A guide to studying human dermal adipocytes in situ

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Prof. Thomas Luger
Editorial Board Member
Experimental Dermatology

Manchester, March 12, 2018

RE: EXD-17-0432

Dear Prof. Luger, lieber Thomas,

We thank you and your referees for the time and effort spent reviewing our methods review by Nicu et al. entitled ‘A guide to studying human dermal adipocyte in situ’.

We are pleased to submit a revised manuscript that fully implements the valuable suggestions made by your expert reviewers. All the changes have been acknowledged and detailed in our point-by-point reply.

As explained below, we are in full agreement with both reviewers and their helpful feedback. We hope that you shall consider our revised manuscript acceptable for publication, and we look forward to hearing back from you soon.

Kind regards,

Ralf

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Nicu et al – Point-by-point Reply

Reviewer: 1

Suggestions for Authors
In this method review, Nicu et al well summarized the available techniques for studying human dermal fat, and also provide detailed guidelines for dermal fat study. This review would be of great help to the investigators of dermal fat study.

We are pleased that the reviewer finds this review to be of “great help to investigators” in the field.

Minor suggestions are listed below:

- Human dermal fat is different from murine one, no panniculus carnosus layer exist in human skin to separate the dermal fat layer and subcutaneous fat layer. The primary adipocytes that mentioned in the “Dermal Adipocyte Culture” Section (Page 11), the Lonza derived adipocytes, was isolated from the whole subcutaneous fat of human skin. As a result, Lonza preadipocytes are the mixture of preadipocytes from both dermal fat and subcutaneous fat. The author should make it clear in this review.

Thanks a lot – we absolutely agreed that this needs to be made clearer. We already discuss the distinction between human and mouse skin (page 2, section “Human DWAT anatomy and biology in a nutshell”, and mention that, perhaps the platysma colli of human neck skin may be somewhat comparable to the pannicus carnosus that divides the subcutis of murine skin) (Larson et al, 2014). As requested, we now further clarify that the Lonza pre-adipocytes used in the current study are derived from whole subcutaneous human fat.

- As Magnetic resonance imaging (MRI) is a good way for fat imaging. MRI method could be included in this review.

We have incorporated the reviewer’s helpful suggestion into our manuscript by adding a section on imaging adipocytes using MRI after the ‘Transmission Electron Microscopy’ paragraph, as well as adding MRI as an imaging technique in Table 1, along with appropriate references.

Reviewer: 2

Suggestions for Authors
Over the last years, several research groups have focus their activities on the study of subcutaneous and visceral fat, trying to understand the role of these adipose tissues in the development of metabolic diseases (insulin resistance, type 2 diabetes, cardiovascular diseases, liver diseases). Interestingly, adipose cells are found in several other compartments of the body and are now known to participate in the development of other diseases.

This is the case for dermal adipocytes, that are now known to play key roles in regulating several biological processes in the skin. In this manuscript, Carina et al. review the common techniques used to study dermal adipocytes. The timing for this review is good considering the growing interest in dermal fat cells. Overall, this review is interesting and well written. I liked the enthusiasm of the authors toward the study of this fat depot. I only have minor comments that should be addressed before the publication of the manuscript.

We thank the reviewer for this encouraging feedback.
1) P5, line 38. There is an emerging literature on bright fat cells. The authors should at least mention that these cells exist.

The reviewer makes an excellent point. As requested, we have now discussed bright fat cells in our ‘Human DWAT anatomy and biology in a nutshell’ section, along with appropriate references, in which details can be further explored.

2) P5. Please define what HF refers to. I presume that this means hair follicle but I could not find the information.

We apologize for this omission, which has now been corrected. Yes, indeed: HF is an abbreviation of hair follicle.

3) Table 1. This table is interesting but several changes should be made. First, the section 'adipocyte size and number' could be fused with the 'Mature adipocyte' section. The information presented in these sections is the same.

We thank the reviewer for their valuable comments on Table 1. Based on this feedback, we have made extensive changes to Table 1. Among these, we have amalgamated “adipocyte size and number” with “mature adipocyte” as suggested.

4) Table 1 (and reference in the text, p.9-10). I was surprised to see that CD68, PLIN1, and caveolin are used as marker of apoptosis. I agree that adipose cell death often produces necrotic sections and crown like structures. However, dying adipocytes will not necessarily be associated with CD68 staining. Moreover, the presence of macrophages (CD68) in fat does not mean that adipose cells are dying. This section should be corrected as it can be misleading.

Thank you for encouraging us to clarify this. We meant that adipocyte death could be measured using a double stain of CD68 coupled with either PLIN1 or Caveolin1 to mark both macrophages and dermal adipocytes – namely those that are completely surrounded, and thereby supposedly phagocytosed, by CD68+ macrophages. We have now added this information since it features prominently in several mouse adipocyte research papers (Berry et al, 2014; Martinez-Santibanez et al, 2014; Cinti et al, 2005; Giordano et al, 2013), which are now cited in the revised review. However, we understand that dying adipocytes can express CD68+ crown-like structures only during necrosis and pyroptosis (and have phrased this accordingly in the core text under ‘Apoptosis markers’). We have made the appropriate changes in both Table 1 and the core text under ‘Apoptosis markers’ to avoid any confusion with the readers.

5) Table 1. It would be important to add some staining in the table. In the text, the authors refer to Masson Trichrome and Picrosirius Red to stain fibrosis. Unfortunately, we do not find any reference to these staining in Table 1.

6) Table 1. The same applies for Methylene blue.

Thank you for helping us to improve the table regarding Points 5) and 6). These markers have now been added to Table 1.

7) Table 1. The importance of including the sections 'lipid synthesis' and 'lipolysis' in Table 1 is debatable. It is not accurate to measure lipolysis by looking at ATGL, MGL and HSL protein levels by IF. Lipolysis is a
complex biological process that involves rapid phosphorylation /dephosphorylation steps and looking at the total levels of enzyme does not mean anything in terms of enzymatic activity. The same comment applies for lipid synthesis. The levels of FAS, DGAT and LPL can be a marker of differentiation (mature vs immature fat cells) but these proteins cannot be used by IF to measure lipogenesis.

The reviewer raises an excellent point. ATGL, MGL and HSL are regulated at the post-transcriptional level, meaning that there can be a lack of correlation between their levels and the process of lipolysis. Thus, we have eliminated ATGL and MGL from Table 1 as markers of lipolysis, and instead have included phospho-Perilipin1 and phospho-HSL which, in their phosphorylated form, constitute a direct readout of PKA activity (Schweiger et al, 2014) and could be used to semi-quantitatively study levels of lipolysis by IF. Furthermore, we have transferred FAS, DGAT and LPL markers to the mature adipocyte section in Table 1 and, instead, have added ex vivo BODIPY C1/C12 uptake as an efficient measure of DWAT lipogenesis to Table 1; we have also added BODIPY C1/C12 uptake assay in human abdominal organ culture as an additional supplementary figure (Suppl. Fig. 6a). We also include free glycerol released by adipocytes into media as a readout parameter for lipolysis ex vivo alongside an example of quantification for this assay (Suppl. Fig 6b). Moreover, in the lipogenesis/lipolysis section of Table 1 we highlight a simple method to measure these parameters in situ via quantifying changes in adipocyte size/volume. In addition, we have referred the readers to reviews which explain how to efficiently measure lipogenesis/lipolysis in activity-based assays (Schweiger et al, 2014) (Section ‘Studying lipogenesis/lipolysis in human DWAT’) and now explicitly acknowledge that lipolysis is a complex biological process that involves rapid phosphorylation /dephosphorylation steps so that looking at the total protein levels of enzymes involved in this complex process is uninstructive in terms of assessing enzymatic activity (Lass et al, 2011; Nielsen et al, 2014) (section ‘Examining lipogenesis/lipolysis in situ and using activity-based techniques’).

8) P.7 It would be great to say few words about the timing for the fixation steps. Many people over or under fix their samples, which greatly impairs the staining process.

Yes, that makes perfect sense, as fixation time can be critical. We now recommend fixation times in the core text in the ‘Fixation’ section for both paraffin-embedded and cryopreserved tissue. Thank you!

9) Generally, it would be important to carefully review the abbreviations used throughout the review. I have seen many abbreviations that are never fully described in the text. FABP4, DAG, MGL, LPL, PPARG, CEBP, CD68, IF, DGAT, FASN, LD, NEFA, PKA…

Thank you for pointing out this error. We have now surveyed the entire text and made the appropriate changes. Where dispensable, abbreviations were deleted and words spelled-out in full.

10) Table 2. The line that describes pCEBPB. Please mention that this protein promotes terminal adipocyte differentiation together with CEBPA (not CEBPD).

Thank you for drawing attention to this mistake in Table 2 – this has now been amended.

11) The lipidomics, proteomics and metabolomics sections should be either i) removed or ii) improved. These sections are extremely short and underdeveloped. These sections also appear out of place. Personally, I would take them out.

The aim of these sections was to present the readers with alternative approaches to study dermal fat in addition to immunohistochemistry, ISH and TEM. We agree that these sections were underdeveloped and we have now
removed them from the manuscript, as recommended. In addition, we clarify in the introduction that the review will not discuss techniques associated with lipidomics and metabolomics – instead, we point the readers to a few useful reviews in case they wish to delve into these complex topics after reading our review.

We are genuinely grateful to this expert reviewer for the excellent advice we have received on making our review maximally useful – thank you very much!
Methods Review

A guide to studying human dermal adipocytes *in situ*

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Abstract

Dermal white adipose tissue (DWAT) is a main component of human skin, composed of individual lipid-laden mesenchymal cells known as dermal adipocytes (DAs). Besides their well-known role in lipid storage and release, DAs also promote skin immunity, wound healing and hair follicle cycling and are important players in cutaneous neuro-endocrinology. The ever-growing insights into DWAT functions, albeit mostly in mice, have invited speculation that it may be involved in multiple skin diseases ranging from fibrosis to alopecia and psoriasis, thus designating human DWAT a clinically relevant, but as yet insufficiently investigated skin compartment. Therefore, this practical, user-friendly guide aims to introduce the techniques available to study human DWAT in situ and ex vivo, including immunohistochemistry, immunofluorescence microscopy and analysis via quantitative immunohistomorphometry. Here we provide information on a collection of stains comprising pre-adipocyte (Pref1) and mature adipocyte markers (Perilipin1, Caveolin1), as well as various lipid (OilRedO, BODIPY) and histochemical stains (H&E, Trichrome) available for use on human DWAT. We offer the reader guidelines on fixation, tissue processing and staining human DAs and highlight caveats and solutions to common problems that one may encounter when studying this fascinating skin compartment. We also suggest standard methods for conducting quantitative immunohistomorphometry on human DWAT and its individual adipocytes in order to quantify cell size, number, lipid content and fluorescence intensity of adipose-specific markers. Finally, we briefly introduce in situ hybridisation, transmission electron microscopy, and essentials of MRI imaging as additional tools for instructively interrogating this largest, but still least-known compartment of human skin.

Key Words

white adipose tissue; immunohistochemistry; immunofluorescence
Introduction

Historically often viewed as just an inert, energy-storing and thermo-insulating spacer material [1], dermal white adipose tissue (DWAT) is making a resounding scientific comeback as increasingly more studies are emerging which highlight its important and diverse functions in cutaneous biology and beyond [2–9]. It is now recognized that the dermal adipose depot is involved in multiple additional activities, including protection against bacterial infections [4], controlling hair cycle wave patterns [6, 7, 10], thermogenesis [1], promoting wound healing [1, 11–13] and hypothesized neuroendocrine activities [14, 15]. Human DWAT as well as deeper subcutaneous fat layers also harbour heterogeneous, but multi-potent adipose-derived stem cell populations that have been used in the context of cell-based autologous cell therapy, thus finding widespread entry into clinical practice [3, 16–21].

Multiple reviews have appeared on the functions and biology of DWAT in mice [2, 8, 9, 22–25], including reviews on how to image murine adipose tissue [26, 27]. However, there is no comprehensive guide available on staining and imaging techniques of human DAs in situ. This constitutes a major open question since the anatomy of human and murine skin adipose layers (and quite likely their physiology) are distinct, calling for independent characterisation.

The present Methods Review attempts to close the literature gap by firstly providing a brief introduction into DA biology in humans. We then present a compendium of histological stains and immunohistochemistry markers available to study human DWAT functions, ranging from lipid droplet biology to distinguishing between pre-and mature adipocytes. We also present data from a primary pre-adipocyte cell line that has potential to differentiate and act as a positive control for DWAT markers. Although we do not discuss key adipocyte techniques associated with lipidomics, metabolomics or proteomics, we direct the readers towards specialized reviews on the subject [28–37]. We aim to provide advice to both enthusiasts and newcomers in the skin adipocyte field.

Human DWAT anatomy and biology in a nutshell

Adipose tissue is found within multiple body sites and can be divided into brown and white fat, the first thought to be involved predominantly in thermogenesis whereas the latter is recognized as a key player in whole-body metabolism [1, 8, 9, 38–40]. An additional type of fat cell exists i.e. beige/bright adipocytes that undergo transdifferentiation from white fat [41–43]. Recently, there has been an important paradigm shift in how “fat” and what used to be called the “subcutis” or “hypodermis” [44] is viewed, since it has turned out that functionally and embryologically distinct layers of adipose tissue are distinguishable in mammalian skin (Fig. 1a) [45].

Whilst murine DWAT is clearly divided from the underlying subcutaneous white adipose tissue by a distinctive muscle layer, the panniculus carnosus [46–48], it remains unclear how to morphologically distinguish between human DWAT and the subcutaneous fat layer (Fig. 1a,b) [49]. However, one notable exception described on human cadavers is that of the platysma colli muscle that separates the subcutis into two fat compartments in the neck, the distal (upper) which may be related to human DWAT [50]. Interestingly, human DWAT exhibits a unique cone geometry, clustering around the pilosebaceous units and forming so-called ‘dermal cones’ (Fig. 1c).
DAs typically envelope the connective tissue sheath of hair follicles (HFs) in scalp and abdominal skin, and can stretch from underneath the HF matrix up to eccrine sweat glands and the bulge area of HFs [51, 52] (Fig. 1c).

*Dermal Adipocytes*

DWAT contains a variety of components including fibroblast-like progenitor adipocytes, mature lipid-laden adipocytes and other cell types such as fibroblasts, macrophages, pericytes, mast cells, endothelial cells, adipose-derived stem cells, as well as an extensive vascular network (Fig 1f) [27]. The main building blocks of DWAT are dermal adipocytes (DAs) – unilocular spherical cells which function to store and release energy via lipogenesis and lipolysis [53]. Their typical shape is the result of the resident lipid droplet, the main site of triglyceride and cholesterol storage which occupies >90% of the cell volume [24]. With their characteristic honeycomb-shape, adipocytes can oscillate in size from 50µm up to 150µm, thus representing the largest cells in the skin. Their cytoplasmic structures and nucleus are compactly located in a thin layer on the inner rim of the cell (Fig 1.d,e).

**Clinical relevance of DWAT**

At a whole-body level, adipose tissue is a complex endocrine organ system involved in regulating immunity, inflammation, metabolism, insulin sensitivity and overall tissue homeostasis via the secretion of hormones, metabolites and signalling molecules referred to as adipokines [38, 39, 54, 55].

DAs stimulate wound repair by their own regeneration from myofibroblasts and by promoting fibroblast migration and vessel regeneration [3, 11, 13, 56]. DWAT also defends against *S. aureus* skin infections by producing the antimicrobial peptide Cathelicidin [4], and DAs are thought to play a key role in skin fibrosis via adipocyte-myofibroblast transition [5, 57]. This mechanism is speculated to promote development of fibrotic structures associated with hair follicles in androgenetic alopecia [40], supported by higher plasma leptin levels being associated with an increased incidence of androgenetic alopecia [58].

Apart from classical dermatoses that affect the adipocyte layers of human skin (e.g., panniculitis, lipogranulomatoses, lipomata, lipoatrophy, lipodystrophy) [59, 60] DAs are likely involved in other cutaneous diseases. Studies have shown fluctuating levels of adipokines in psoriasis patients [61, 62], and the use of therapeutics in this condition has been associated with fluctuations in DWAT thickness [63–65]. Moreover, a recent study supports the role of DAs in promoting melanoma aggressiveness [19].

**Overview of visualisation methods to consider**

When studying DWAT, there are a number of methods that one can choose from. Standard stains such as Haematoxylin and Eosin (H&E) and Masson’s Trichrome allow the visualization of adipocyte morphology and any changes in tissue or individual cell expansion or contraction. Lipid stains such as Oil Red O (ORO) and BODIPY can be used to visualize and analyse lipid droplet content, size and accumulation within DAs. Lastly, immunohistochemical/immunofluorescence techniques are widely employed to localize and detect changes in proteins of interest within DWAT by immunohistomorphometry.
**Key considerations prior to human DA visualization in situ**

The predominant approach to assessing DWAT is via histology/immunohistochemistry, however, prior to staining there are some key considerations to be made (Table 1).

**Paraffin-embedded versus cryopreserved tissue**

Although paraffin embedding offers excellent morphological detail of DWAT, lipids are displaced during paraffin processing rendering lipid staining protocols impossible. In contrast, cryosectioning offers many advantages including time-efficient protocols and the ability to conduct lipid stains. However, if tissue is scarce and multiple sections are required, sectioning via the microtome is preferable to obtain sufficient thin sections.

**Section thickness**

When the primary aim is to study lipid content, it is advisable to cut cryopreserved samples at a thickness of 15 – 30μm to maintain the cellular lipid content (Suppl Fig. 1). Sections may be quickly visualized using a microscope, though must not be allowed to thaw before storage at -80°C in order to preserve the lipids. For immunohistochemical-based approaches thinner sections are preferred, however, when collecting sections from cryopreserved DWAT, maintaining a temperature equal to or lower than -30°C within the cryostat is crucial to obtain thin (7μm) sections of high quality.

**Fixation**

Choice of fixative and its duration can greatly affect the quality of tissue sections and subsequent staining. Though neutral-buffered formalin (NBF) is the universal fixative used for tissues aimed to undergo paraffin processing, our observations suggest that it is a harsh fixative for DWAT. One alternative fixative is Zinc Formalin which better preserves DA morphology as recommended by [26]. Alternative fixation methods include 4% paraformaldehyde (PFA), 20% normal-buffered formalin (NBF) and 99% ethanol (EtOH), however these have very different effects upon RNA quality, morphology and staining intensities [66]. For fatty samples that are to be embedded in paraffin we recommend 24hr fixation prior to processing.

For cryopreserved tissue, multiple fixatives can be employed including 4% PFA, ethanol, acetone and methanol; however, alcohol-based fixatives should be avoided when the aim is to examine lipid droplet content as these solvents displace lipids [67]. Instead, 4%PFA or ethanol:glacial acetic acid can be successfully used to obtain high-quality lipid stains (Suppl Fig. 2). We recommend performing the fixation step in 4%PFA immediately after thawing the tissue. For cryosections containing fat we have found 20-30min to be an optimal fixation time.

Following fixation and sectioning, DWAT can be stained using a range of techniques including immunohistochemistry and immunofluorescence stains. To help our readers, we have compiled a list of suggestions to quickly and easily combat common problems when working with human DWAT (Box 1).

**Histochemistry**

Standard haematoxylin and eosin (H&E) staining may be employed to visualize DWAT structure within skin, and around pilosebaceous units (Fig 1b,c; Fig 2a). H&E is useful when assessing human biopsies because it
shows gross changes in morphology and may point towards further areas for closer examination [68]. For example, one can infer whether human DWAT thickness or the amount of fibroblast networks surrounding DAs changes in samples from healthy, alopecia areata and sebaceous nevus patients [69]. Moreover, H&E can be used to pinpoint whether expansion or reduction occurs within DWAT as highlighted in S. aureus infection in mice [4]. Using H&E, the exact distribution and changes in individual cell size and number can be assessed using Adiposoft and Cell Counter plugins in ImageJ (Suppl. Fig 3 a,b).

Another histological stain that proves useful when studying adipocytes is Masson’s Trichrome (Fig 2b) which stains fibrillar collagens I and III surrounding DWAT. Masson’s Trichrome can highlight ectopic collagen deposition between individual DAs, a marker of DWAT fibrosis [70], and also infers collagen deposition following organ culture of skin biopsies (Fig. 2b), a simple method to study fibrosis in DWAT [57, 71–73]. An alternative stain for collagen fibrils is Picrosirius Red (Fig. 2c).

Methylene Blue (MB) is a vital dye that can be used to stain the skin as a whole [74]. MB stains human skin compartments such as the epidermis, follicular units and the dermis but is not absorbed within DAs apart from in the nucleus [75, 76]. An advantage of MB compared to H&E is that it can be performed as a wholemount technique, allowing the dissection and isolation of DWAT by providing contrast to the surrounding dermis and follicular unit (Fig. 2d).

**Lipid stains**

Oil red O (ORO) is a hydrophobic dye used to visualize lipid droplet localization, morphology and accumulation within adipose tissue [67, 77]. ORO stains neutral lipids (triacylglycerols, diacylglycerols) and cholesteryl esters (Fig 2e), but not polar lipids on the lipid droplet membrane such as ceramides, sphingolipids and phospholipids [78]. ORO content in DAs can be quantified using ImageJ as detailed in Suppl. Fig 3c. ORO staining can prove difficult to quantify if sections are too thin (Suppl. Fig 2) or not stored and fixed appropriately following cryosectioning. Precautions should be taken when choosing the reagent in which to dissolve ORO as certain chemicals may result in staining of non-adipogenic cells [79].

Other chemicals that stain lipid within DAs include Nile Red [80], Sudan Red [81], and Osmium Tetroxide which is routinely used in electron microscopy, but may result in adipocyte swelling [82].

In order to quantify changes in lipid accumulation in DAs, fluorescent lipid stains on cryosections such as BODIPY 493/503 (Fig. 2f) or LipidTOX (Suppl. Fig. 1) can be used as an alternative to ORO. BODIPY 493/503 and LipidTOX are cell-permeable lipophilic neutral dyes that can be employed for both microscopy and flow cytometry [2, 83–90]. In our studies BODIPY (Fig 2f) appears superior in terms of fluorescence intensity given that LipidTOX rapidly bleaches and does not display the lipid droplet morphology described in other studies (Suppl. Fig. 1) [2, 7].

Cell Mask Plasma Membrane (CPM) stain (Suppl. Fig. 1) is an amphipathic molecule that anchors into, and histochemically defines, the plasma membrane of human DAs and can aid in visualizing DAs in conjunction with lipid stains. This double stain also allows the correct identification of adipocyte nuclei, located between the lipid droplet and the adipocyte plasma membrane [26].
**Immunohistochemistry and immunofluorescence microscopy**

When staining adipocytes using IHC or immunofluorescence (IF) techniques, various protein markers such as pC/EBPβ, PPARγ2, Caveolin1, Perilipin1 and Pref1 can be employed to stain progenitor and mature adipocytes (Table 2). Overall, markers including Perilipin1, Pref1 and PPARγ2 exclusively stain human DAs. Although sebocytes are cells of the epithelial lineage derived from outer root sheath keratinocytes [91], and embryologically distinct from DAs, they share common antigens. In our stains human sebocytes provide an internal positive control within the skin for adipocyte markers such as phospho-C/EBPβ (pC/EBPβ), Caveolin1, DGAT1 and FASN.

**Pre-adipocyte and mature adipocyte markers**

Perilipin1, the ubiquitous marker of mature DAs in mice, has been used in multiple studies to demarcate the lipid droplet and cytoplasm of DWAT [4, 7, 92]. Perilipin1 also marks human DAs and can be assessed in both cryosections and paraffin sections (Fig 2g). Caveolin1, an intracellular scaffolding protein, is abundant in murine adipocytes where it occupies 30% of their plasma membrane, and is involved in lipid uptake [93, 94]. In human skin, Caveolin1 shows the expected pattern in IF and IHC, staining the cell membrane of DAs (Fig. 2h).

Two markers that stain both differentiating pre-adipocytes and mature DAs are Peroxisome proliferator-activated receptor gamma 2 (PPARγ2) and CCAAT-enhancer-binding protein beta (C/EBPβ) [95]. PPARγ2 is the master regulator of adipogenesis [96, 97], and exhibits a nuclear staining pattern (Fig. 2i). C/EBPβ is an early marker of pre-adipocytes, and its phosphorylation is crucial for the expression of downstream adipogenic markers such as adiponectin and CCAAT-enhancer-binding protein alpha (C/EBPα) [98]. Phosphorylated C/EBPβ (pC/EBPβ) shows nuclear staining in human adipocytes (Fig 2j). In contrast, Preadipocyte Factor 1 (Pref1) (Fig. 2k) is a well-known inhibitor of adipocyte differentiation, thus acting as a bone fide human pre-adipocyte marker [99–102].

Overall, it is crucial to utilize appropriate positive and negative controls for each marker in order to ensure the staining is reliable (see Table 1 for details on internal and tissue positive controls). For detailed reviews on imaging adipose tissue using fluorescence and confocal microscopy, the reader is directed towards specialized reviews [26, 27].

**Analysis by quantitative immunohistomorphometry**

Depending on the scientific question asked, various staining techniques (Table 1) as well as adipocyte-specific functional markers (Table 2) can be utilized as described above.

For example, changes in adipocyte size/volume as well as cell number can be quantified via H&E or by using mature adipocyte markers such as Perilipin1 and Caveolin1 using ImageJ (Fig. 2g-h; Suppl Fig. 3a). Alternatively, lipid droplet stains can be employed to quantify adipocyte size, although they require tissue processing via cryopreservation and sectioning at approx. 30μm (Table 1). The reader is also directed towards automated techniques of quantifying size and number of individual cells in adipose tissue using software such as MetaMorph, Adiposoft and ImageJ [103, 104].
Changes in lipid content can be inferred from ORO/NileRed/Sudan Black stains or fluorescent BODIPY/LipidTOX stains, quantified also using ImageJ (Fig. 2, Suppl Fig 1,2,3b). An alternative option for measuring adipocyte differentiation in cell lines is by using AdipoRed – a Nile Red kit (Lonza) that is quantified using a microplate reader (Suppl. Fig. 4) [105].

**Apoptosis markers**

Cell death in skin adipocytes has historically proven difficult to capture since classical apoptosis markers such as TUNEL and Cleaved Caspase-3 routinely escape detection in murine studies [106, 107]. Given their considerable size and relatively small cytoplasmic area, most DAs in one section will not have a visible nucleus, making reliable quantification difficult (Suppl Fig. 5 a-d). Moreover, distinguishing between nuclei pertaining to mature adipocytes or surrounding cells can also complicate TUNEL staining and its subsequent quantification. We also confirm the lack of TUNEL+ or Cleaved Caspase3+ adipocytes in control human scalp skin (Suppl. Fig. 4 a-d).

In obesity adipocyte cell death is driven by necrosis [108] and pyroptosis [109] with macrophages forming crown-like structures around Perilipin1 –ve adipocytes [26, 110]. Thus, loss of Perilipin1 or Caveolin1 (Suppl. Fig. 4e,f) can be used as a marker of adipocyte necrosis, since dying adipocytes lose Perilipin1 and Caveolin1 and are surrounded by macrophages [108, 111–113].

**Proliferation markers**

Mature adipocytes are post-mitotic under physiological conditions [114]. Any newly-produced adipocytes normally undergo differentiation from resident progenitor cells [115]. Therefore, proliferation markers such as Ki-67 (Fig. 2l) or Proliferating cell nuclear antigen (PCNA) can be employed to quantify proliferation of all resident cells within DWAT, and only estimate the amount of pre-adipocyte proliferation within DWAT (Fig. 2l). Rather, a more specific approach is measuring co-localization of pre-adipocyte markers such as Pref1 and Zinc finger protein 521 (Zfp521) (the human homologue of murine Zfp423) with a proliferative marker as described in murine DWAT [4].

Other techniques such as EdU and BrdU incorporation can also be utilized to study proliferation within DWAT [7]. To do so in preclinical studies of human skin ex vivo, one can resort to full-thickness human skin organ culture [116]. The same techniques and markers for cell cycle analysis ex vivo can be employed for human DWAT that we previously described for human hair follicle organ culture [117, 118].

**Examining lipogenesis/lipolysis in situ and using activity-based techniques**

Metabolic processes are central to white adipocytes which function to store and release energy [24]. Various key enzymes involved in lipid synthesis and breakdown such as Diacylglycerol O-acyltransferase 1/2 (DGAT1/2) (Fig. 2m), Fatty acid Synthase (FASN) (Fig 2n), lipoprotein lipase (LPL) [119, 120], adipose triglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL) [121–123] can be used to stain human DAs in situ (Fig. 2b). However, lipolysis is a complex biological process that involves rapid phosphorylation/dephosphorylation steps [124–126] during which ATGL, MGL and HSL are regulated at the
post-transcriptional level [127]. Therefore, quantifying total protein levels of enzymes involved in this complex process is uninformative in terms of assessing enzymatic activity. Furthermore, when studying adipocyte differentiation, the simple presence of lipid cannot attest to the full metabolic capacity of the cell, which can be tested in situ using the activity of other enzymes including NADH, NADP, esterase, succinate dehydrogenase and α-glycerol phosphate dehydrogenase [128–130].

A number of activity-based assays can be used to study lipogenesis/lipolysis in a more quantitative manner. For instance, lipogenesis can be measured in human DWAT ex vivo by using BODIPY C1/C12, a fluorescent fatty acid analogue that is taken up by cells undergoing lipogenesis (Suppl. Fig. 6a) [131–134]. Lipolysis can also be assessed ex vivo by measuring levels of glycerol and free fatty acids released into the culture media (Suppl. Fig 6b). For more detailed measurements of lipogenesis and lipolysis the reader is directed towards specialised reviews on these topics [127, 135–138].

**In situ hybridisation**

When antibody stains for secreted factors prove difficult to optimize, in situ hybridization (ISH) can be used to measure messenger RNA concentration within adipose tissue e.g. [6] (Fig. 2o). In addition, when one is not certain whether an immunostain is specific, if ISH detects no corresponding mRNA, then immunoreactivity is likely non-specific. The reader is directed towards the methods review by [139] for detailed protocols on conducting ISH on adipose tissue. A commercially available kit is the RNAscope assay (ACD CellBio) which can be used to conduct ISH on human DAs (Fig. 2o).

**Confocal microscopy**

In addition to fluorescence and brightfield microscopy, confocal microscopy is a widespread tool used to image adipose tissue either in wholemount, thick sections or adipose cell lines [26, 27, 140]. Confocal microscopy is an advantageous tool because of its enhanced optical resolution and capacity to create serial images throughout 5-50μm-thick sections, allowing visual 3D tissue reconstruction [141]. Confocal imaging is particularly advantageous to visualize features of interest within human DAs that may otherwise escape detection due to the thin cytoplasm in relation to the lipid droplet (Fig. 2p) [142]. Furthermore, confocal imaging can be used in conjunction with other techniques for a more in-depth study of human DWAT in large skin samples [143].

**Transmission electron microscopy**

Using transmission electron microscopy (TEM), one can observe unparalleled detail of intracellular structures (Fig. 2q-s) and quantify changes in adipocytes such as general cellular degeneration, glycogen deposits, cytoplasmic thickness, mitochondrial area and density, extracellular matrix, as well as the presence of crown-like structures reminiscent of adipose pyroptosis [109]. Various transmission and scanning electron microscopy studies have focused on human white adipocytes [144] following liposuction [145], facial autologous graft [146] as well as functional studies of secreted factors [147], white adipocyte de-differentiation into a stem-cell-like state [148] and cell behaviour following adrenergic stimulation [149].
Magnetic Resonance Imaging

Used widely both in clinic and lab-based settings, magnetic resonance imaging (MRI) is a versatile, non-invasive tool for 3D imaging extensively used for studying various fat depots including DWAT [150–155]. MRI could be employed to measure parameters such as thermogenic potential, thickness and true volume of DWAT or individual adipocytes [156, 157], since measuring adipocyte area using 2D methods is prone to error [158, 159]. MRI could also be used to determine the exact division between dermal and subcutaneous adipose compartments in human skin [160], which currently remains uncharacterized. An alternative to MRI would be computed tomography which has also been used for 3D imaging of adipose depots [151, 152, 161–164].

Studying DWAT in human skin organ culture

Although human DWAT tends to degenerate rapidly in standard skin organ culture, it can be kept vital for several days in serum-free William’s E medium supplemented with insulin, hydrocortisone and L-glutamine [116]. DWAT viability depends on how freshly the tissue is obtained, how gently it is handled, how fast it is placed into culture and how much trauma is applied during tissue trimming and organ culture set-up. We recommend using 4-6 mm punch biopsies to facilitate tissue perfusion/oxygenation and standardization of the organ-cultured tissue volume (Suppl Fig. 7 a,b). Alternatively, human DWAT can be dissected from scalp/abdominal skin and cultured in the same conditions described above as a mini-organ on its own (Suppl. Fig. 7 c,d). Placing the biopsies onto a pre-hydrated extracellular matrix support structure, such as porcine skin-derived gelatine sponges [165] may prolong DWAT survival ex vivo. Tissue viability can be monitored by measuring LDH release into the medium [116].

Dermal adipocyte culture

An alternative to studying human DAs in situ is the in vitro culture of human white adipocytes [166–169]. However, this removes DAs from the physiological concert of diverse stimuli (systemic, cell-cell, cell-matrix, vascular and neural) that they are exposed to within their normal tissue habitat and subjects them to unphysiological stressors, including the typical hypoxic, reactive oxygen species-generating conditions of cell culture. Therefore, one is well-advised to assume that some results obtained with isolated DAs in vitro might represent cell culture artefacts under conditions of oxidative stress. The unphysiological nature of isolated DA studies can be partly overcome by adopting suitable 3D designs [170–175].

One advantage of adipose cell lines over in situ analysis is that they provide a model for studying adipogenesis, trans-differentiation [148], formation of lipid droplets [176] or their interaction with other cells in co-culture experiments [10, 177]. In addition, adipocyte cell lines facilitate manipulation of the tissue ex vivo that would not otherwise be possible in situ such as adding inhibitors/stimulators, performing siRNA knockdowns or measuring secretion of various factors into the culture media.

The most widely-used murine cell line for adipogenesis studies is 3T3-L1 [178], with human pre-adipocytes cell lines developed from various depots across the body including abdominal and gluteal tissue [179], peripheral blood mononuclear cells [180], stromal cell fraction of subcutaneous adipocytes [181, 182] and even liposarcomas (SW872 cells) [183, 184]. A widely used model for studying human fat biology are SGBS pre-
adipocytes [185–187]. Human DAs can also be studied by differentiating white adipocytes from human pluripotent stem cells [188] or by reprogramming human dermal fibroblasts [189].

A useful tool to study human adipocytes in vitro are primary subcutaneous pre-adipocytes (Lonza) that are isolated from whole human fat i.e. containing both dermal and subcutaneous pre-adipocytes. These cells have the capacity to differentiate into mature lipid-laden adipocytes (Lonza). Seven days following stimulation, differentiated adipocytes express the same biomarkers seen in mature adipocyte cells such as Perilipin1, Caveolin1 and lipids shown using ORO and BODIPY (Fig. 3). In primary cells, Perilipin1 is expressed seven days following maturation, with no expression observed in pre-adipocytes (Fig. 3). Primary pre-adipocytes commence expressing Caveolin1 after three days in culture, while phospho-CEBPβ stains nuclei of both pre- and differentiated adipocytes [95] (Fig. 3). The bone fide pre-adipocyte marker Pref1, appears to stain both pre- and mature adipocytes (Fig. 3), highlighting the artificial conditions of adipocyte culture in vitro.

Overall, we confirm that these commercially available primary subcutaneous pre-adipocytes are an instructive tool for studying human DAs given that they express the expected markers and can be easily differentiated as an alternative to the other human adipose cell lines available.

Summary and perspective

In this practical guide, we have reviewed the routine methods available to study the human DWAT in situ and ex vivo including immunohistochemistry, immunofluorescence microscopy and analysis via quantitative immunohistomorphometry. We have discussed some particularly useful stains/markers that cover wide-ranging aspects of human adipocyte biology (e.g. OilRedO, Trichrome, Perilipin1, Caveolin1, BODIPY) and histochemical stains (H&E, Trichrome) available for use on human DWAT, along with guidelines on fixation, tissue processing and quantitative immuno-histomorphometry of human DAs in situ. We have also highlighted caveats and solutions to common problems frequently encountered when studying DWAT, including the challenges one faces when assessing adipocyte proliferation and apoptosis. For more advanced analyses, we have briefly covered ISH, TEM, DWAT ex vivo and in vitro culture, and essentials of MRI imaging of adipocytes”.

It is our intention that this guide greatly facilitates future DWAT analysis in human skin and encourages newcomers to this field to join the growing number of investigators that have begun to (re)-appreciate DWAT as one of the last great frontiers in human skin research. Thus, the current guide is meant to complement the rich clinical dermatology and pathology literature on diseases that affect the human subcutis [59, 60].

While the existing textbook literature documents that clinicians have long appreciated the clinical importance of subcutaneous disease, skin research into the physiology of human DWAT has yet to be fully dissected. Currently, medical therapy only deals with the clinical consequences of DWAT pathology, without attempting to target DWAT functions and thus impact on the function of skin and its appendages. One notable exception remains the use of adipose-derived autologous stem cells [18, 177, 190]. Hopefully, stimulated by the recent progress that several landmark papers have swept into both murine [3, 191, 192] and human DWAT research [19, 20, 193], this will change in future, with the current guide serving as encouragement and a laboratory companion for furthering these almost entirely mouse-based concepts in the human system.
Acknowledgements

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Author contributions

Photographs and images were provided by C.N. and L.B. The review was written by C.N. with full scientific and writing contributions from all co-authors. The review was designed and edited by RP.

Conflict of interests

The authors declare no conflict of interest. However, for the record, JP and RB are employees of Unilever Plc., and RP is founder and owner of Monasterium Laboratory, Münster/Germany.
Methods Review

A guide to studying human dermal adipocytes \textit{in situ}

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Abstract

Dermal white adipose tissue (DWAT) is a main component of human skin, composed of individual lipid-laden mesenchymal cells known as dermal adipocytes (DAs). Besides their well-known role in lipid storage and release, DAs also promote skin immunity, wound healing and hair follicle cycling and are important players in cutaneous neuro-endocrinology. The ever-growing insights into DWAT functions, albeit mostly in mice, have invited speculation that it may be involved in multiple skin diseases ranging from fibrosis to alopecia and psoriasis, thus designating the human DWAT a clinically relevant, but as yet insufficiently investigated skin compartment. Therefore, this practical, user-friendly guide aims to introduce the techniques available to study the human DWAT in situ and ex vivo, including immunohistochemistry, immunofluorescence microscopy and analysis via quantitative immunohistomorphometry. Here we provide information on a collection of stains comprising pre-adipocyte (Pref1) and mature adipocyte markers (Perilipin1, Caveolin1), as well as various lipid (OilRedO, BODIPY) and histochemical stains (H&E, Trichrome) available for use on human DWAT. We offer the reader guidelines on fixation, tissue processing and staining human DAs and highlight caveats and solutions to common problems that one may encounter when studying this fascinating skin compartment. We also suggest standard methods for conducting quantitative immunohistomorphometry on the human DWAT and its individual adipocytes in order to quantify cell size, number, lipid content and fluorescence intensity of adipose-specific markers. Finally, we also briefly introduce in situ hybridisation, transmission electron microscopy, and essentials of DWAT-related "-omics", as additional tools for instructively interrogating this largest, but still least-known compartment of human skin.

Key Words

white adipose tissue; immunohistochemistry; immunofluorescence
Introduction

Historically often viewed as just an inert, energy-storing and thermo-insulating spacer material [1], dermal white adipose tissue (DWAT) is making a resounding scientific comeback as increasingly more studies are emerging which highlight its important and diverse functions in cutaneous biology and beyond [2–9]. It is now recognized that the dermal adipose depot is involved in multiple additional activities, including protection against bacterial infections [4], controlling hair cycle wave patterns [6, 7, 10], thermogenesis [1], promoting wound healing [1, 11–13] and hypothesized neuroendocrine activities [14, 15]. Human DWAT as well as deeper subcutaneous fat layers also harbour heterogeneous, but multi-potent adipose-derived stem cell populations that have been used in the context of cell-based autologous cell therapy, thus finding widespread entry into clinical practice [3, 16–21].

Multiple reviews have appeared on the functions and biology of DWAT in mice [2, 8, 9, 22–25], including reviews on how to image murine adipose tissue [26, 27]. However, there is no comprehensive guide available on staining and imaging techniques of human DAs \textit{in situ}. This constitutes a major open question since the anatomy of human and murine skin adipose layers (and quite likely their physiology) are distinct, calling for independent characterisation.

The present Methods Review attempts to close the literature gap by firstly providing a brief introduction into DA biology in humans. We then present a compendium of histological stains and immunohistochemistry markers available to study human DWAT functions, ranging from lipid droplet biology to distinguishing between pre- and mature adipocytes. We also present data from a primary pre-adipocyte cell line that has potential to differentiate and act as a positive control for DWAT markers. \textbf{Although we do not discuss key adipocyte techniques associated with lipidomics, metabolomics or proteomics, we direct the readers towards specialized reviews on the subject [28–37], We aim to provide advice to both enthusiasts and newcomers in the skin adipocyte field.}

Human DWAT anatomy and biology in a nutshell

Adipose tissue is found within multiple body sites and can be divided into brown and white fat, the first thought to be involved predominantly in thermogenesis whereas the latter is recognized as a key player in whole-body metabolism [1, 8, 9, 38–40]. \textbf{An additional type of fat cell exists i.e. beige/bright adipocytes that undergo transdifferentiation from white fat [41–43].} Recently, there has been an important paradigm shift in how “fat” and what used to be called the “subcutis” or “hypodermis” [44] is viewed, since it has turned out that functionally and embryologically distinct layers of adipose tissue are distinguishable in mammalian skin (Fig. 1a) [45].

Whilst murine DWAT is clearly divided from the underlying subcutaneous white adipose tissue by a distinctive muscle layer, the \textit{panniculus carnosus} [46–48], it remains unclear how to morphologically distinguish between human DWAT and the subcutaneous fat layer (Fig. 1a,b) [49]. \textbf{However, one notable exception described on human cadavers is that of the platysma colli muscle that separates the subcutis into two fat compartments in the neck, the distal (upper) which may be related to human DWAT [50]. Interestingly, human DWAT exhibits a unique cone geometry, clustering around the pilosebaceous units and forming so-called ‘dermal cones’ (Fig. 1c).}
DA(s) typically envelope the connective tissue sheath of hair follicles (HFs) in scalp and abdominal skin, and can stretch from underneath the HF matrix up to eccrine sweat glands and the bulge area of HFs [51, 52] (Fig. 1c).

**Dermal Adipocytes**

DWAT contains a variety of components including fibroblast-like progenitor adipocytes, mature lipid-laden adipocytes and other cell types such as fibroblasts, macrophages, pericytes, mast cells, endothelial cells, adipose-derived stem cells, as well as an extensive vascular network (Fig 1f) [27]. The main building blocks of DWAT are dermal adipocytes (DAs) – unilocular spherical cells which function to store and release energy via lipogenesis and lipolysis [53]. Their typical shape is the result of the resident lipid droplet, the main site of triglyceride and cholesterol storage which occupies >90% of the cell volume [24]. With their characteristic honeycomb-shape, adipocytes can oscillate in size from 50\(\mu\)m up to 150\(\mu\)m, thus representing the largest cells in the skin. Their cytoplasmic structures and nucleus are compactly located in a thin layer on the inner rim of the cell (Fig 1.d,e).

**Clinical relevance of DWAT**

At a whole-body level, adipose tissue is a complex endocrine organ system involved in regulating immunity, inflammation, metabolism, insulin sensitivity and overall tissue homeostasis via the secretion of hormones, metabolites and signalling molecules referred to as adipokines [38, 39, 54, 55].

DAs stimulate wound repair by their own regeneration from myofibroblasts and by promoting fibroblast migration and vessel regeneration [3, 11, 13, 56]. DWAT also defends against *S. aureus* skin infections by producing the antimicrobial peptide Cathelicidin [4], and DAs are thought to play a key role in skin fibrosis via adipocyte-myofibroblast transition [5, 57]. This mechanism is speculated to promote development of fibrotic structures associated with hair follicles in androgenetic alopecia [40], supported by higher plasma leptin levels being associated with an increased incidence of androgenetic alopecia [58].

Apart from classical dermatoses that affect the adipocyte layers of human skin (e.g., panniculitis, lipogranulomatoses, lipoma, lipoatrophy, lipodystrophy) [59, 60] DAs are likely involved in other cutaneous diseases. Studies have shown fluctuating levels of adipokines in psoriasis patients [61, 62], and the use of therapeutics in this condition has been associated with fluctuations in DWAT thickness [63–65]. Moreover, a recent study supports the role of DAs in promoting melanoma aggressiveness [19].

**Overview of visualisation methods to consider**

When studying DWAT, there are a number of methods that one can choose from. Standard stains such as Haematoxylin and Eosin (H&E) and Masson’s Trichrome allow the visualization of adipocyte morphology and any changes in tissue or individual cell expansion or contraction. Lipid stains such as Oil Red O (ORO) and BODIPY can be used to visualize and analyse lipid droplet content, size and accumulation within DAs. Lastly, immunohistochemical/immunofluorescence techniques are widely employed to localize and detect changes in proteins of interest within DWAT by immunohistomorphometry.
Key considerations prior to human DA visualization in situ

The predominant approach to assessing DWAT is via histology/immunohistochemistry, however, prior to staining there are some key considerations to be made (Table 1).

Paraffin-embedded versus cryopreserved tissue

Although paraffin embedding offers excellent morphological detail of DWAT, lipids are displaced during paraffin processing rendering lipid staining protocols impossible. In contrast, cryosectioning offers many advantages including time-efficient protocols and the ability to conduct lipid stains. However, if tissue is scarce and multiple sections are required, sectioning via the microtome is preferable to obtain sufficient thin sections.

Section thickness

When the primary aim is to study lipid content, it is advisable to cut cryopreserved samples at a thickness of 15–30μm to maintain the cellular lipid content (Suppl Fig. 1). Sections may be quickly visualized using a microscope, though must not be allowed to thaw before storage at -80°C in order to preserve the lipids. For immunohistochemical-based approaches thinner sections are preferred, though when collecting sections from cryopreserved DWAT, maintaining a temperature equal to or lower than -30°C within the cryostat is crucial to obtain thin (7μm) sections of high quality.

Fixation

Choice of fixative and its duration can greatly affect the quality of tissue sections and subsequent staining. Though neutral-buffered formalin (NBF) is the universal fixative used for tissues aimed to undergo paraffin processing, our observations suggest that it is a harsh fixative for DWAT. One alternative fixative is Zinc Formalin which better preserves DA morphology as recommended by [26]. Alternative fixation methods include 4% paraformaldehyde (PFA), 20% normal-buffered formalin (NBF) and 99% ethanol (EtOH), however these have very different effects upon RNA quality, morphology and staining intensities [66]. For fatty samples that are to be embedded in paraffin we recommend 24hr fixation prior to processing.

For cryopreserved tissue, multiple fixatives can be employed including 4% PFA, ethanol, acetone and methanol; however, alcohol-based fixatives should be avoided when the aim is to examine lipid droplet content as these solvents displace lipids [67]. Instead, 4%PFA or ethanol:glacial acetic acid can be successfully used to obtain high-quality lipid stains (Suppl Fig. 2). We recommend performing the fixation step in 4%PFA immediately after thawing the tissue. For cryosections containing fat we have found 20-30min to be an optimal fixation time.

Following fixation and sectioning, DWAT can be stained using a range of techniques including immunohistochemistry and immunofluorescence stains. To help our readers, we have compiled a list of suggestions to quickly and easily combat common problems when working with human DWAT (Box 1).

Histochemistry
Standard haematoxylin and eosin (H&E) staining may be employed to visualize DWAT structure within skin, and around pilosebaceous units (Fig 1b,c; Fig 2a). H&E is useful when assessing human biopsies because it shows gross changes in morphology and may point towards further areas for closer examination [68]. For example, one can infer whether human DWAT thickness or the amount of fibroblast networks surrounding DAs changes in samples from healthy, alopecia areata and sebaceous nevus patients [69]. Moreover, H&E can be used to pinpoint whether expansion or reduction occurs within DWAT as highlighted in *S. aureus* infection in mice [4]. Using H&E, the exact distribution and changes in individual cell size and number can be assessed using Adiposoft and Cell Counter plugins in ImageJ (Suppl. Fig 3 a,b).

Another histological stain that proves useful when studying adipocytes is Masson’s Trichrome (Fig 2b) which stains fibrillar collagens I and III surrounding DWAT. Masson’s Trichrome can highlight ectopic collagen deposition between individual DAs, a marker of DWAT fibrosis [70], and also infers collagen deposition following organ culture of skin biopsies (Fig. 2b), a simple method to study fibrosis in DWAT [57, 71–73]. An alternative stain for collagen fibrils is Picrosirius Red (Fig. 2c).

Methylene Blue (MB) is a vital dye that can be used to stain the skin as a whole [74]. MB stains human skin compartments such as the epidermis, follicular units and the dermis but is not absorbed within DAs apart from in the nucleus [75, 76]. An advantage of MB compared to H&E is that it can be performed as a wholemount technique, allowing the dissection and isolation of DWAT by providing contrast to the surrounding dermis and follicular unit (Fig. 2d).

Lipid stains

Oil red O (ORO) is a hydrophobic dye used to visualize lipid droplet localization, morphology and accumulation within adipose tissue [67, 77]. ORO stains neutral lipids (triacylglycerols, diacylglycerols) and cholesteryl esters (Fig 2e), but not polar lipids on the lipid droplet membrane such as ceramides, sphingolipids and phospholipids [78]. ORO content in DAs can be quantified using ImageJ as detailed in Suppl. Fig 3 cb. ORO staining can prove difficult to quantify if sections are too thin (Suppl. Fig 2) or not stored and fixed appropriately following cryosectioning. Precautions should be taken when choosing the reagent in which to dissolve ORO as certain chemicals may result in staining of non-adipogenic cells [79].

Other chemicals that stain lipid within DAs include Nile Red [80], Sudan Red [81], and Osmium Tetroxide which is routinely used in electron microscopy, but may result in adipocyte swelling [82].

In order to quantify changes in lipid accumulation in DAs, fluorescent lipid stains on cryosections such as BODIPY 493/503 (Fig. 2f) or LipidTOX (Suppl. Fig. 1) can be used as an alternative to ORO. BODIPY 493/503 and LipidTOX are cell-permeable lipophilic neutral dyes that can be employed for both microscopy and flow cytometry [2, 83–90]. In our studies BODIPY (Fig 2f) appears superior in terms of fluorescence intensity given that LipidTOX rapidly bleaches and does not display the lipid droplet morphology described in other studies (Suppl. Fig. 1) [2, 7].

Cell Mask Plasma Membrane (CPM) stain (Suppl. Fig. 1) is an amphipathic molecule that anchors into, and histochemically defines, the plasma membrane of human DAs and can aid in visualizing DAs in conjunction
with lipid stains. This double stain also allows the correct identification of adipocyte nuclei, located between the lipid droplet and the adipocyte plasma membrane [26].

**Immunohistochemistry and immunofluorescence microscopy**

When staining adipocytes using IHC or immunofluorescence (IF) techniques, various protein markers such as pC/EBPβ, PPARγ2, Caveolin1, Perilipin1 and Pref1 can be employed to stain progenitor and mature adipocytes (Table 2). Overall, markers including Perilipin1, Pref1 and PPARγ2 exclusively stain human DAs. Although sebocytes are cells of the epithelial lineage derived from outer root sheath keratinocytes [91], and embryologically distinct from DAs, they share common antigens. In our stains human sebocytes provide an internal positive control within the skin for adipocyte markers such as phospho-C/EBPβ (pC/EBPβ), Caveolin1, DGAT1 and FASN.

**Pre-adipocyte and mature adipocyte markers**

Perilipin1, the ubiquitous marker of mature DAs in mice, has been used in multiple studies to demarcate the lipid droplet and cytoplasm of DWAT [4, 7, 92]. Perilipin1 also marks human DAs and can be assessed in both cryosections and paraffin sections (Fig 2g). Caveolin1, an intracellular scaffolding protein, is abundant in murine adipocytes where it occupies 30% of their plasma membrane, and is involved in lipid uptake [93, 94]. In human skin, Caveolin1 shows the expected pattern in IF and IHC, staining the cell membrane of DAs (Fig. 2h).

Two markers that stain both differentiating pre-adipocytes and mature DAs are Peroxisome proliferator-activated receptor gamma 2 (PPARγ2) and CCAAT-enhancer-binding protein beta (C/EBPβ) [95]. PPARγ2 is the master regulator of adipogenesis [96, 97], and exhibits a nuclear staining pattern (Fig. 2i). C/EBPβ is an early marker of pre-adipocytes, and its phosphorylation is crucial for the expression of downstream adipogenic markers such as adiponectin and CCAAT-enhancer-binding protein alpha (C/EBPα) [98]. Phosphorylated C/EBPβ (pC/EBPβ) shows nuclear staining in human adipocytes (Fig 2j). In contrast, Preadipocyte Factor 1 (Pref1) (Fig. 2k) is a well-known inhibitor of adipocyte differentiation, thus acting as a bone fide human pre-adipocyte marker [99–102].

Overall, it is crucial to utilize appropriate positive and negative controls for each marker in order to ensure the staining is reliable (see Table 1 for details on internal and tissue positive controls). For detailed reviews on imaging adipose tissue using fluorescence and confocal microscopy, the reader is directed towards specialized reviews of [26, 27].

**Analysis by quantitative immunohistomorphometry**

Depending on the scientific question asked, various staining techniques (Table 1) as well as adipocyte-specific functional markers (Table 2) can be utilized as described above.

For example, changes in adipocyte size/volume as well as cell number can be quantified via H&E or by using mature adipocyte markers such as Perilipin1 and Caveolin1 using ImageJ (Fig. 2g-h; Suppl Fig. 3a). Alternatively, lipid droplet stains can be employed to quantify adipocyte size, although they require tissue processing via cryopreservation and sectioning at approx. 30μm (Table 1). The reader is also directed towards
automated techniques of quantifying size and number of individual cells in adipose tissue using software such as MetaMorph, Adiposoft and ImageJ [103, 104].

Changes in lipid content can be inferred from ORO/NileRed/Sudan Black stains or fluorescent BODIPY/LipidTOX stains, quantified also using ImageJ (Fig. 2, Suppl Fig 1,2,3b). An alternative option for measuring adipocyte differentiation in cell lines is by using AdipoRed – a Nile Red kit (Lonza) that is quantified using a microplate reader (Suppl. Fig. 4) [105].

**Apoptosis markers**

Cell death in skin adipocytes has historically proven difficult to capture since classical apoptosis markers such as TUNEL and Cleaved Caspase-3 routinely escape detection in murine studies [106, 107]. Given their considerable size and relatively small cytoplasmic area, most DAs in one section will not have a visible nucleus, making reliable quantification difficult (Suppl Fig. 5 a-d). Moreover, distinguishing between nuclei pertaining to mature adipocytes or surrounding cells can also complicate TUNEL staining and its subsequent quantification. We also confirm the lack of TUNEL+ or Cleaved Caspase3+ adipocytes in control human scalp skin (Suppl. Fig. 4 a-d).

In obesity adipocyte cell death is driven by necrosis [108] and pyroptosis [109] with macrophages forming crown-like structures around Perilipin1 –ve adipocytes [26, 110]. Therefore, the best method of visualizing DA cell death is by using macrophage markers such as CD68 or Mac-2 [27]. Alternatively, Thus, loss of Perilipin1 or Caveolin1 (Suppl. Fig. 4e,f) can be used as a marker of adipocyte necrosis, since dying adipocytes lose Perilipin1 and Caveolin1 and are surrounded by macrophages [108, 111–113].

**Proliferation markers**

Mature adipocytes are post-mitotic under physiological conditions [114]. Any newly-produced adipocytes normally undergo differentiation from resident progenitor cells [115]. Therefore, proliferation markers such as Ki-67 (Fig. 2l) or Proliferating cell nuclear antigen (PCNA) can be employed to quantify proliferation of all resident cells within DWAT, and only estimate the amount of pre-adipocyte proliferation within DWAT (Fig. 2l). Rather, a more specific approach is measuring co-localization of pre-adipocyte markers such as Pref1 and Zinc finger protein 521 (Zfp521) (the human homologue of murine Zfp423) with a proliferative marker as described in murine DWAT [4].

Other techniques such as EdU and BrdU incorporation can also be utilized to study proliferation within DWAT [7]. To do so in preclinical studies of human skin *ex vivo*, one can resort to full-thickness human skin organ culture [116]. The same techniques and markers for cell cycle analysis *ex vivo* can be employed for human DWAT that we previously described for human hair follicle organ culture [117, 118].

**Enzyme histochemistry for important lipid metabolism/synthesis enzymes**

Examining lipogenesis/lipolysis *in situ* and using activity-based techniques
Metabolic processes are central to white adipocytes which function to store and release energy [24]. Various key enzymes involved in lipid synthesis and breakdown such as Diacylglycerol O-acyltransferase 1/2 (DGAT1/2) (Fig. 2m), Fatty acid Synthase (FASN) (Fig. 2n), lipoprotein lipase (LPL) [119, 120], and adipose triacylglyceride lipase (ATGL), hormone-sensitive lipase (HSL) and monoglyceride lipase (MGL) [121–123] can be used to stain human DAs in situ (Fig. 2b). These stains are incredibly useful because they can semi-quantitatively determine enzyme activity in situ. However, lipolysis is a complex biological process that involves rapid phosphorylation/dephosphorylation steps [124–126] during which ATGL, MGL and HSL are regulated at the post-transcriptional level [127]. Therefore, quantifying total protein levels of enzymes involved in this complex process is uninstructive in terms of assessing enzymatic activity. Furthermore, when studying adipocyte differentiation, the simple presence of lipid cannot attest to the full metabolic capacity of the cell, which can be tested in situ using the activity of other enzymes including NADH, NADP, esterase, succinate dehydrogenase and α-glycerol phosphate dehydrogenase [128–130].

A number of activity-based assays can be used to study lipogenesis/lipolysis in a more quantitative manner. For instance, lipogenesis can be measured in human DWAT ex vivo by using BODIPY C1/C12, a fluorescent fatty acid analogue that is taken up by cells undergoing lipogenesis (Suppl. Fig. 6a) [131–134]. Lipolysis can also be assessed ex vivo by measuring levels of glycerol and free fatty acids released into the culture media (Suppl. Fig. 6b). For more detailed measurements of lipogenesis and lipolysis the reader is directed towards specialised reviews on these topics [127, 135–138].

**In situ hybridisation**

When antibody stains for secreted factors prove difficult to optimize, in situ hybridization (ISH) can be used to measure messenger RNA concentration within adipose tissue e.g. [6] (Fig. 2o). In addition, when one is not certain whether an immunostain is specific, if ISH detects no corresponding mRNA, then immunoreactivity is likely non-specific. The reader is directed towards the methods review by [139] for detailed protocols on conducting ISH on adipose tissue. A commercially available kit is the RNAscope assay (ACD CellBio) which can be used to conduct ISH on human DAs (Fig. 2o).

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In addition to fluorescence and brightfield microscopy, confocal microscopy is a widespread tool used to image adipose tissue either in wholemount, thick sections or adipose cell lines [26, 27, 140]. Confocal microscopy is an advantageous tool because of its enhanced optical resolution and capacity to create serial images throughout 5-50μm-thick sections, allowing visual 3D tissue reconstruction [141]. Confocal imaging is particularly advantageous to visualize features of interest within human DAs that may otherwise escape detection due to the thin cytoplasm in relation to the lipid droplet (Fig. 2p) [142]. Furthermore, confocal imaging can be used in conjunction with other techniques for a more in-depth study of human DWAT in large skin samples [143].

**Transmission electron microscopy**
Using transmission electron microscopy (TEM), one can observe unparalleled detail of intracellular structures (Fig. 2q-s) and quantify changes in adipocytes such as general cellular degeneration, glycogen deposits, cytoplasmic thickness, mitochondrial area and density, extracellular matrix, as well as the presence of crown-like structures reminiscent of adipose pyroptosis [109]. Various transmission and scanning electron microscopy studies have focused on human white adipocytes [144] following liposuction [145], facial autologous graft [146] as well as functional studies of secreted factors [147], white adipocyte de-differentiation into a stem-cell-like state [148] and cell behaviour following adrenergic stimulation [149].

**Magnetic Resonance Imaging**

Used widely both in clinic and lab-based settings, magnetic resonance imaging (MRI) is a versatile, non-invasive tool for 3D imaging extensively used for studying various fat depots including DWAT [150–155]. MRI could be employed to measure parameters such as thermogenic potential, thickness and true volume of DWAT or individual adipocytes [156, 157], since measuring adipocyte area using 2D methods is prone to error [158, 159]. MRI could also be used to determine the exact division between dermal and subcutaneous adipose compartments in human skin [160], which currently remains uncharacterized. An alternative to MRI would be computed tomography which has also been used for 3D imaging of adipose depots [151, 152, 161–164].

**Studying DWAT in human skin organ culture**

Although human DWAT tends to degenerate rapidly in standard skin organ culture, it can be kept vital for several days in serum-free William’s E medium supplemented with insulin, hydrocortisone and L-glutamine [116]. DWAT viability depends on how freshly the tissue is obtained, how gently it is handled, how fast it is placed into culture and how much trauma is applied during tissue trimming and organ culture set-up. We recommend using 4-6 mm punch biopsies to facilitate tissue perfusion/oxygenation and standardization of the organ-cultured tissue volume (Suppl Fig. 76 a,b). Alternatively, human DWAT can be dissected from scalp/abdominal skin and cultured in the same conditions described above as a mini-organ on its own (Suppl. Fig. 76 c,d). Placing the biopsies onto a pre-hydrated extracellular matrix support structure, such as porcine skin-derived gelatine sponges [165] may prolong DWAT survival ex vivo. Tissue viability can be monitored by measuring LDH release into the medium [116].

**Dermal adipocyte culture**

An alternative to studying human DAs in situ is the in vitro culture of human white adipocytes [166–169]. However, this removes DAs from the physiological concert of diverse stimuli (systemic, cell-cell, cell-matrix, vascular and neural) that they are exposed to within their normal tissue habitat and subjects them to unphysiological stressors, including the typical hypoxic, reactive oxygen species-generating conditions of cell culture. Therefore, one is well-advised to assume that some results obtained with isolated DAs in vitro might represent cell culture artefacts under conditions of oxidative stress. The unphysiological nature of isolated DA studies can be partly overcome by adopting suitable 3D designs [170–175].

One advantage of adipose cell lines over in situ analysis is that they provide a model for studying adipogenesis, trans-differentiation [148], formation of lipid droplets [176] or their interaction with other cells in co-culture.
experiments [10, 177]. In addition, adipocyte cell lines facilitate manipulation of the tissue *ex vivo* that would not otherwise be possible *in situ* such as adding inhibitors/stimulators, performing siRNA knockdowns or measuring secretion of various factors into the culture media.

The most widely-used murine cell line for adipogenesis studies is 3T3-L1 [178], with human pre-adipocytes cell lines developed from various depots across the body including abdominal and gluteal tissue [179], peripheral blood mononuclear cells [180], stromal cell fraction of subcutaneous adipocytes [181, 182] and even liposarcomas (SW872 cells) [183, 184]. A widely used model for studying human fat biology are SGBS pre-adipocytes [185–187]. Human DAs can also be studied by differentiating white adipocytes from human pluripotent stem cells [188] or by reprogramming human dermal fibroblasts [189].

A useful tool to study human adipocytes *in vitro* are primary subcutaneous pre-adipocytes (Lonza) that are isolated from whole human fat i.e. containing both dermal and subcutaneous pre-adipocytes. These cells which have the capacity to differentiate into mature lipid-laden adipocytes (Lonza). Seven days following stimulation, differentiated adipocytes express the same biomarkers seen in mature adipocyte cells such as Perilipin1, Caveolin1 and lipids shown using ORO and BODIPY (Fig. 3). In primary cells, Perilipin1 is expressed seven days following maturation, with no expression observed in pre-adipocytes (Fig. 3). Primary pre-adipocytes commence expressing Caveolin1 after three days in culture, while phospho-CEBPβ stains nuclei of both pre- and differentiated adipocytes [95] (Fig. 3). The bone fide pre-adipocyte marker Pref1, appears to stain both pre- and mature adipocytes (Fig. 3), highlighting the artificial conditions of adipocyte culture *in vitro*.

Overall, we confirm that these commercially available primary subcutaneous pre-adipocytes are an instructive tool for studying human DAs given that they express the expected markers and can be easily differentiated as an alternative to the other human adipose cell lines available.

**Summary and perspective**

In this practical guide, we have reviewed the routine methods available to study the human DWAT *in situ* and *ex vivo* including immunohistochemistry, immunofluorescence microscopy and analysis via quantitative immunohistomorphometry. We have discussed some particularly useful stains/markers that cover wide-ranging aspects of human adipocyte biology (e.g. OilRedO, Trichrome, Perilipin1, Caveolin1, BODIPY) and histochemical stains (H&E, Trichrome) available for use on human DWAT, along with guidelines on fixation, tissue processing and quantitative immuno-histomorphometry of human DAs *in situ*. We have also highlighted caveats and solutions to common problems frequently encountered when studying DWAT, including the challenges one faces when assessing adipocyte proliferation and apoptosis. For more advanced analyses, we have briefly covered JSH, TEM, DWAT *ex vivo* and *in vitro* culture, and essentials of MRI imaging of adipocytes of DWAT-related “omics”.

It is our intention that this guide greatly facilitates future DWAT analysis in human skin and encourages newcomers to this field to join the growing number of investigators that have begun to (re)-appreciate the DWAT as one of the last great frontiers in human skin research. Thus, the current guide is meant to complement the rich clinical dermatology and pathology literature on diseases that affect the human subcutis [59, 60].
While the existing textbook literature documents that clinicians have long appreciated the clinical importance of subcutaneous disease, skin research into the physiology of the human DWAT has yet to be fully dissected. Currently, medical therapy only deals with the clinical consequences of DWAT pathology, without attempting to target DWAT functions and thus impact on the function of skin and its appendages. One notable exception remains the use of adipose-derived autologous stem cells [18, 177, 190]. Hopefully, stimulated by the recent progress that several landmark papers have swept into both murine [3, 191, 192] and human DWAT research [19, 20, 193], this will change in future, with the current guide serving as encouragement and a laboratory companion for furthering these almost entirely mouse-based concepts in the human system.

Acknowledgements

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Author contributions

Photographs and images were provided by C.N. and L.B. The review was written by C.N. with full scientific and writing contributions from all co-authors. The review was designed and edited by RP.

Conflict of interests

The authors declare no conflict of interest. However, for the record, JP and RB are employees of Unilever Plc., and RP is founder and owner of Monasterium Laboratory, Münster/Germany.
Figure 1. Structure of dermal white adipose tissue and its components. Whilst murine DWAT is divided from subcutaneous adipocytes by the panniculus carnosus, human DWAT is found as a continuous layer in the human dermis (a) with no equivalent muscle structure separating it from subcutaneous white adipose tissue (SWAT) (a). DWAT surrounds the bottom half of hair follicles in the form of so-called ‘dermal cones’ (b-c). At the ultrastructural level (d-e), the cellular components of dermal adipocytes become clearer – most of the cell volume is occupied by the centrally-located lipid droplet (LD) which is surrounded by a thin sheath of cytoplasm (represented between the dotted lines); the cytoplasm is enveloped by the plasma membrane on the outside. DWAT is composed of various cell types including mature adipocytes, fibroblast-like pre-adipocytes, macrophages, pericytes, endothelial cells and a multitude of blood vessels (f). Scale bar is 2μm in (d) and 500nm in (e).
Figure 2. Stains for observing and quantifying human dermal white adipose tissue *in situ*. Adipocytes can be examined using histological stains such as (a) Haematoxylin and Eosin (H&E), (b) Masson’s Trichrome, (b) Picrosirius Red and (d) Methylene blue (MB). The lipid within DAs can be measured using (e) Oil Red O (ORO), or fluorescently via (f) BODIPY493/503. Furthermore, various markers are available to study pre- and mature adipocytes including (g) Perilipin1 (PLIN1), (h) Caveolin1 (CAV1), (i) PPARγ2, (j) p-C/EBPβ and (k) PREF-1. In addition, proliferation (l) via Ki67 as well as lipid synthesis enzymes such as (m) diacylglycerol acetyl transferase (DGAT1) and (n) fatty acid synthase (FASN) can be used to stain DAs in situ. Alternative methods of studying adipocyte *in situ* include (o) *in situ* hybridization (ISH) to study synthesis of individual strands of mRNA, and various cellular processes at the ultrastructural level (q-s) using transmission electron microscopy (TEM). Scale bar is 50μm across all images except the last two TEM images.
Figure 3. Human primary subcutaneous adipocytes as a model for studying human DAs in vitro. Primary pre-adipocytes can be differentiated over 7 days into differentiated adipocytes that express PLIN1, p-CEBPβ, CAV1 and PREF1. Lipid stains such as Oil Red O and BODIPY also stain differentiated adipocytes. Thus, primary cells can also serve as positive and negative controls for antibody stains conducted in situ. Scale bar is 50μm.
<table>
<thead>
<tr>
<th>What should I choose when studying…?</th>
<th>How to process the tissue</th>
<th>Stains / Markers</th>
<th>Target / Antigen Localization</th>
<th>Positive Control Tissue</th>
<th>Imaging and Analysis</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>DWAT Morphology</td>
<td>FFPE Cryo</td>
<td>H&amp;E</td>
<td>n/a</td>
<td>n/a</td>
<td>BF</td>
<td>[68, 68, 194]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>BODIPY, LipidTOX</td>
<td>LD</td>
<td>SG, SC, Breast</td>
<td>IFM, CFM</td>
<td></td>
</tr>
<tr>
<td>Mature Adipocyte</td>
<td>FFPE Cryo</td>
<td>PLIN1</td>
<td>LD Membrane</td>
<td>SC, BC</td>
<td>BF/IFM</td>
<td></td>
</tr>
<tr>
<td>Adipocyte size and number</td>
<td></td>
<td>CAV1</td>
<td>Cell membrane</td>
<td>KCs, FBs, HeLa Cells</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>FABP4</td>
<td>Cytoplasm</td>
<td>KCs, Lymph node</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>H&amp;E</td>
<td>n/a</td>
<td>n/a</td>
<td>BF</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>DGAT1/2</td>
<td>Cytoplasm - ER</td>
<td>KCs, Colon</td>
<td>BF/IFM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>FASN</td>
<td>Cytoplasm</td>
<td>KCs, PC, BC</td>
<td>BF/IFM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LPL</td>
<td>Membrane</td>
<td>Intestine</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adipocyte differentiation</td>
<td>FFPE Cryo</td>
<td>pC/EBPββ</td>
<td>Nucleus</td>
<td>SG, BC</td>
<td>BF/IFM</td>
<td>[7, 195]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PPARγ</td>
<td>Nucleus</td>
<td>SG, BC</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pre-adipocytes</td>
<td>FFPE Cryo</td>
<td>Pref1</td>
<td>Membrane / Nucleus</td>
<td>Placenta</td>
<td>BF/IFM</td>
<td>[189, 197]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zip521</td>
<td>Nucleus</td>
<td>Bone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adipocyte apoptosis</td>
<td>FFPE Cryo</td>
<td>PLIN1</td>
<td>LD Membrane</td>
<td>SC, BC</td>
<td>Loss of Perilipin1</td>
<td>[27, 194, 198]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAV1</td>
<td>Cell membrane</td>
<td>KCs, FBs, HeLa Cells</td>
<td>Loss of Caveolin1</td>
<td></td>
</tr>
<tr>
<td>Pre-adipocyte proliferation</td>
<td>FFPE Cryo</td>
<td>Pref1 + Ki67/ EdU</td>
<td>Nucleus</td>
<td>Placenta</td>
<td>BF/IFM</td>
<td>[7, 115, 195]</td>
</tr>
<tr>
<td>Lipid synthesis (lipogenesis)</td>
<td>FFPE Cryo</td>
<td>Mature DA stain to measure change in size (see above)</td>
<td>Various</td>
<td>Dependent on stain chosen</td>
<td>BF/IFM</td>
<td>See sections above</td>
</tr>
</tbody>
</table>

Table 1. A guide to choosing the right processing route and stain for analysing dermal adipocytes.

<table>
<thead>
<tr>
<th>Section</th>
<th>FFPE/Cryo</th>
<th>phospho-Perilipin</th>
<th>Cytoplasm</th>
<th>Adipocyte cell line stimulated to undergo lipolysis</th>
<th>BF/IFM</th>
<th>phospho-HSL</th>
<th>Cytoplasm</th>
<th>TEM</th>
<th>OsO4/Uranyl Acetate</th>
<th>Only for immuno-EM</th>
<th>n/a</th>
<th>TEM</th>
<th>[127, 201, 202]</th>
</tr>
</thead>
<tbody>
<tr>
<td>Adipocyte Ultrastructure</td>
<td>EM-specific</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>mRNA</td>
<td>FFPE/Cryo</td>
<td>Fluorescent or chromogenic ISH</td>
<td>Cytoplasm</td>
<td>Dependent upon gene of interest PPIB; HeLa cells</td>
<td>ISH</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>[139, 205]</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>FFPE</td>
<td>Masson’s Trichrome/ Picrosirius Red</td>
<td>Fibrillar Collagens I and III</td>
<td>n/a</td>
<td>ISH</td>
<td>Dermal components other than DAs</td>
<td>n/a</td>
<td>BF</td>
<td>Methylene Blue</td>
<td>Dermal components other than DAs</td>
<td>n/a</td>
<td>BF</td>
<td>[70, 206, 207]</td>
</tr>
</tbody>
</table>
Table 2. Markers of human dermal adipocytes and their functional importance.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Target Localization</th>
<th>Function</th>
<th>Murine References</th>
<th>Human References</th>
</tr>
</thead>
<tbody>
<tr>
<td>Perilin1</td>
<td>LD membrane, ER</td>
<td>Master regulator of lipolysis. Upon phosphorylation by PKA, Perilin1 allows HSL to translocate to the adipocyte lipid droplet and commence lipolysis.</td>
<td>[7, 17, 49, 140, 195, 196, 207, 208, 209]</td>
<td>[102, 170, 176, 210, 211]</td>
</tr>
<tr>
<td>Caveolin1</td>
<td>Plasma membrane</td>
<td>Endo- and exocytosis Component of caveolae which are sites of triacylglycerol synthesis</td>
<td>[212, 213]</td>
<td>[214]</td>
</tr>
<tr>
<td>PPARγ2</td>
<td>Nucleus</td>
<td>Master regulator of adipogenesis</td>
<td>[95]</td>
<td>[102, 219]</td>
</tr>
<tr>
<td>pC/EBPβ</td>
<td>Nucleus</td>
<td>Promotes terminal adipocyte differentiation together with C/EBPβ.</td>
<td>[216]</td>
<td>[217, 218]</td>
</tr>
<tr>
<td>Pref1/Dlk1</td>
<td>Plasma Membrane</td>
<td>Inhibits pre-adipocyte proliferation in its membrane-bound form Prevents adipocyte differentiation (adipogenesis)</td>
<td>[99, 219–223]</td>
<td>[100–102]</td>
</tr>
<tr>
<td>Zfp423/Znf521</td>
<td>Nucleus</td>
<td>Induces PPARγ expression thus initiating preadipocyte commitment</td>
<td>[224–226]</td>
<td>[228]</td>
</tr>
<tr>
<td>DGAT 1/2</td>
<td>Cytoplasm - ER</td>
<td>Catalyses addition of third fatty acid to diacylglycerol</td>
<td>[119, 227, 228]</td>
<td>[120]</td>
</tr>
<tr>
<td>FASN</td>
<td>Cytoplasm</td>
<td>Uses acetylCoA, malonyl-CoA, and NADPH for de novo synthesis of long chain saturated fatty acids.</td>
<td>[229, 230]</td>
<td>[120, 231, 232]</td>
</tr>
<tr>
<td>LPL</td>
<td>Adipocyte plasma membrane</td>
<td>Hydrolyses lipoproteins at capillary endothelium that are triglyceride-rich to generate NEFA</td>
<td>[233, 234]</td>
<td>[120, 234]</td>
</tr>
<tr>
<td>ATGL</td>
<td>LD membrane</td>
<td>Hydrolyses TAG to DAG</td>
<td>[235–237]</td>
<td>[236, 237]</td>
</tr>
<tr>
<td>HSL</td>
<td>Cytosol, LD membrane</td>
<td>Hydrolyses TAG to DAG, and DAG to MAG</td>
<td>[237–240]</td>
<td>[237, 241]</td>
</tr>
<tr>
<td>MGL</td>
<td>Cytosol</td>
<td>Hydrolyses MAG to glycerol and a fatty acid.</td>
<td>[236, 237]</td>
<td>[237]</td>
</tr>
</tbody>
</table>
Box 1. Pitfalls and tricks when working with dermal adipose tissue.

<table>
<thead>
<tr>
<th>Issue</th>
<th>Solution(s)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Poor quality DWAT in cryosections</td>
<td>• Cut thicker sections&lt;br&gt;• Lower the cryostat temperature to -35 degrees&lt;br&gt;• Gently apply coverslips upon tissue sections</td>
</tr>
<tr>
<td>Lipid is displaced from adipocytes in cryosections</td>
<td>• Lower the cryostat temperature to -35 degrees&lt;br&gt;• Store slides in cryostat without allowing them to thaw&lt;br&gt;• Cut sections of up to 30μm thickness&lt;br&gt;• Change fixative to 4% PFA or Ethanol:Glacial Acetic Acid</td>
</tr>
<tr>
<td>Lipid is absent in paraffin sections</td>
<td>• Switch to cryosections – lipids are damaged during processing</td>
</tr>
<tr>
<td>Poor quality of adipocytes in paraffin sections</td>
<td>• Set waterbath temperature to 35 degrees (adipocytes tend to burst at higher temperatures)&lt;br&gt;• Switch to a more gentle fixative (Zinc Formalin instead of 10% NBF)&lt;br&gt;• Apply coverslips gently upon tissue sections&lt;br&gt;• Ensure the tissue is surrounded by enough fixative</td>
</tr>
<tr>
<td>Uncertainty as to whether adipocyte stain worked</td>
<td>• Check staining within positive control (human tissue distinct to skin)&lt;br&gt;• Check whether there is staining in sebaceous gland (there are many shared markers/lipid stains between sebocytes and DAs)</td>
</tr>
<tr>
<td>Antibody optimization of secreted factors repeatedly fails</td>
<td>• Try Tyramide Signal Amplification method&lt;br&gt;• Resort to detecting mRNA of adipose-derived factors using ISH</td>
</tr>
<tr>
<td>Low resolution of target protein in immunofluorescence</td>
<td>• Try confocal microscopy instead (also available for tissue that is 20μm or thicker)&lt;br&gt;• Attempt immuno-EM (as a last resort as the technique is very difficult)</td>
</tr>
<tr>
<td>Identifying adipocyte nuclei within sections</td>
<td>• Cut thicker sections (the volume of adipocyte renders few nuclei pertaining to mature adipocytes)&lt;br&gt;• Double stain with a lipid stain (e.g. BODIPY) and plasma membrane stain (e.g. CPM) to identify adipocyte-specific nuclei</td>
</tr>
<tr>
<td>Difficulty in co-localising target antigen with actual adipose cells</td>
<td>• Conduct double stain with an adipocyte marker e.g. Perilipin1/BODIPY&lt;br&gt;• Use IHC instead of IF stain</td>
</tr>
<tr>
<td>Difficulty in detecting dying adipocytes in situ</td>
<td>• Stain for a macrophage marker (CD68/Mac2) to detect crown-like structures around adipocytes&lt;br&gt;• Quantify loss of Perilipin1/CAY1&lt;br&gt;• Do an ultrastructure test for apoptosis using TEM</td>
</tr>
</tbody>
</table>
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<td>n/a</td>
<td>n/a</td>
<td>BF</td>
<td>[68, 69, 194]</td>
</tr>
<tr>
<td>Mature Adipocyte</td>
<td>FFPE Cryo</td>
<td>PLIN1</td>
<td>LD Membrane</td>
<td>SC, BC</td>
<td>BF/IFM</td>
<td></td>
</tr>
<tr>
<td>Adipocyte size and number</td>
<td>FFPE Cryo</td>
<td>CAV1</td>
<td>Cell membrane</td>
<td>KCs, FBs, HeLa Cells</td>
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<td></td>
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<td></td>
<td>FABP4</td>
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<td>KCs, Lymph node</td>
<td>BF/IFM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>CPM</td>
<td>Cell membrane</td>
<td>All cells, Any tissue</td>
<td>IFM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>H&amp;E</td>
<td>n/a</td>
<td>n/a</td>
<td>BF</td>
<td>[7, 101, 108, 163–166, 169–173]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>DGAT1/2</td>
<td>Cytoplasm - ER</td>
<td>KCs, Colon</td>
<td>BF/IFM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>FASN</td>
<td>Cytoplasm</td>
<td>KCs, PC, BC</td>
<td>BF/IFM</td>
<td></td>
</tr>
<tr>
<td></td>
<td></td>
<td>LPL</td>
<td>Membrane</td>
<td>Intestine</td>
<td>BF/IFM</td>
<td></td>
</tr>
<tr>
<td>Adipocyte differentiation</td>
<td>FFPE Cryo</td>
<td>pC/EBPβ</td>
<td>Nucleus</td>
<td>SG, BC</td>
<td>BF/IFM</td>
<td>[7, 195]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>PPARγ</td>
<td>Nucleus</td>
<td>SG, BC</td>
<td>BF/IFM</td>
<td></td>
</tr>
<tr>
<td>Pre-adipocytes</td>
<td>FFPE Cryo</td>
<td>Pref1</td>
<td>Membrane / Nucleus</td>
<td>Placenta</td>
<td>BF/IFM</td>
<td>[195, 197]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Zfp521</td>
<td>Nucleus</td>
<td>Bone</td>
<td>BF/IFM</td>
<td></td>
</tr>
<tr>
<td>Adipocyte apoptosis</td>
<td>FFPE Cryo</td>
<td>PLIN1</td>
<td>LD Membrane</td>
<td>SC, BC</td>
<td>Loss of Perilipin1</td>
<td>[27, 194, 198]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>CAV1</td>
<td>Cell membrane</td>
<td>KCs, FBs, HeLa Cells</td>
<td>Loss of Caveolin1</td>
<td></td>
</tr>
<tr>
<td>Pre-adipocyte proliferation</td>
<td>FFPE Cryo</td>
<td>Pref1 + Ki67/ BrdU / EdU</td>
<td>Nucleus</td>
<td>Placenta</td>
<td>BF/IFM</td>
<td>[7, 115, 195]</td>
</tr>
<tr>
<td>Lipid synthesis (lipogenesis)</td>
<td>Unfixed tissue</td>
<td>BODIPY C1/C12</td>
<td>Cytoplasm - ER</td>
<td>Adipocytes</td>
<td>IFM</td>
<td>[132, 134, 199, 200]</td>
</tr>
<tr>
<td></td>
<td>FFPE Cryo</td>
<td>Mature DA stain to measure change in size (see above)</td>
<td>Various</td>
<td>Dependent on stain chosen</td>
<td>BF/IFM</td>
<td>See section above</td>
</tr>
<tr>
<td>Lipid metabolism (lipolysis)</td>
<td>FFPE Cryo</td>
<td>phospho-Perilipin1</td>
<td>Cytoplasm</td>
<td>Adipocyte cell line stimulated to undergo lipolysis</td>
<td>BF/IFM</td>
<td>[127, 201, 202]</td>
</tr>
<tr>
<td>-----------------------------</td>
<td>-----------</td>
<td>-------------------</td>
<td>-----------</td>
<td>--------------------------------------------------</td>
<td>--------</td>
<td>----------------</td>
</tr>
<tr>
<td></td>
<td></td>
<td>phospho-HSL</td>
<td>Cytoplasm</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Adipocyte Ultrastructure</td>
<td>EM-specific</td>
<td>OsO4 Uranyl Acetate</td>
<td>Only for immuno-EM</td>
<td>n/a</td>
<td>TEM</td>
<td>[203] [109, 144, 148, 193, 204] [176]</td>
</tr>
<tr>
<td>3D Volume and Thickness</td>
<td>As per MRI/μCT protocol</td>
<td>Various</td>
<td>n/a</td>
<td>n/a</td>
<td>MRI μCT</td>
<td>[150–157, 160–164]</td>
</tr>
<tr>
<td>mRNA</td>
<td>FFPE Cryo</td>
<td>Fluorescent or chromogenic ISH</td>
<td>Cytoplasm</td>
<td>Dependent upon gene of interest PPIB; HeLa cells</td>
<td>ISH</td>
<td>[139] [205]</td>
</tr>
<tr>
<td>Fibrosis</td>
<td>FFPE</td>
<td>Masson’s Trichrome/Picrosirius Red</td>
<td>Fibrillar Collagens I and III</td>
<td>n/a</td>
<td>BF</td>
<td>[70, 206, 207]</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Methylene Blue</td>
<td>Dermal components other than DAs</td>
<td>n/a</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
### Table 2. Markers of human dermal adipocytes and their functional importance.

<table>
<thead>
<tr>
<th>Marker</th>
<th>Target Localization</th>
<th>Function</th>
<th>Murine References</th>
<th>Human References</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Perilipin1</strong></td>
<td>LD membrane, ER</td>
<td>Master regulator of lipolysis. Upon phosphorylation by PKA, Perilipin1 allows HSL to translocate to the adipocyte lipid droplet and commence lipolysis.</td>
<td>[7, 69, 140, 195, 196, 207, 208]</td>
<td>[102, 170, 176, 210, 211]</td>
</tr>
<tr>
<td><strong>Caveolin1</strong></td>
<td>Plasma membrane</td>
<td>Endo- and exocytosis. Component of caveolae which are sites of triacylglycerol synthesis.</td>
<td>[212, 213]</td>
<td>[214]</td>
</tr>
<tr>
<td><strong>PPARγ2</strong></td>
<td>Nucleus</td>
<td>Master regulator of adipogenesis.</td>
<td>[95]</td>
<td>[102, 215]</td>
</tr>
<tr>
<td><strong>pC/EBPβ</strong></td>
<td>Nucleus</td>
<td>Promotes terminal adipocyte differentiation together with C/EBPα.</td>
<td>[216]</td>
<td>[217, 218]</td>
</tr>
<tr>
<td><strong>Pref1/Dlk1</strong></td>
<td>Plasma membrane</td>
<td>Inhibits pre-adipocyte proliferation in its membrane-bound form. Prevents adipocyte differentiation (adipogenesis).</td>
<td>[99, 219–223]</td>
<td>[100–102]</td>
</tr>
<tr>
<td><strong>Zfp423/Znf521</strong></td>
<td>Nucleus</td>
<td>Induces PPARγ expression thus initiating preadipocyte commitment.</td>
<td>[224–226]</td>
<td>[228]</td>
</tr>
<tr>
<td><strong>DGAT 1/2</strong></td>
<td>Cytoplasm, ER</td>
<td>Catalyses addition of third fatty acid to diacylglycerol.</td>
<td>[119, 227, 228]</td>
<td>[120]</td>
</tr>
<tr>
<td><strong>FASN</strong></td>
<td>Cytoplasm</td>
<td>Uses acetylCoA, malonyl-CoA, and NADPH for de novo synthesis of long chain saturated fatty acids.</td>
<td>[229, 230]</td>
<td>[120, 231, 232]</td>
</tr>
<tr>
<td><strong>LPL</strong></td>
<td>Adipocyte plasma membrane</td>
<td>Hydrolyses lipoproteins at capillary endothelium that are triglyceride-rich to generate NEFA.</td>
<td>[233, 234]</td>
<td>[120, 234]</td>
</tr>
<tr>
<td><strong>ATGL</strong></td>
<td>LD membrane</td>
<td>Hydrolyses TAG to DAG.</td>
<td>[235–237]</td>
<td>[236, 237]</td>
</tr>
<tr>
<td><strong>HSL</strong></td>
<td>Cytosol, LD membrane</td>
<td>Hydrolyses TAG to DAG, and DAG to MAG.</td>
<td>[237–240]</td>
<td>[237, 241]</td>
</tr>
<tr>
<td><strong>MGL</strong></td>
<td>Cytosol</td>
<td>Hydrolyses MAG to glycerol and a fatty acid.</td>
<td>[236, 237]</td>
<td>[237]</td>
</tr>
</tbody>
</table>
Box 1. Pitfalls and tricks when working with dermal adipose tissue.

<table>
<thead>
<tr>
<th>Issue</th>
<th>Solution(s)</th>
</tr>
</thead>
</table>
| Poor quality DWAT in cryosections                                   | • Cut thicker sections  
• Lower the cryostat temperature to -35 degrees  
• Gently apply coverslips upon tissue sections                                                                                                    |
| Lipid is displaced from adipocytes in cryosections                  | • Lower the cryostat temperature to -35 degrees  
• Store slides in cryostat without allowing them to thaw  
• Cut sections of up to 30μm thickness  
• Change fixative to 4%PFA or Ethanol:Glacial Acetic Acid                                                                                       |
| Lipid is absent in paraffin sections                                | • Switch to cryosections – lipids are damaged during processing                                                                                                                                           |
| Poor quality of adipocytes in paraffin sections                     | • Set waterbath temperature to 35 degrees (adipocytes tend to burst at higher temperatures)  
• Switch to a more gentle fixative (Zinc Formalin instead of 10% NBF)  
• Apply coverslips gently upon tissue sections  
• Ensure the tissue is surrounded by enough fixative                                                                                             |
| Uncertainty as to whether adipocyte stain worked                    | • Check staining within positive control (human tissue distinct to skin)  
• Check whether there is staining in sebaceous gland (there are many shared markers/lipid stains between sebocytes and DAs)                                                                                   |
| Antibody optimization of secreted factors repeatedly fails          | • Try Tyramide Signal Amplification method  
• Resort to detecting mRNA of adipose-derived factors using ISH                                                                                                                                           |
| Low resolution of target protein in immunofluorescence              | • Try confocal microscopy instead (also available for tissue that is 20μm or thicker)  
• Attempt immuno-EM (as a last resort as the technique is very difficult)                                                                                                                               |
| Identifying adipocyte nuclei within sections                        | • Cut thicker sections (the volume of adipocyte renders few nuclei pertaining to mature adipocytes)  
• Double stain with a lipid stain (e.g. BODIPY) and plasma membrane stain (e.g. CPM) to identify adipocyte-specific nuclei                                                                 |
| Difficulty in co-localising target antigen with actual adipose cells | • Conduct double stain with an adipocyte marker e.g. Perilipin1/BODIPY  
• Use IHC instead of IF stain                                                                                                                      |
| Difficulty in detecting dying adipocytes in situ                   | • Stain for a macrophage marker (CD68/Mac2) to detect crown-like structures around adipocytes  
• Quantify loss of Perilipin1/CAV1  
• Do an ultrastructure test for apoptosis using TEM                                                                                             |
Supplementary Figures

Suppl. Fig. 1. Section thickness determines lipid droplet content and morphology in dermal adipocytes. Although integrity of adipocyte cytoplasm and plasma membrane is maintained in samples sectioned at 7μm, most of the lipid droplet remains intact only when cutting the tissue at a thickness of 15-30μm as revealed by various lipid stains such as Lipid TOX (a-c), BODIPY (d-f) and OilRedO (g-i). BODIPY (d-f) appears a more robust fluorescent lipid stain in comparison with LipidTOX that fades fairly quickly (a-c). The advantage of fluorescent lipid stains (a-f) over chromogenic ones such as OilRedO (g-i) is that they can be paired with a fluorescent plasma membrane marker such as Cell Plasma Membrane stain (CPM). Scale bar = 50μm.
Suppl. Fig. 2. Effect of fixation upon lipid content of human dermal adipocytes. The choice of fixative has a great impact upon the lipid content of human dermal adipocytes. Whilst acetone displaces the lipid in the sebaceous gland (SG) and DWAT (a, b) thus making it an unsuitable choice for fixation prior to BODIPY or ORO staining, Ethanol:Glacial Acetic Acid (Eth:GlacAc) (c,d) and 4%PFA (e,f) are both suitable fixatives for subsequent lipid stains. Note how BODIPY also stains the lipid-filled sebocytes within the sebaceous gland (SG), rendering the SG as a positive control. Scale bar = 50μm.
Suppl. Fig. 3. Quantifying adipocyte size/volume and lipid content in ImageJ. Different tools can be employed to measure adipocyte size/volume in ImageJ (a) such as freehand selection, elliptical tool and polygon selection. We recommend utilizing the polygon selection tool (a) to measure adipocyte size/volume, which allows a more efficient tracing of the adipocyte plasma membrane, generating more consistent measurements of individual surface area/volume. Alternatively, adipocyte size can be measured automatically/manually using an ImageJ plugin called ‘Adiposoft’. Quantifying adipocyte number (b) can be done using the Cell Counter Tool in ImageJ, whilst lipid stains such as OilRedO (c) can also be quantified using ImageJ. However, before quantifying staining intensity, the ORO stain needs to be inverted. Scale bar = 50µm.
Suppl. Fig. 4. Quantifying adipocyte differentiation in culture using the AdipoRed assay. Human primary subcutaneous adipocytes (Lonza) in culture can be differentiated in 7-10 days following addition of differentiation medium to display multiple bead-like lipid droplets within their cytoplasm (a). The level of differentiation can be measured using the AdipoRed kit (Lonza) which acts like a lipophilic dye similar to Nile Red that stains the lipid droplets of adipocytes. Maturation can be quantified using a plate reader. Displayed are the levels of adipocyte maturation at distinct passages (b,c) and also when cells are thawed and cultured following cryopreservation (d).
Suppl. Fig. 5. Cell death of skin adipocytes. Conventional methods for detecting apoptosis such as TUNEL (a,b) and Cleaved Caspase 3 (CASP3) (c,d) show no cell death within human DAs. Instead, apoptosis can be measured via different approaches, for example by measuring loss of Perilipin1 (c) or Caveolin1 (d) in dermal adipocytes within abdominal skin following 10 days in culture. Asterisks in the images denote the adipocytes that have lost Perilipin1 (c) and Caveolin1 (d) expression. The percentage of skin adipocytes that are negative for Perilipin1 (c) or Caveolin1 (d) out of total adipocytes per field of view (FOV) can be calculated in ImageJ using the Cell Counter. Results can then be represented in a graph style when comparing different methods of treatment. Scale bar = 50μm.
Suppl. Fig. 6. Measuring lipogenesis and lipolysis in human DWAT ex vivo. One of the methods that can be used to assess fatty acid uptake ex vivo is by adding BODIPY C1/C12 (green or red) to the media of abdominal skin organ culture (a). For instance, BODIPY C1/C12 can be added to adipocyte culture either as a single or double labelling with green or red BODIPY to observe the cells that are undergoing continuous or dynamic changes in lipid uptake over a certain period of time (a). Studying lipolysis in abdominal skin organ culture can be done by a colorimetric kit that detects free glycerol released into the media over a certain time period; such a test can be used to determine the effect of different medias or treatments upon adipocyte lipolysis (b). Scale bar in (a) is 50 μm.
Suppl. Fig. 7 Culturing human DWAT ex vivo. Several methods can be used to culture human DWAT ex vivo such as 4mm punch biopsies collected from human abdominal skin (a,b) or human DWAT carefully dissected from scalp skin. Depending on the depth of the punch biopsy, significant amounts of subcutaneous adipose tissue may be present at the bottom of the punch, whereas DWAT is present mostly around follicular units in abdominal skin. Scale bar = 1mm