone to fibroblast growth medium to prepare adipocyte differentiation medium.

8.3. Replace medium of the cultured cells with adipocyte differentiation medium and let cells differentiate for 14 days. Exchange medium after day 2 with reduced adipogenesis medium containing 5 µg/mL insulin and 5 µM troglitazone. Replenish medium every 3rd or 4th day.

8.4. Fix cells with 4% PFA for 20 min at ambient temperature (20–22 °C) at differentiation day 14. Wash wells with 60% isopropanol and let it evaporate completely. Stain cells with 5 mM Oil Red O (filter prior to use) for 20 min. Wash stained cells four times with distilled water. Proceed to perform microscopy.

CAUTION: PFA is toxic and harmful. Handle carefully.

NOTE: The protocol can be paused here. Fixed cells can be stored in 1x PBS at 4 °C until Oil Red O staining is performed. Cover dish with paraffin film prior to storage to avoid evaporation.

8.5. Image cells immersed in water with an inverted microscope at 10x magnification with transmitted light.

REPRESENTATIVE RESULTS:

An overview of the main steps for processing skin tissue to obtain a single cell suspension is shown in **Figure 1**, displaying the dermatoming of the skin (**Figure 1A**), different dermal layers (**Figure 1B**), removal of the subcutaneous fat layer (**Figure 1C**) and the separation of the epidermis and papillary dermis (**Figure 1D**), as well as the different steps of the manual and enzymatic tissue dissociation (**Figure 1E,F**). A scheme of the three dermal layers is provided in **Figure 2**.

Figure 3 displays the gating strategy of a flow cytometry staining panel for the analysis of different fibroblast subsets from human skin. Additional cell surface markers that are not expressed on fibroblasts permit the exclusion of various other cells present in the skin such as

immune cells, epidermal cells, mesenchymal stem cells (MSCs), red blood cells or endothelial cells to attain maximal purity in the isolated populations. It is not critical to use the identical FACS panel used in **Figure 2** for the identification of these fibroblast subpopulations, however this is our recommendation. One may use antibodies labelled with different fluorescent dyes or alter the combination of exclusion markers.

Of note, this protocol enables the identification of three fibroblast populations in human skin which present with different intradermal localization, gene expression profiles and also functions (**Figure 4**). FAP+CD90- are enriched in the papillary dermis whereas FAP+CD90+ and FAP-CD90+ are more abundant in the reticular dermis (**Figure 4A**). All three subpopulations exhibit the typical fibroblast morphology upon sorting in cell culture (**Figure 4B**). Interestingly, they differ regarding their ability to differentiate into adipocytes which is a hallmark for reticular fibroblasts. Combining these results with gene expression profiling via real-time polymerase chain reaction (RT-PCR)[16] (**Figure 4C**), showing that FAP+CD90- cells express high levels of markers commonly attributed to papillary fibroblasts such as CD26, NTN and PDPN while CD90+ cells express the known reticular markers such as CD36, ACTA2 and PPARγ at high levels, we conclude that FAP+CD90- cells belong to the papillary and CD90+ cells belong to the reticular lineage.

FIGURE AND TABLE LEGENDS:

Figure 1: **Isolation of dermal single cell suspension from intact human skin.** (**A**) Skin is sliced with an electric dermatome into papillary and reticular dermis. (**B**) Papillary dermis with adjacent epidermis is 300 µm thick (top) while upper reticular dermis is 700 µm thick (bottom). (**C**) Subcutaneous fat layer is scraped-off of lower reticular dermis with a scalpel. (**D**) After incubation of papillary dermis in dissociation enzyme solution at 37 °C for 1 h, the epidermis can easily be removed with forceps. (**E**) Different dermal layers are minced with scissors and transferred into an enzyme digestion mix consisting of collagenase I, II and IV and hyaluronidase. (**F**) After 1 h of dissociation at 37 °C, tissue digestion is stopped and the suspension is poured through a tea strainer to remove undigested skin pieces. (**G**) Cell suspension is filtered once more through a 70 µm cell strainer and centrifuged at 500 x g at 4 °C for 10 min. (**H**) After centrifugation, the cell pellet is washed with 1x PBS with 10% FCS and is ready for FACS staining.

Figure 2: Sectioning of human skin dermis into papillary and reticular layers with a dermatome. Scheme showing the three dermal layers obtained by slicing full thickness skin into papillary dermis (including epidermis; 0–300 µm; dermal layer 1), upper reticular (300–1000 µm; dermal layer 2) and lower reticular dermis (>1000 µm; dermal layer 3) with a dermatome.

Figure 3: FACS gating strategy for human dermal fibroblast subpopulations. (**A–E**) First, cells are gated on single (**B**) and viable (DAPI-) cells (**C**). Immune cells (CD45+), mesenchymal stem cells (CD106+), red blood cells (CD235ab+) are excluded (**C**) and Pacific blue negative cells are gated further on E-cadherin (Ecad) and ITGA6 double negative cells (PE channel, **D**). E-cadherin and ITGA6 are markers expressed on epidermal cells. Next, CD31-FITC positive cells (endothelial and lymphatic cells) are excluded (**D**) resulting in three fibroblast populations expressing either one

or both of the two cell surface fibroblast markers FAP and CD90: FAP+CD90-, FAP+CD90+ and FAP-CD90+ (**E**).

Figure 4: FAP+CD90-, FAP+CD90+ and FAP-CD90+ fibroblasts differ in dermal localization, gene expression and functionality. (**A**) Representative FACS plots of gated fibroblasts isolated from papillary, upper reticular and lower reticular dermis. Gating strategy is explained in **Figure 3**. FAP+CD90- fibroblasts are enriched in the papillary dermis (19.2%) compared to lower reticular dermis (0.83%). FAP+CD90+ cells can be found throughout the dermis but their highest abundance is in the upper reticular dermis (40.7%). FAP-CD90+ are enriched in the lower reticular dermis (64.4 %). (**B**) Of note, all three sorted subpopulations display typical fibroblast morphology upon culture for 7 days (left). Interestingly, they behave differently in an adipogenesis assay. After 14 days in culture, FAP+CD90- do not differentiate into adipocytes while FAP+CD90+ and FAP-CD90+ readily undergo adipogenesis (right; Oil Red O stains lipid-bearing cells red). Scale bars represent 1000 µm. (**C**) Directly sorted FAP+CD90- fibroblasts express the papillary fibroblast markers CD26, NTN1 and PDPN, while FAP-CD90+ cells express CD36, ACTA2 and PPARγ, known to be expressed by the reticular lineage. * $p \leq 0.05$; *** $p \leq 0.0005$ compared to FAP+/CD90- cells; $^{\#}$ $p \leq 0.05$; $^{\#\#\#}$ $p \leq 0.0005$ compared to FAP-/CD90+ cells.

DISCUSSION:

In this article, we describe a method for the isolation of papillary and reticular fibroblasts from human skin. CD90 has been widely used for the identification or isolation of dermal fibroblasts[18,20,21]. However, we have demonstrated that besides CD90+ fibroblasts, human dermis also harbors a CD90- fibroblast population expressing FAP[16], which has been established as a marker for activated fibroblasts and cancer-associated fibroblasts (CAFs)[22-25]. Importantly, we were able to identify three fibroblast subpopulations FAP+CD90-, FAP+CD90+ and FAP-CD90+ in skin biopsies from all healthy human donors. We therefore conclude that FAP is not only a marker for activated fibroblasts or CAFs but also normal tissue fibroblasts.

Of note, the FAP-CD90- cell population remaining after application of the above-described exclusion- and gating strategy does not contain fibroblasts, since these cells do not proliferate in fibroblast cultivation medium in vitro, but most likely a mixed cell population including lymphatic cells and pericytes amongst others[16].

The cell yield obtained by use of the above-described protocol can vary depending on the body-part that the skin piece used for the isolation originates from. Dermis from different body parts differs regarding its structure, thickness as well as collagen composition. For example, skin from the face or the upper arm is much thinner than skin from the belly or the thigh, which also frequently display a thicker subcutaneous fat layer. Additionally, age and sex of the skin donors may further not only impact the tissue dissociation efficiency, but may also affect the distribution of the three fibroblast subpopulations (**Figure 3**) when isolated from full thickness skin. This results from the fact that the papillary dermis shrinks and that total fibroblast numbers decrease with age[11,26-28]. Furthermore, the cell pellet from papillary dermis will probably be larger than from reticular dermis, since the upper dermis is more densely populated by fibroblasts than the reticular dermis. Besides, the lower dermis is also tougher and more densely packed with

collagen, making it harder to dissociate the tissue and to release the fibroblasts. Of note, the cell pellet may appear very red, which is why red blood cell lysis is recommended.

In addition to the identification of three subpopulations in intact human skin, we also show that in dermatomed skin, each fibroblast subset is enriched either in papillary or reticular dermis[16]. Precise slicing of the skin with the dermatome is critical to obtain a proper enrichment of each subpopulation from different dermal layers. Since the papillary dermis is very thin, the dermatomed slice representing it should not exceed a thickness of 300 µm. Upper reticular and lower reticular fibroblasts both represent the reticular lineage and display similar functions and gene signatures, therefore, one could also consider not separating them.

Importantly, all three fibroblasts populations are found throughout the dermis and are not exclusively present in one layer, which is why explant cultures from papillary or reticular dermis result in mixed fibroblast cultures. However, FAP+CD90- papillary fibroblast are most abundant in the papillary dermis and follow a gradient from superficial to lower dermal layers while FAP+CD90+ and FAP-CD90+ fibroblasts follow an inverse gradient from the lower to the superficial layers[16]. Furthermore, the majority of CD90+ fibroblasts of the papillary dermis are almost exclusively found surrounding blood vessels and express the perivascular fibroblast marker CD146[29], and thus probably exhibit different functions than the remaining CD146- reticular fibroblasts[16]. CD146 could be used as an additional marker in the gating strategy to exclude this population.

Following the dissociation of dermal layers, the isolated cells are stained with a specially designed antibody cocktail containing various antibodies for the exclusion of immune cells, endothelial and lymphatic cells, epidermal cells, erythrocytes and MSCs to obtain pure fibroblast populations. Of note, choosing a marker for the identification and exclusion of MSCs may be tricky because of the high number of published MSC markers[30,31]. Since MSCs express CD90 like fibroblasts, additional MSC markers such as CD105 or CD271 could prove useful for their identification. However, MSCs only represent a very low percentage of all dermal cells and since CD90+ fibroblasts display typical morphological features of fibroblasts upon sorting, one could argue that the exclusion of MSCs by use of distinct cell surface markers might be unnecessary.

Importantly, we analyzed FAP and CD90 gene expression after keeping the cells in culture for 7-14 days after sorting (data not shown) and found that expression of both markers is upregulated in the respective sorted single positive (FAP+CD90- or FAP-CD90+) cells[16]. We therefore emphasize that the above-described marker sets and protocol permit the isolation of primary fibroblast subsets directly from the tissue but not from previously cultured mixed fibroblast populations.

Nevertheless, we demonstrate that the functionality of all three subpopulations is retained in cell culture regardless of the alteration of cell surface marker expression, since fibroblasts sorted as FAP+CD90- papillary fibroblasts do not acquire the ability to undergo adipogenesis after a longer period of culture, while fibroblasts sorted as FAP+CD90+ or FAP-CD90+ reticular fibroblasts maintain their ability to differentiate into adipocytes [16]. Importantly, we also found that papillary

and reticular-specific genes are still expressed to a higher extent in FAP+CD90- and CD90+ respectively.

In conclusion, we have established a protocol for the isolation of functionally distinct fibroblast subsets via FACS that for the first time permits the isolation and analysis of pure and naïve fibroblast subpopulations from human skin dermis. This method establishes a major advancement to the commonly used fibroblast explant culture isolation protocol from upper and lower dermis as (i) an opposing gradient of papillary and reticular fibroblasts exists from the skin surface to the hypodermis and (ii) fibroblasts change their gene signature in vitro.

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DISCLOSURES:

The authors have nothing to disclose.

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Figure

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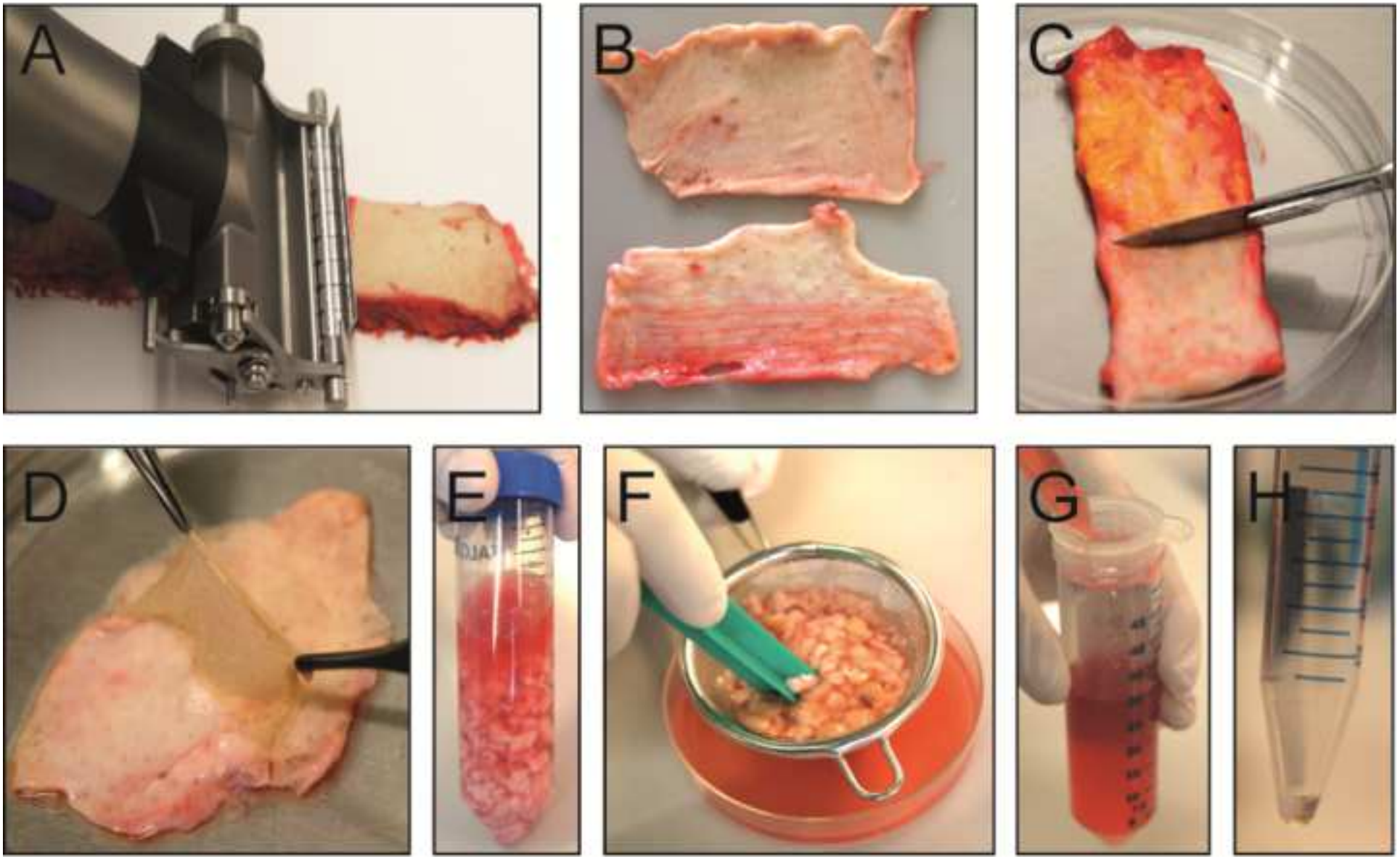

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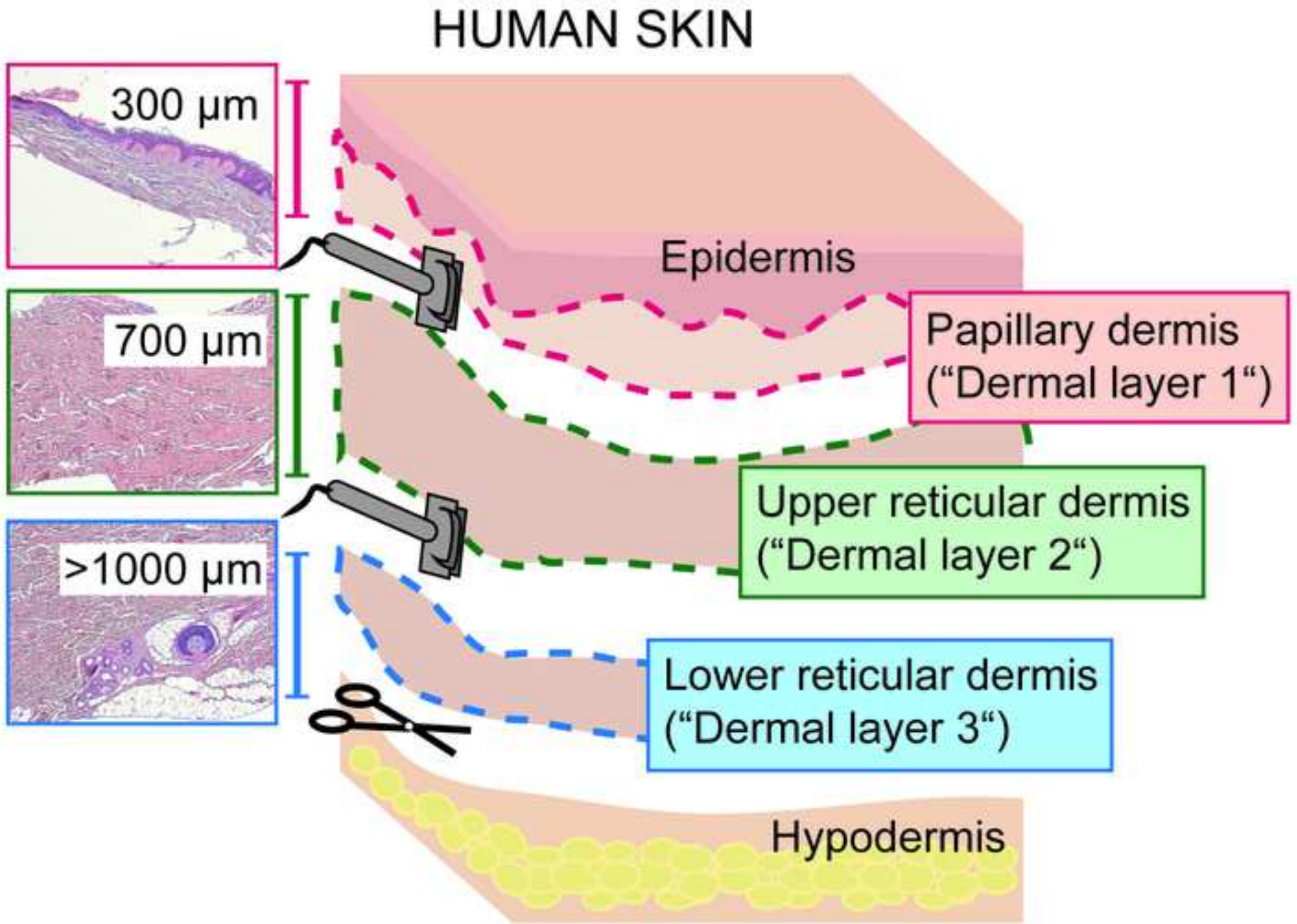

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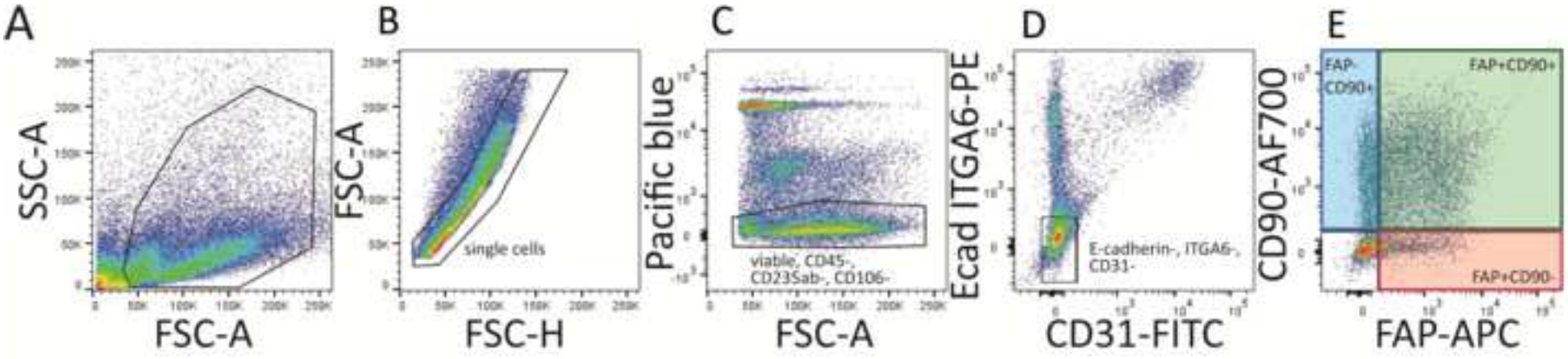

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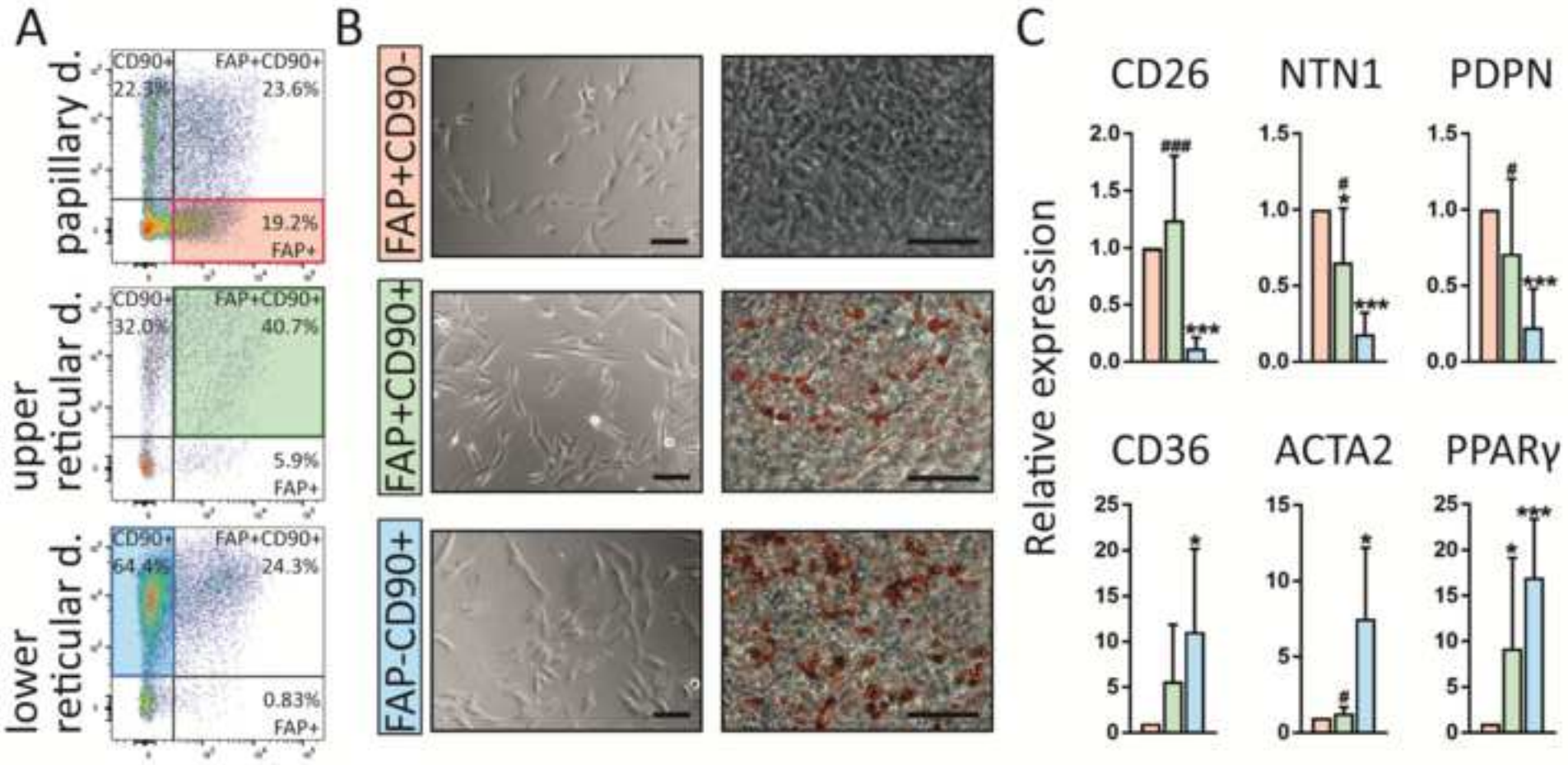

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Name of Material/ Equipment	Company	Catalog Number
Material:		
3-isobutyl-1-methylxanthine	Sigma	I5879
Ammonium chloride	Sigma	9718
Amniomax-C100 (1x) Basal Medium	Gibco	17001-074
β-Mercaptoethanol	Scharlau	ME0095
C100 Supplement	Thermo Scientific	12556015
Collagenase I	Thermo Scientific	17100017
Collagenase II	Gibco	17101015
Collagenase IV	Sigma	C5138
DAPI	Thermo Scientific	62248
Dexamethasone	Sigma	D8893
Dispase II	Roche	4942078001
Dulbecco‘s Modified Eagle Medium + GlutaMAX	Gibco	31966-021
EDTA disodium salt	Sigma	E1644
Fetal bovine serum (heat inactivated)	Gibco	10500-064
Hyaluronidase	Sigma	H4272
Insulin	Sigma	I5500
Isopropanol	Merck	1,096,341,011
OilRed O	Sigma	O-0625
Paraformaldehyd	Sigma	158127
Penicillin/Streptomycin	Gibco	15070-063
Phosphate-buffered saline without Ca++ & Mg++	Lonza	BE17-512F
Potassium bicarbonate	Sigma	237205
PureLink RNA MicroKit	Invitrogen	12183-016
SuperScript III First-Strand Synthesis SuperMix for qRT-PCR	Invitrogen	11752
Taqman 2xUniversal PCR Master Mix	Applied Biosystems	4324018
Troglitazone	Sigma	T2573
Flow cytometry Antibodies:		
anti human CD31-AF488	Biolegend	303109
anti human CD45-Pacific blue	Biolegend	304029

anit human CD49f/ITGA6-PE	Serotec	MCA699PE
anti human CD90/Thy-1-AF700	Biolegend	328120
anti human CD106-AF421	BD	744309
anti human CD235ab-Pacific blue	Biolegend	306611
anti-human E-cadherin-PE	Biolegend	147304
anti human FAP-APC	R&D	FAB3715A
Human TruStain FcX	Biolegend	422302

Equipment:

1.4 mL micronic tubes	Thermo Scientific	4140
1.5 mL screw cap micro tube	Sarstedt	72,692,405
5 mL Polystyrene Round Bottom Tube with cell strainer cap	Falcon	352235
48-well cell culture cluster	Costar	3548
50 mL polypropylene canonical tubes	Falcon	352070
70 µm cell strainer nylon	Falcon	352350
Aesculap Acculan 3Ti Dermatome	VWR	AESCGA670
Aesculap Dermatome blades	VWR	AESCGB228R
MicroAmp Fast Optical 96well	Applied Biosystems	4346906
Primary cell culture dish	Corning	353803
Scalpel blades	F.S.T	10022-00
Tea strainer	x	x

Fibroblast growth medium:

AmnioMAX basal medium with AmnioMAX C-100 Supplement, 10 % FCS and 1 % P/S

Comments/Description	Dilution:	Lot:	RRID:
used for selfmade ACK Lysis Buffer	1:30	B213986	AB_493075
used for fibroblast growth medium	1:20	B218608	AB_2174123
! CAUTION			
used for fibroblast growth medium			
! CAUTION			
! CAUTION			
! CAUTION			
! CAUTION			
used for selfmade ACK Lysis Buffer			
! CAUTION			
! CAUTION Cancerogenic. Skin and eye irritant. Handle with care.			
used for selfmade ACK Lysis Buffer			
! CAUTION Skin and eye irritant.			

1:20	0109	AB_566833
1:30	B217250	AB_2203302
1:100	7170537	x
1:1000	B224563	AB_2248153
1:20	B197481	AB_2563040
1:20	AEHI0117111	x
1:20	B235080	x

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Author(s): HOROSEC, FRECH, LICHTENBERGER

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Vienna, 18-Dec-2018

Dear Drs. Upponi and Dsouza,

Many thanks again for inviting us to submit our manuscript entitled "Isolation of papillary and reticular fibroblasts from human skin by FACS sorting" (JoVE59372) to JoVE, and for giving us the opportunity to revise the manuscript.
We have changed the text and figures according to the editor's and reviewers' suggestions. Please find the point-by-point response to these comments attached. Our response is labelled in blue.

We hope that the manuscript is now acceptable for publication at JoVE and look forward to hearing from you soon.

Yours sincerely,

Beate Lichtenberger

POINT-BY-POINT RESPONSE

Editorial comments:
Changes to be made by the author(s) regarding the manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.
We have proofread the text thoroughly and corrected typos etc..
2. Please shorten the Summary to no more than 50 words.
The summary is now shorter than 50 words.
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Commercial terms in the text have been replaced.
4. Please place the ethics statement (lines 434-437) before the numbered protocol steps, indicating that the protocol follows the guidelines of your institution's human research ethics committee.
The position of the ethics statement has been changed accordingly.
5. Please revise the protocol text to avoid the use of any personal pronouns (e.g., "we", "you", "our" etc.).
Personal pronouns have been removed.
6. The Protocol should be made up almost entirely of discrete steps without large paragraphs of text between sections. Please simplify the Protocol so that individual steps contain only 2-3 actions per step and a maximum of 4 sentences per step. Use sub-steps as necessary. Please move the discussion about the protocol to the Discussion.
We have changed the protocol according to the editor's suggestions and moved any discussion sentences from the protocol to the discussion.
7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. See examples below.
We have added more details wherever necessary.
8. Line 116: Please specify the source of skin piece epidermis.
The source has been added.
9. 4.3: Please specify the incubation temperature.
This has been specified.
10. 5.4: Please provide the composition of ACK Lysis buffer.
This has now been provided.
11. 8.1: Please specify the cell numbers.
Cell numbers have been added.
12. 8.3: At what temperature are the cells fixed?
At ambient temperature.
13. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.
The text for the filming has been highlighted.
14. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Notes cannot usually be filmed and should be excluded from the highlighting.
15. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.
16. Figure 2: Please label different panels to facilitation their explanation in the figure legend.
This has been changed.
17. Please revise the Discussion to explicitly cover the critical steps within and protocol and any limitations of the technique.
The discussion has been revised.
18. References: Please do not abbreviate journal titles.
We have used the endnote output style provided by JoVE. Although in the style options the

term ‘use full journal name” is ticked, some Journal titles are abbreviated.
19. Table of Materials: Please provide lot numbers and RRIDs of antibodies, if available, and sort the items in alphabetical order according to the name of material/equipment.
The materials list has been changed accordingly.

Reviewers' comments:

Reviewer #1:

Manuscript Summary:
This manuscript describes a clear and useful protocol to isolate human skin fibroblasts from reticular and papillary dermis by enzymatic digestion followed by FACS (avoiding performing explant culture). It is a improvement from what is described so far in the field. Authors are experts who have troubleshooted a great new technique.
We are glad that this reviewer thinks our protocol is an improvement compared to other available methods, and are grateful for his/her minor comments, which we addressed to improve the manuscript.

Major Concerns:
No major concern.

Minor Concerns:

In the introduction regarding the structure of the skin, hypodermis should be mentioned.
This has been added.
Overnight storage of other layer samples could be clarified : floating on PBS or immersed in PBS?
This has been clarified in the revised manuscript.
Similarly, lanes 352/353, section 3.1 "place the epidermis/papillary dermis from 2.1. with the epidermis facing upward..." from 2.1 or from 1, correct?
This has been corrected.
Finally, in section 4.3 providing a ratio of weight to volume of digestion mix could be helpful
This has been added.

Reviewer #2:

Manuscript Summary:
The protocol presented by Korosec et al describes the step-wise isolation of human papillary and reticular fibroblast using FACS sorting. This is the first protocol to isolate distinct fibroblast populations with very high purity from human skin. Given the importance of fibroblasts in tissue homeostasis and disease, and analyzing human tissue, this protocol is highly valuable. The protocol itself is clearly explained and good to follow (the very few improvement suggestions are given below). Taken together, I recommend publication of this protocol in JoVE.
We are grateful to this reviewer for recommending publication of our manuscript and his/her comments and suggestions of how to improve the manuscript.

Major Concerns:
None.

Minor Concerns:

Suggestions for minor textual changes to further improve clarity:

Lines 117-120:
Authors wrote "Then slice the tissue into 5 mm wide strips in order to facilitate the penetration of the Dispase II solution (cf. 3. Separation of epidermis and dermis) into the tissue before putting the skin pieces into a petri dish if epidermis needs to be separated from the whole dermis. Please skip

2. Sectioning of human skin papillary and reticular layers with a dermatome and directly refer to 3. Separation of epidermis and dermis."

Suggested change:
Then slice the tissue into 5 mm wide strips before putting into a petri dish. This facilitates the penetration of the Dispase II (cf. 3. Separation of epidermis and dermis), and is used if the epidermis needs to be separated from the entire dermis. Please skip 2. Sectioning of human skin papillary and reticular layers with a dermatome and directly refer to 3. Separation of epidermis and dermis.

We have introduced these changes as suggested.

Line 124: Perhaps some readers would be concerned if cleaning the tissue with ethanol would cause lots of cell death in the tissue. Could you please make a comment here (if true) that the caused death of cells is minor (or something similar).

A comment concerning cell death has been added.

Line 152: In 3.1 it would add to clarity to say: 3.1. Prepare a 3 U/mL Dispase II solution in sterile 1xPBS and place the 5 mm skin stripes (from 1.), or the epidermis/papillary dermis (from 2.1.), with the epidermis facing upward in a 10 cm petri dish with 10 mL Dispase II solution....

This has been changed according to the reviewer's suggestion.

Line 168: It may be helpful to introduce "Dermal layer 1, 2 and 3 (or similar)" in the points 2.2, 2.3 and 3.2 (see below), so that here you could refer to: "Mince all dermal layers (Dermal layers 1, 2, and 3) separately with scissors/scalpel as thoroughly.....". This would have helped me not to search back in the text which exact steps/layers/tissue parts were meant.

For example:
2.2. Repeat 2.1. by adjusting the dermatome to a cutting thickness of 700 μm and slice the remaining dermis. Place upper slice which is defined as the upper reticular dermis ("Dermal layer 2") into a separate petri dish.

2.3. Scrape away the subcutaneous fat layer with a scalpel from the residual lower reticular dermal layer (> 1000 μm) and discard it. Collect the lower reticular dermis ("Dermal layer 3") in another petri dish.

3.2. After incubation, transfer the epidermis/papillary dermis to the dry lid of the petri dish and separate epidermis from upper dermis ("Dermal layer 1") with two forceps each holding the edge of either epidermis or dermis.

Alternatively (if this is an option for JoVE) you could show a simple cartoon marking the 3 layers.

We have added a cartoon showing the three dermal layers (Figure 2) and changed the text according to this reviewers suggestions.

Line 212: Since you give an exact time for the lysis, this statement may lead to confusions. Either it would need one more sentence like: you can adjust the time for erythrocyte lysis if it would appear incomplete; however, don't keep cells too long in buffer .... Also, I could not easily find the self-made ACK Lysis buffer (i.e. contents); could you please refer to it?

The contents of the ACK lysis buffer has been added and we have changed the text according to the suggestions.

Since it is apparent that the protocol is new/first time of its nature, it feels not necessary to use the word "novel" repeated times; a suggestion is to use it only in the abstract and remove elsewhere (lines 90, 103, 416), but this is of course free for the authors to decide.

We have removed repeated claims of novelty.