A guide to studying human dermal adipocytes in situ

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A guide to studying human dermal adipocytes *in situ*

Prof. Thomas Luger

Editorial Board Member

Experimental Dermatology

Manchester, March 12, 2018

RE: EXD-17-0432_2

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

Ralf Paus, MD, FRSB

Professor of Cutaneous Medicine

The Centre for Dermatology Research,

University of Manchester,

Manchester, United Kingdom

ralf.paus@manchester.ac.uk

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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 (Page11), 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 *panniculus 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 section is the same.

We thank the reviewer for their valuable comments on Table 1. Based on this feedback we have made extensive changes to Table1. 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 Table1.

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 Table1.

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, HF, 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

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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*

Carina Nicu[1,2], Jennifer Pople[3], Laura Bonsell[1,2], Ranjit Bhogal[3] David M Ansell[1,2] and Ralf Paus[1,2]

[1] Centre for Dermatology Research, The University of Manchester, Manchester, UK

[2] NIHR Manchester Biomedical Research Centre and Manchester Academic Health Science Centre, UK

[3] Unilever R&D Colworth, Colworth Science Park, Bedford, U.K

Corresponding author: ralf.paus@manchester.ac.uk

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 fibrillary 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 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).

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

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 *in situ*

Carina Nicu[1,2], Jennifer Pople[3], Laura Bonsell[1,2], Ranjit Bhogal[3] David M Ansell[1,2] and Ralf Paus[1,2]

[1] Centre for Dermatology Research, The University of Manchester, Manchester, UK

[2] NIHR Manchester Biomedical Research Centre and Manchester Academic Health Science Centre, UK

[3] Unilever R&D Colworth, Colworth Science Park, Bedford, U.K

Corresponding author: ralf.paus@manchester.ac.uk

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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 *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.

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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~~ 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 fibrillary 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 3cb. 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 the 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

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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 ~~in obesity~~ 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 triglyceride 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, Wwhen 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].

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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].

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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 ISH, 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.

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).

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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) diacyglycerol 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-CEPBβ, 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.

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Table 1. A guide to choosing the right processing route and stain for analysing dermal adipocytes.
LD – lipid droplet; BC – breast carcinoma; BF – brightfield; CFM –confocal microscopy; DA – dermal adipocyte; FB – fibroblasts; FFPE – formalin fixed paraffin embedded; H&E – haematoxylin and eosin; IFM – immunofluorescence microscopy; KC - keratinocyte; LD – lipid droplet; MP – macrophage; PC- prostate carcinoma; SC – stratum corneum; SG – sebaceous gland; TEM – transmission electron microscopy

What should I choose when studying…?	How to process the tissue	Stains/ Markers	Target / Antigen Localization	Positive Control Tissue	Imaging and Analysis	References
DWAT Morphology	FFPE Cryo	H&E	n/a	n/a	BF	[68, 69, 194]
Lipid droplet content and Morphology	Cryo	Oil Red O, Nile Red	LD	SG, SC, Breast	BF	[3, 7, 105, 115, 140, 148, 183, 194–196]
		BODIPY, LipidTOX	LD	SG, SC, Breast	IFM CFM	
Mature Adipocyte Adipocyte size and number	FFPE Cryo	PLIN1	LD Membrane	SC, BC	BF/IFM	[7, 101, 108, 163-166, 169-173]
		CAV1	Cell membrane	KCs, FBs, HeLa Cells		
		FABP4	Cytoplasm	KCs, Lymph node		
		CPM	Cell membrane	All cells, Any tissue	IFM	
		H&E	n/a	n/a	BF	
		DGAT1/2	Cytoplasm - ER	KCs, Colon	BF/IFM	
		FASN	Cytoplasm	KCs, PC, BC		
		LPL	Membrane	Intestine		
Adipocyte differentiation	FFPE Cryo	pC/EBPβ	Nucleus	SG, BC	BF/IFM	[7, 195]
		PPARγ	Nucleus	SG, BC		
Pre-adipocytes	FFPE Cryo	Pref1	Membrane / Nucleus	Placenta	BF/IFM	[195, 197]
		Zfp521	Nucleus	Bone		
Adipocyte apoptosis	FFPE Cryo	PLIN1	LD Membrane	SC, BC	Loss of Perilipin1	[27, 194, 198]
		CAV1	Cell membrane	KCs, FBs, HeLa Cells	Loss of Caveolin1	
Pre-adipocyte proliferation	FFPE Cryo	Pref1 + Ki67/ BrdU/ EdU	Nucleus	Placenta	BF/IFM	[7, 115, 195]
Lipid synthesis (lipogenesis)	Unfixed tissue	BODIPY C1/C12	Cytoplasm - ER	Adipocytes	IFM	[132, 134, 199, 200]
	FFPE Cryo	Mature DA stain to meaure change in size (see above)	Various	Dependent on stain chosen	BF/IFM	See section above

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Lipid metabolism (lipolysis)	FFPE Cryo	phospho-Perilipin1	Cytoplasm	Adipocyte cell line stimulated to undergo lipolysis	BF/IFM	[127, 201, 202]
		phospho-HSL	Cytoplasm			
Adipocyte Ultrastructure	EM-specific	OsO4 Uranyl Acetate	Only for immuno-EM	n/a	TEM	[203] [109, 144, 148, 193, 204] [176]
3D Volume and Thickness	As per MRI/μCT protocol	Various	n/a	n/a	MRI μCT	[150–157, 160–164]
mRNA	FFPE Cryo	Fluorescent or chromogenic ISH	Cytoplasm	Dependent upon gene of interest PPIB; HeLa cells	ISH	[139] [205]
Fibrosis	FFPE	Masson's Trichrome/ Picrosirius Red	Fibrillary Collagens I and III	n/a	BF	[70, 206, 207]
		Methylene Blue	Dermal components other than DAs	n/a		

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Table 2. Markers of human dermal adipocytes and their functional importance.

Marker	Target Localization	Function	Murine References	Human References
Perilipin1	LD membrane ER	Master regulator of lipolysis Upon phosphorylation by PKA, Perilipin1 allows HSL to translocate to the adipocyte lipid droplet and commence lipolysis	[7, 69, 140, 195, 196, 207, 208] [209]	[102, 170, 176, 210, 211]
Caveolin1	Plasma membrane	Endo- and exocytosis Component of caveolae which are sites of triacylglycerol synthesis	[212, 213]	[214]
PPARγ2	Nucleus	Master regulator of adipogenesis	[95]	[102, 215]
pC/EBPβ	Nucleus	Promotes terminal adipocyte differentiation together with C/EBPα	[216]	[217, 218]
Pref1/Dlk1	Plasma Membrane	Inhibits pre-adipocyte proliferation in its membrane-bound form Prevents adipocyte differentiation (adipogenesis)	[99, 219–223]	[100–102]
Zfp423/ Znf521	Nucleus	Induces PPARγ expression thus initiating preadipocyte commitment	[224–226]	[226]
DGAT 1/2	Cytoplasm - ER	Catalyses addition of third fatty acid to diacylglycerol	[119, 227, 228]	[120]
FASN	Cytoplasm	Uses acetylCoA, malonyl-CoA, and NADPH for *de novo* synthesis of long chain saturated fatty acids.	[229, 230]	[120, 231, 232]
LPL	Adipocyte plasma membrane	Hydrolyses lipoproteins at capillary endothelium that are triglyceride-rich to generate NEFA	[233, 234]	[120, 234]
ATGL	LD membrane	Hydrolyses TAG to DAG	[235–237]	[236, 237]
HSL	Cytosol, LD membrane	Hydrolyses TAG to DAG, and DAG to MAG	[237–240]	[237, 241]
MGL	Cytosol	Hydrolyses MAG to glycerol and and a fatty acid.	[236, 237]	[237]

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Box 1. Pitfalls and tricks when working with dermal adipose tissue.

Issue	Solution(s)
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

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References

1.   Alexander CM, Kasza I, Yen C-LE, et al (2015) Dermal white adipose tissue: a new component of the thermogenic response. J Lipid Res 56:2061–9 . doi: 10.1194/jlr.R062893

2.   Shook B, Rivera Gonzalez G, Ebmeier S, et al (2016) The Role of Adipocytes in Tissue Regeneration and Stem Cell Niches. Annu Rev Cell Dev Biol. doi: 10.1146/annurev-cellbio-111315-125426

3.   Plikus M V., Guerrero-Juarez CF, Ito M, et al (2017) Regeneration of fat cells from myofibroblasts during wound healing. Science (80-) 355:748–752 . doi: 10.1126/science.aai8792

4.   Zhang L -j., Guerrero-Juarez CF, Hata T, et al (2015) Dermal adipocytes protect against invasive Staphylococcus aureus skin infection. Science (80-) 347:67–71 . doi: 10.1126/science.1260972

5.   Marangoni RG, Lu TT (2017) The roles of dermal white adipose tissue loss in scleroderma skin fibrosis. Curr Opin Rheumatol 1 . doi: 10.1097/BOR.0000000000000437

6.   Plikus M V., Mayer JA, de la Cruz D, et al (2008) Cyclic dermal BMP signalling regulates stem cell activation during hair regeneration. Nature 451:340–344 . doi: 10.1038/nature06457

7.   Festa E, Fretz J, Berry R, et al (2011) Adipocyte lineage cells contribute to the skin stem cell niche to drive hair cycling. Cell 146:761–71 . doi: 10.1016/j.cell.2011.07.019

8.   Guerrero-Juarez CF, Plikus M V. (2018) Emerging nonmetabolic functions of skin fat. Nat Rev Endocrinol 14:163–173 . doi: 10.1038/nrendo.2017.162

9.   Zwick RK, Guerrero-Juarez CF, Horsley V, Plikus M V (2018) Anatomical, Physiological, and Functional Diversity of Adipose Tissue. Cell Metab 27:68–83 . doi: 10.1016/j.cmet.2017.12.002

10.   Yang C-C, Sheu H-M, Chung P-L, et al (2015) Leptin of dermal adipose tissue is differentially expressed during the hair cycle and contributes to adipocyte-mediated growth inhibition of anagen-phase vibrissa hair. Exp Dermatol 24:57–60 . doi: 10.1111/exd.12566

11.   Fu X, Fang L, Li H, et al (2007) Adipose tissue extract enhances skin wound healing. Wound Repair Regen 15:540–8 . doi: 10.1111/j.1524-475X.2007.00262.x

12.   Kim W-S, Park B-S, Sung J-H, et al (2007) Wound healing effect of adipose-derived stem cells:

a critical role of secretory factors on human dermal fibroblasts. J Dermatol Sci 48:15–24 . doi: 10.1016/j.jdermsci.2007.05.018

13. Schmidt BA, Horsley V (2013) Intradermal adipocytes mediate fibroblast recruitment during skin wound healing. Development 140:1517–27 . doi: 10.1242/dev.087593

14. Procaccini C, La Rocca C, Carbone F, et al (2017) Leptin as immune mediator: Interaction between neuroendocrine and immune system. Dev Comp Immunol 66:120–129 . doi: 10.1016/j.dci.2016.06.006

15. Greenhill C (2017) Adipose tissue: Crosstalk between adipocytes and neurons. Nat Rev Endocrinol 13:438–438 . doi: 10.1038/nrendo.2017.79

16. Klar AS, Zimoch J, Biedermann T (2017) Skin Tissue Engineering: Application of Adipose-Derived Stem Cells. Biomed Res Int 2017:9747010

17. Owczarczyk-Saczonek A, Wociór A, Placek W, et al (2017) The Use of Adipose-Derived Stem Cells in Selected Skin Diseases (Vitiligo, Alopecia, and Nonhealing Wounds). Stem Cells Int. 2017

18. Pak J, Lee JH, Park KS, et al (2017) Current use of autologous adipose tissue-derived stromal vascular fraction cells for orthopedic applications. J Biomed Sci 24:9 . doi: 10.1186/s12929-017-0318-z

19. Lazar I, Clement E, Dauvillier S, et al (2016) Adipocyte Exosomes Promote Melanoma Aggressiveness through Fatty Acid Oxidation: A Novel Mechanism Linking Obesity and Cancer. Cancer Res 76:4051–4057 . doi: 10.1158/0008-5472.CAN-16-0651

20. Shih BB, Nirmal AJ, Headon DJ, et al (2017) Derivation of marker gene signatures from human skin and their use in the interpretation of the transcriptional changes associated with dermatological disorders. J Pathol 241:600–613 . doi: 10.1002/path.4864

21. Wang Y, Zhang L, Yu J, et al (2017) A Co-Drug of Butyric Acid Derived from Fermentation Metabolites of the Human Skin Microbiome Stimulates Adipogenic Differentiation of Adipose-Derived Stem Cells: Implications in Tissue Augmentation. J Invest Dermatol 137:46–56 . doi: 10.1016/j.jid.2016.07.030

22. Rivera-Gonzalez G, Shook B, Horsley V (2014) Adipocytes in skin health and disease. Cold Spring Harb Perspect Med 4: . doi: 10.1101/cshperspect.a015271

23. Driskell RR, Watt FM (2015) Understanding fibroblast heterogeneity in the skin. Trends Cell Biol 25:92–9 . doi: 10.1016/j.tcb.2014.10.001

24. Rutkowski JM, Stern JH, Scherer PE (2015) The cell biology of fat expansion. J Cell Biol 208:501–12 . doi: 10.1083/jcb.201409063

25. Kruglikov IL, Scherer PE, Wollina U (2016) Are dermal adipocytes involved in psoriasis? Exp Dermatol n/a-n/a . doi: 10.1111/exd.12996

26. Berry R, Church CD, Gericke MT, et al (2014) Imaging of adipose tissue. Methods Enzymol 537:47–73 . doi: 10.1016/B978-0-12-411619-1.00004-5

27. Martinez-Santibañez G, Cho KW, Lumeng CN (2014) Imaging white adipose tissue with confocal microscopy. Methods Enzymol 537:17–30 . doi: 10.1016/B978-0-12-411619-1.00002-1

28. Stanley EG, Jenkins BJ, Walker CG, et al (2017) Lipidomic profiling of human adipose tissue identifies a pattern of lipids associated with fish oil supplementation. J Proteome Res acs.jproteome.7b00161 . doi: 10.1021/acs.jproteome.7b00161

29. Pietil??inen KH, R??g T, Sepp??nen-Laakso T, et al (2011) Association of Lipidome remodeling in the Adipocyte membrane with acquired obesity in humans. PLoS Biol 9: . doi: 10.1371/journal.pbio.1000623

30. Baker RC, Nikitina Y, Subauste AR (2014) Analysis of adipose tissue lipid using mass spectrometry. Methods Enzymol 538:89–105 . doi: 10.1016/B978-0-12-800280-3.00006-2

31. López-Bascón MA, Calderón-Santiago M, Sánchez-Ceinos J, et al (2017) Influence of sample preparation on lipidomics analysis of polar lipids in adipose tissue. Talanta. doi: 10.1016/j.talanta.2017.09.017

32. Camera E, Ludovici M, Tortorella S, et al (2016) Use of lipidomics to investigate sebum dysfunction in juvenile acne. J Lipid Res 57:1051–1058 . doi: 10.1194/jlr.M067942

33. Shields KJ, Wu C (2017) Differential Adipose Tissue Proteomics. In: Methods in molecular biology (Clifton, N.J.)

34. Kirkwood JS, Miranda CL, Bobe G, et al (2016) 18O-Tracer metabolomics reveals protein turnover and CDP-choline cycle activity in differentiating 3T3-L1 pre-adipocytes. PLoS One 11: . doi: 10.1371/journal.pone.0157118

35. Suárez-Zamorano N, Fabbiano S, Chevalier C, et al (2015) Microbiota depletion promotes browning of white adipose tissue and reduces obesity. Nat Med 21:1497–1501 . doi: 10.1038/nm.3994

36. Sanmiguel C, Gupta A, Mayer EA (2015) Gut Microbiome and Obesity: A Plausible Explanation for Obesity. Curr. Obes. Rep. 4:250–261

37. Schugar RC, Shih DM, Warrier M, et al (2017) Erratum: The TMAO-Producing Enzyme Flavin-Containing Monooxygenase 3 Regulates Obesity and the Beiging of White Adipose Tissue (Cell Reports (2017) 19(12) (2451–2461) (S2211124717307519) (10.1016/j.celrep.2017.05.077)). Cell Rep. 20:279

38. Carobbio S, Pellegrinelli V, Vidal-Puig A (2017) Adipose tissue function and expandability as determinants of lipotoxicity and the metabolic syndrome. In: Advances in Experimental Medicine and Biology. pp 161–196

39. Reilly SM, Saltiel AR (2017) Adapting to obesity with adipose tissue inflammation. Nat Rev Endocrinol. doi: 10.1038/nrendo.2017.90

40. Kruglikov IL, Scherer PE (2017) Adipocyte-myofibroblast transition as a possible pathophysiological step in androgenetic alopecia. Exp. Dermatol. 26:522–523

41. Wu J, Boström P, Sparks LM, et al (2012) Beige adipocytes are a distinct type of thermogenic fat cell in mouse and human. Cell 150:366–76 . doi: 10.1016/j.cell.2012.05.016

42. Giralt M, Villarroya F (2013) White, Brown, Beige/Brite: Different Adipose Cells for Different Functions? Endocrinology 154:2992–3000 . doi: 10.1210/en.2013-1403

43. Wang QA, Scherer PE (2016) Human Beige Adipocytes: Epiphenomenon or Drivers of Metabolic Improvements? Trends Endocrinol Metab 27:244–246 . doi: 10.1016/j.tem.2016.03.008

44. Driskell RR, Jahoda CAB, Chuong CM, et al (2014) Defining dermal adipose tissue. Exp. Dermatol. 23:629–631

45. Driskell RR, Lichtenberger BM, Hoste E, et al (2013) Distinct fibroblast lineages determine dermal architecture in skin development and repair. Nature 504:277–81 . doi: 10.1038/nature12783

46. DURWARD A, RUDALL KM (1949) Studies on hair growth in the rat. J Anat 83:325–35, 4 pl

47. Hausman GJ, Campion DR, Richardson RL, Martin RJ (1981) Adipocyte development in the rat hypodermis. Am J Anat 161:85–100 . doi: 10.1002/aja.1001610107

48. Driskell RR, Jahoda CAB, Chuong C-M, et al (2014) Defining dermal adipose tissue. Exp Dermatol 23:629–631 . doi: 10.1111/exd.12450

49. Schneider MR (2014) Coming home at last: Dermal white adipose tissue. Exp. Dermatol. 23:634–635

50. Larson JD, Tierney WS, Ozturk CN, Zins JE (2014) Defining the Fat Compartments in the Neck: A Cadaver Study. Aesthetic Surg J 34:499–506 . doi: 10.1177/1090820X14526406

51. Poblet E, Jiménez-Acosta F, Hardman JA, et al (2016) Is the eccrine gland an integral, functionally important component of the human scalp pilosebaceous unit? Exp Dermatol 25:149–150 . doi: 10.1111/exd.12889

52. Poblet E, Jimenez-Acosta F, Escario-Travesedo E, et al (2018) Eccrine sweat glands associate with the human hair follicle within a defined compartment of dermal white adipose tissue. Br J Dermatol. doi: 10.1111/bjd.16436

53. Saely CH, Geiger K, Drexel H (2012) Brown versus White Adipose Tissue: A Mini-Review. Gerontology 58:15–23 . doi: 10.1159/000321319

54. Kwok KHM, Lam KSL, Xu A (2016) Heterogeneity of white adipose tissue: molecular basis and clinical implications. Exp Mol Med 48:e215 . doi: 10.1038/emm.2016.5

55. Sun K, Kusminski CCM, Scherer PEP (2011) Adipose tissue remodeling and obesity. J Clin … 121:2094–2101 . doi: 10.1172/JCI45887.2094

56. Horsley V, Watt F (2017) Repeal and Replace: Adipocyte Regeneration in Wound Repair. Cell Stem Cell 20:424–426 . doi: 10.1016/j.stem.2017.03.015

57. Divoux A, Tordjman J, Lacasa D, et al (2010) Fibrosis in Human Adipose Tissue : Composition , Distribution , and Link With Lipid Metabolism and Fat. Diabetes 59:2817–2825 . doi: 10.2337/db10-0585.A.D.

58. Yang C-C, Chung P-L, Lin L-Y, et al (2017) Higher plasma leptin is associated with higher risk of androgenetic alopecia in men. Exp Dermatol 26:524–526 . doi: 10.1111/exd.13369

59. Chen G (2016) Chapter 100: "Other acquired disorders of subcutaneous fat." Rook's Textb dermatology Wiley-Blackwell 9:67–82

60. Fung (2017) Chapter 2 "Panniculitis." Dermatopathology 2nd editio:Elsevier/Saunders, Philadelphia

61. Toussirot É, Aubin F, Dumoulin G (2014) Relationships between adipose tissue and psoriasis, with or without arthritis. Front. Immunol. 5

62. Kruglikov I, Wollina U (2017) Local effects of adipose tissue in psoriasis and psoriatic arthritis. Psoriasis Targets Ther Volume 7:17–25 . doi: 10.2147/PTT.S122959

63. Kruglikov IL (2017) Interfacial Adipose Tissue in Systemic Sclerosis. Curr Rheumatol Rep 19:1–8 . doi: 10.1007/s11926-017-0627-y

64. Lynch M, Ahern T, Sweeney CM, et al (2017) Adipokines, psoriasis, systemic inflammation, and endothelial dysfunction. Int. J. Dermatol. 56:1103–1118

65. Cheung L, Fisher RM, Kuzmina N, et al (2016) Psoriasis Skin Inflammation-Induced microRNA-26b Targets NCEH1 in Underlying Subcutaneous Adipose Tissue. J Invest Dermatol 136:640–648 . doi: 10.1016/j.jid.2015.12.008

66. Matsuda Y, Fujii T, Suzuki T, et al (2011) Comparison of Fixation Methods for Preservation of Morphology, RNAs, and Proteins From Paraffin-Embedded Human Cancer Cell-Implanted Mouse Models. J Histochem Cytochem 59:68–75 . doi: 10.1369/jhc.2010.957217

67. Mehlem A, Hagberg CE, Muhl L, et al (2013) Imaging of neutral lipids by oil red O for analyzing the metabolic status in health and disease. Nat Protoc 8:1149–1154 . doi: 10.1038/nprot.2013.055

68. Ezure T, Amano S (2015) Increment of subcutaneous adipose tissue is associated with decrease of elastic fibres in the dermal layer. Exp Dermatol 24:924–929 . doi: 10.1111/exd.12816

69. Donati G, Proserpio V, Lichtenberger BM, et al (2014) Epidermal Wnt/ -catenin signaling regulates adipocyte differentiation via secretion of adipogenic factors. Proc Natl Acad Sci 111:E1501–E1509 . doi: 10.1073/pnas.1312880111

70. Halberg N, Khan T, Trujillo ME, et al (2009) Hypoxia-inducible factor 1alpha induces fibrosis and insulin resistance in white adipose tissue. Mol Cell Biol 29:4467–4483 . doi: 10.1128/MCB.00192-09

71. Fleischmajer R, Damiano V, Nedwich A (1971) Scleroderma and the subcutaneous tissue.

Science 171:1019–21

72. Huang C, Murphy GF, Akaishi S, Ogawa R (2013) Keloids and hypertrophic scars: update and future directions. Plast Reconstr surgery Glob open 1:e25 . doi: 10.1097/GOX.0b013e31829c4597

73. Marangoni RG, Korman BD, Wei J, et al (2015) Myofibroblasts in Murine Cutaneous Fibrosis Originate From Adiponectin-Positive Intradermal Progenitors. Arthritis Rheumatol 67:1062–1073 . doi: 10.1002/art.38990

74. HARRIS JE, Peters A (1953) Experiments on vital staining with methylene blue. Q J Microsc Sci 3:113–124

75. Genina EA, Bashkatov AN, Tuchin V V. (2004) Methylene blue diffusion in skin tissue. In: ALT'03 International Conference on Advanced Laser Technologies: Biomedical Optics. pp 315–323

76. Tchoukalova YD, Koutsari C, Karpyak M V, et al (2008) Subcutaneous adipocyte size and body fat distribution. Am J Clin Nutr 87:56–63

77. Ramírez-Zacarías JL, Castro-Muñozledo F, Kuri-Harcuch W (1992) Quantitation of adipose conversion and triglycerides by staining intracytoplasmic lipids with oil red O. Histochemistry 97:493–497 . doi: 10.1007/BF00316069

78. Fowler SD, Greenspan P (1985) Application of Nile red, a fluorescent hydrophobic probe, for the detection of neutral lipid deposits in tissue sections: comparison with oil red O. J Histochem Cytochem 33:833–836 . doi: 10.1177/33.8.4020099

79. Kinkel AD, Fernyhough ME, Helterline DL, et al (2004) Oil red-O stains non-adipogenic cells: A precautionary note. Cytotechnology 46:49–56 . doi: 10.1007/s10616-004-3903-4

80. Greenspan P, Mayer EP, Fowler SD (1985) Nile red: a selective fluorescent stain for intracellular lipid droplets. J Cell Biol 100:965–973 . doi: 10.1083/jcb.100.3.965

81. Mendel D (1912) Behavior of Fat-Soluble Dyes

82. Hirsch J, Gallian E (1968) Methods for the determination of adipose cell size in man and animals. J Lipid Res 9:110–119

83. Spangenburg EE, Pratt SJP, Wohlers LM, Lovering RM (2011) Use of BODIPY (493/503) to visualize intramuscular lipid droplets in skeletal muscle. J Biomed Biotechnol 2011: . doi:

10.1155/2011/598358

84. Qiu B, Simon M (2016) BODIPY 493/503 Staining of Neutral Lipid Droplets for Microscopy and Quantification by Flow Cytometry. BIO-PROTOCOL 6: . doi: 10.21769/BioProtoc.1912

85. Jiang M, Gu X, Lam JWY, et al (2017) Two-photon AIE bio-probe with large Stokes shift for specific imaging of lipid droplets. Chem Sci 8:5440–5446 . doi: 10.1039/C7SC01400G

86. Tang J, Zhang Y, Yin H-Y, et al (2017) Precise Labeling and Tracking of Lipid Droplets in Adipocytes Using a Luminescent ZnSalen Complex. Chem - An Asian J 12:2533–2538 . doi: 10.1002/asia.201701010

87. Jester J V., Potma E, Brown DJ (2016) PPARγ Regulates Mouse Meibocyte Differentiation and Lipid Synthesis. Ocul Surf 14:484–494 . doi: 10.1016/j.jtos.2016.08.001

88. Dutta P, Haller E, Sharp A, Nanjundan M (2016) MIR494 reduces renal cancer cell survival coinciding with increased lipid droplets and mitochondrial changes. BMC Cancer 16:33 . doi: 10.1186/s12885-016-2053-3

89. Ding J, Liu Y, Sullivan DA (2015) Effects of insulin and high glucose on human meibomian gland epithelial cells. Investig Ophthalmol Vis Sci 56:7814–7820 . doi: 10.1167/iovs.15-18049

90. Wang H, Sreenivasan U, Sreenevasan U, et al (2011) Perilipin 5, a lipid droplet-associated protein, provides physical and metabolic linkage to mitochondria. J Lipid Res 52:2159–68 . doi: 10.1194/jlr.M017939

91. Hinde E, Haslam IS, Schneider MR, et al (2013) A practical guide for the study of human and murine sebaceous glands in situ. Exp. Dermatol. 22:631–637

92. Bayati V, Abbaspour MR, Dehbashi FN, et al (2016) A dermal equivalent developed from adipose-derived stem cells and electrospun polycaprolactone matrix: an in vitro and in vivo study. Anat Sci Int 1–12 . doi: 10.1007/s12565-016-0352-z

93. Fan JY, Carpentier JL, van Obberghen E, et al (1983) Morphological changes of the 3T3-L1 fibroblast plasma membrane upon differentiation to the adipocyte form. J Cell Sci 61:219–30

94. Kwok KHM, Lam KSL, Xu A (2016) Heterogeneity of white adipose tissue: molecular basis and clinical implications. Exp Mol Med 48:e215 . doi: 10.1038/emm.2016.5

95. Lefterova MI, Zhang Y, Steger DJ, et al (2008) PPAR and C/EBP factors orchestrate adipocyte biology via adjacent binding on a genome-wide scale. Genes Dev 22:2941–2952 . doi:

10.1101/gad.1709008

96. Shao X, Wang M, Wei X, et al (2016) Peroxisome Proliferator-Activated Receptor-γ: Master Regulator of Adipogenesis and Obesity. Curr Stem Cell Res Ther 11:282–9 . doi: 10.2174/1574888X10666150528144905

97. Tontonoz P, Hu E, Devine J, et al (1995) PPAR gamma 2 regulates adipose expression of the phosphoenolpyruvate carboxykinase gene. Mol Cell Biol 15:351–357 . doi: 10.1128/MCB.15.1.351

98. Park B-H, Qiang L, Farmer SR (2004) Phosphorylation of C/EBPbeta at a consensus extracellular signal-regulated kinase/glycogen synthase kinase 3 site is required for the induction of adiponectin gene expression during the differentiation of mouse fibroblasts into adipocytes. Mol Cell Biol 24:8671–80 . doi: 10.1128/MCB.24.19.8671-8680.2004

99. Hudak CS, Sul HS (2013) Pref-1, a gatekeeper of adipogenesis. Front Endocrinol (Lausanne) 4:79 . doi: 10.3389/fendo.2013.00079

100. Mitterberger MC, Lechner S, Mattesich M, et al (2012) DLK1(PREF1) is a negative regulator of adipogenesis in CD105+/CD90+/CD34+/CD31−/FABP4− adipose-derived stromal cells from subcutaneous abdominal fat pats of adult women. Stem Cell Res 9:35–48 . doi: 10.1016/j.scr.2012.04.001

101. Zwierzina ME, Ejaz A, Bitsche M, et al (2015) Characterization of DLK1(PREF1)+/CD34+ cells in vascular stroma of human white adipose tissue. Stem Cell Res 15:403–418 . doi: 10.1016/j.scr.2015.08.004

102. Marques AP, Rosmaninho-Salgado J, Estrada M, et al (2017) Hypoxia mimetic induces lipid accumulation through mitochondrial dysfunction and stimulates autophagy in murine preadipocyte cell line. Biochim Biophys Acta - Gen Subj 1861:673–682 . doi: 10.1016/j.bbagen.2016.12.005

103. Galarraga M, Campión J, Muñoz-Barrutia A, et al (2012) Adiposoft: automated software for the analysis of white adipose tissue cellularity in histological sections. J Lipid Res 53:2791–2796 . doi: 10.1194/jlr.D023788

104. Parlee SD, Lentz SI, Mori H, MacDougald OA (2014) Quantifying size and number of adipocytes in adipose tissue. Methods Enzymol 537:93–122 . doi: 10.1016/B978-0-12-411619-1.00006-9

105. Gadupudi G, Gourronc FA, Ludewig G, et al (2015) PCB126 inhibits adipogenesis of human preadipocytes. Toxicol Vitr 29:132–141 . doi: 10.1016/j.tiv.2014.09.015

106. Wojciechowicz K, Gledhill K, Ambler CA, et al (2013) Development of the mouse dermal adipose layer occurs independently of subcutaneous adipose tissue and is marked by restricted early expression of FABP4. PLoS One 8:e59811 . doi: 10.1371/journal.pone.0059811

107. Prins JB, Walker NI, Winterford CM, Cameron DP (1994) Human adipocyte apoptosis occurs in malignancy. Biochem Biophys Res Commun 205:625–630

108. Cinti S (2005) Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res 46:2347–2355 . doi: 10.1194/jlr.M500294-JLR200

109. Giordano A, Murano I, Mondini E, et al (2013) Obese adipocytes show ultrastructural features of stressed cells and die of pyroptosis. J Lipid Res 54:2423–2436 . doi: 10.1194/jlr.M038638

110. Cinti S, Mitchell G, Barbatelli G, et al (2005) Adipocyte death defines macrophage localization and function in adipose tissue of obese mice and humans. J Lipid Res 46:2347–2355 . doi: 10.1194/jlr.M500294-JLR200

111. Feng D, Tang Y, Kwon H, et al (2011) High-fat diet-induced adipocyte cell death occurs through a cyclophilin D intrinsic signaling pathway independent of adipose tissue inflammation. Diabetes 60:2134–2143 . doi: 10.2337/db10-1411

112. Lumeng CN, Delproposto JB, Westcott DJ, Saltiel AR (2008) Phenotypic switching of adipose tissue macrophages with obesity is generated by spatiotemporal differences in macrophage subtypes. Diabetes 57:3239–3246 . doi: 10.2337/db08-0872

113. Lumeng CN, Bodzin JL, Saltiel AR (2007) Obesity induces a phenotypic switch in adipose tissue macrophage polarization. J Clin Invest 117:175–184 . doi: 10.1172/JCI29881

114. Simon G (1965) Adipose Tissue. Handb Physiol 5:101–107

115. Rodeheffer MS, Birsoy K, Friedman JM (2008) Identification of white adipocyte progenitor cells in vivo. Cell 135:240–9 . doi: 10.1016/j.cell.2008.09.036

116. Lu Z, Hasse S, Bodo E, et al (2006) Towards the development of a simplified long-term organ culture method for human scalp skin and its appendages under serum-free conditions. doi: 10.1111/j.1600-0625.2006.00510.x

117. Langan EA, Philpott MP, Kloepper JE, Paus R (2015) Human hair follicle organ culture: theory, application and perspectives. Exp. Dermatol. 24:903–911

118. Purba TS, Haslam IS, Shahmalak A, et al (2015) Mapping the expression of epithelial hair follicle stem cell-related transcription factors LHX2 and SOX9 in the human hair follicle. Exp Dermatol 24:462–7 . doi: 10.1111/exd.12700

119. Yen CL, Stone SJ, Koliwad S, et al (2000) DGAT enzymes and triacylglycerol biosynthesis. J Lipid Res . doi: 10.1194/jlr.R800018-JLR200

120. Ranganathan G, Unal R, Pokrovskaya I, et al (2006) The lipogenic enzymes DGAT1, FAS, and LPL in adipose tissue: effects of obesity, insulin resistance, and TZD treatment. J Lipid Res 47:2444–2450 . doi: 10.1194/jlr.M600248-JLR200

121. Girousse A, Langin D (2012) Adipocyte lipases and lipid droplet-associated proteins: insight from transgenic mouse models. Int J Obes 36:581–594 . doi: 10.1038/ijo.2011.113

122. Yang X, Lu X, Lombès M, et al (2010) The G(0)/G(1) switch gene 2 regulates adipose lipolysis through association with adipose triglyceride lipase. Cell Metab 11:194–205 . doi: 10.1016/j.cmet.2010.02.003

123. Morimoto C, Kameda K, Tsujita T, Okuda H (2001) Relationships between lipolysis induced by various lipolytic agents and hormone-sensitive lipase in rat fat cells. J Lipid Res 42:120–7

124. Lass A, Zimmermann R, Oberer M, Zechner R (2011) Lipolysis - a highly regulated multi-enzyme complex mediates the catabolism of cellular fat stores. Prog Lipid Res 50:14–27 . doi: 10.1016/j.plipres.2010.10.004

125. Nielsen TS, Jessen N, Jørgensen JOL, et al (2014) Dissecting adipose tissue lipolysis: molecular regulation and implications for metabolic disease. J Mol Endocrinol 52:R199-222 . doi: 10.1530/JME-13-0277

126. Martin S, Okano S, Kistler C, et al (2009) Spatiotemporal regulation of early lipolytic signaling in adipocytes. J Biol Chem 284:32097–107 . doi: 10.1074/jbc.M109.002675

127. Schweiger M, Eichmann TO, Taschler U, et al (2014) Measurement of lipolysis. Methods Enzymol 538:171–93 . doi: 10.1016/B978-0-12-800280-3.00010-4

128. Society RM (1965) Enzyme histochemistry of developing human skin in tissue culture. 84:317–322

129. Danse HJC (1974) 脂褐素是氧化终产物Enzyme Histochemical S t u d i e s of Adipose Tissue in Porcine Yellow Fat Disease. 476:465–476

130. Hausman GJ (1984) Histochemistry of connective tissue cells in subcutaneous adipose tissue of normal and decapitated pig fetuses. Acta Anat (Basel) 118:147–52

131. Li H, Black PN, DiRusso CC (2005) A live-cell high-throughput screening assay for identification of fatty acid uptake inhibitors. Anal Biochem 336:11–19 . doi: 10.1016/J.AB.2004.09.025

132. Li H, Black PN, Chokshi A, et al High-throughput screening for fatty acid uptake inhibitors in humanized yeast identifies atypical antipsychotic drugs that cause dyslipidemias. doi: 10.1194/jlr.D700015-JLR200

133. Liao J, Sportsman R, Harris J, Stahl A (2005) Real-time quantification of fatty acid uptake using a novel fluorescence assay. J Lipid Res 46:597–602 . doi: 10.1194/jlr.D400023-JLR200

134. Kolahi K, Louey S, Varlamov O, Thornburg K (2016) Real-Time Tracking of BODIPY-C12 Long-Chain Fatty Acid in Human Term Placenta Reveals Unique Lipid Dynamics in Cytotrophoblast Cells. PLoS One 11:e0153522 . doi: 10.1371/journal.pone.0153522

135. Iglesias J, Lamontagne J, Erb H, et al (2015) Simplified assays of lipolysis enzymes for drug discovery and specificity assessment of of known inhibitors. J Lipid Res 57:131–141 . doi: 10.1194/jlr.D058438

136. Previs SF, McLaren DG, Wang SP, et al (2014) New methodologies for studying lipid synthesis and turnover: Looking backwards to enable moving forwards. Biochim. Biophys. Acta - Mol. Basis Dis. 1842:402–413

137. Murphy EJ (2006) Stable isotope methods for the in vivo measurement of lipogenesis and triglyceride metabolism. J. Anim. Sci. 84 Suppl

138. Akie TE, Cooper MP (2015) Determination of Fatty Acid Oxidation and Lipogenesis in Mouse Primary Hepatocytes. J Vis Exp 2–7 . doi: 10.3791/52982

139. Grino M (2008) Study of adipose tissue gene expression by in situ hybridization. Methods Mol Biol 456:271–283 . doi: 10.1007/978-1-59745-245-8_20

140. Moore HPH, Silver RB, Mottillo EP, et al (2005) Perilipin targets a novel pool of lipid droplets for lipolytic attack by hormone-sensitive lipase. J Biol Chem 280:43109–43120 . doi: 10.1074/jbc.M506336200

141. Jonkman J, Brown CM (2015) Any Way You Slice It - A Comparison of Confocal Microscopy Techniques. J Biomol Tech 26:54–65 . doi: 10.7171/jbt.15-2602-003

142. Malide (2001) Confocal Microscopy of Adipocytes. Adipose Tissue Protoc Methods Mol Biol 155:

143. Wilke K, Wick K, Keil FJ, et al (2008) A strategy for correlative microscopy of large skin samples: Towards a holistic view of axillary skin complexity. Exp. Dermatol. 17:73–80

144. Sbarbati A, Accorsi D, Benati D, et al (2010) Subcutaneous adipose tissue classification. Eur J Histochem 54:48 . doi: 10.4081/ejh.2010.e48

145. Conti G, Benati D, Bernardi P, et al (2014) The post-adipocytic phase of the adipose cell cycle. Tissue Cell 46:520–526 . doi: 10.1016/j.tice.2014.09.005

146. Bertossi D (2003) Lipofilling of the lips: ultrastructural evaluation by transmission electron microscopy of injected adipose tissue. Arch facial … 5:392–398 . doi: 10.1001/archfaci.5.5.392

147. Kranendonk MEG, Visseren FLJ, Van Balkom BWM, et al (2014) Human adipocyte extracellular vesicles in reciprocal signaling between adipocytes and macrophages. Obesity 22:1296–1308 . doi: 10.1002/oby.20679

148. Maurizi G, Poloni A, Mattiucci D, et al (2017) Human White Adipocytes Convert Into "Rainbow" Adipocytes In Vitro. J Cell Physiol 232:2887–2899 . doi: 10.1002/jcp.25743

149. Frontini A, Cinti S (2010) Distribution and Development of Brown Adipocytes in the Murine and Human Adipose Organ. Cell Metab. 11:253–256

150. Mirrashed F, Sharp JC, Krause V, et al (2004) Pilot study of dermal and subcutaneous fat structures by MRI in individuals who differ in gender, BMI, and cellulite grading. Ski Res Technol 10:161–168 . doi: 10.1111/j.1600-0846.2004.00072.x

151. Juan Y-H, Saboo SS, Tirumani SH, et al (2014) Malignant Skin and Subcutaneous Neoplasms in Adults: Multimodality Imaging With CT, MRI, and ^{18}F-FDG PET/CT. Am J Roentgenol 202:W422–W438 . doi: 10.2214/AJR.13.11424

152. Kudawara I, Ueda T, Yoshikawa H, et al (1999) Granular cell tumor of the subcutis: CT and MRI findings. Skelet Radiol 28:

153. Deng J, Schoeneman SE, Zhang H, et al (2015) MRI characterization of brown adipose tissue in obese and normal-weight children. Pediatr Radiol 45:1682–1689 . doi: 10.1007/s00247-015-

3391-z

154. Poonawalla AH, Sjoberg BP, Rehm JL, et al (2013) Adipose tissue MRI for quantitative measurement of central obesity. J Magn Reson Imaging 37:707–716 . doi: 10.1002/jmri.23846

155. Lindenberg KS, Weydt P, Müller H-P, et al (2014) Two-Point Magnitude MRI for Rapid Mapping of Brown Adipose Tissue and Its Application to the R6/2 Mouse Model of Huntington Disease. PLoS One 9:e105556 . doi: 10.1371/journal.pone.0105556

156. Kasza I, Hernando D, Roldán-Alzate A, et al (2016) Thermogenic profiling using magnetic resonance imaging of dermal and other adipose tissues. JCI insight 1:e87146 . doi: 10.1172/jci.insight.87146

157. Gronemeyer SA, Steen RG, Kauffman WM, et al (2000) Fast adipose tissue (FAT) assessment by MRI. Magn Reson Imaging 18:815–818 . doi: 10.1016/S0730-725X(00)00168-5

158. Nerlekar N, Baey Y-W, Brown AJ, et al (2018) Poor Correlation, Reproducibility, and Agreement Between Volumetric vs. Linear Epicardial Adipose Tissue Measurement: A 3D Computed Tomography vs. 2D Echocardiography Comparison. JACC Cardiovasc Imaging. doi: 10.1016/J.JCMG.2017.10.019

159. Hu HH, Nayak KS, Goran MI (2011) Assessment of abdominal adipose tissue and organ fat content by magnetic resonance imaging. Obes Rev 12:e504-15 . doi: 10.1111/j.1467-789X.2010.00824.x

160. Hu HH, Chen J, Shen W (2016) Segmentation and quantification of adipose tissue by magnetic resonance imaging. MAGMA 29:259–76 . doi: 10.1007/s10334-015-0498-z

161. Kim WH, Kim CG, Kim D-W (2012) Optimal CT Number Range for Adipose Tissue When Determining Lean Body Mass in Whole-Body F-18 FDG PET/CT Studies. Nucl Med Mol Imaging (2010) 46:294–9 . doi: 10.1007/s13139-012-0175-3

162. Judex S, Luu YK, Ozcivici E, et al (2010) Quantification of adiposity in small rodents using micro-CT. Methods 50:14–9 . doi: 10.1016/j.ymeth.2009.05.017

163. Marchadier A, Vidal C, Tafani J-P, et al (2011) Quantitative CT imaging for adipose tissue analysis in mouse model of obesity Quantitative CT imaging for adipose tissue analysis in mouse model of obesity Quantitative CT imaging for adipose tissue analysis in mouse model of obesity. SPIE Med Imaging

164. Kullberg J, Hedström A, Brandberg J, et al (2017) Automated analysis of liver fat, muscle and adipose tissue distribution from CT suitable for large-scale studies. Sci Rep 7:10425 . doi: 10.1038/s41598-017-08925-8

165. Li L, Margolis LB, Paus R, Hoffman RM (1992) Hair shaft elongation, follicle growth, and spontaneous regression in long-term, gelatin sponge-supported histoculture of human scalp skin. Proc Natl Acad Sci U S A 89:8764–8 . doi: 10.1073/pnas.89.18.8764

166. Gesta S, Lolmède K, Daviaud D, et al (2003) Culture of human adipose tissue explants leads to profound alteration of adipocyte gene expression. Horm Metab Res = Horm und Stoffwechselforsch = Horm métabolisme 35:158–63 . doi: 10.1055/s-2003-39070

167. Carswell KA, Lee M-J, Fried SK (2012) Culture of isolated human adipocytes and isolated adipose tissue. Methods Mol Biol 806:203–14 . doi: 10.1007/978-1-61779-367-7_14

168. Skurk T, Hauner H (2012) Primary culture of human adipocyte precursor cells: expansion and differentiation. Methods Mol Biol 806:215–26 . doi: 10.1007/978-1-61779-367-7_15

169. Ruiz-Ojeda F, Rupérez A, Gomez-Llorente C, et al (2016) Cell Models and Their Application for Studying Adipogenic Differentiation in Relation to Obesity: A Review. Int J Mol Sci 2016, Vol 17, Page 1040 17:1040 . doi: 10.3390/IJMS17071040

170. Huber B, Link A, Linke K, et al (2016) Integration of Mature Adipocytes to Build-Up a Functional Three-Layered Full-Skin Equivalent. Tissue Eng Part C Methods 22:756–764 . doi: 10.1089/ten.tec.2016.0141

171. Lequeux C, Auxenfans C, Thépot A, et al (2011) A simple way to reconstruct a human 3-D hypodermis: A useful tool for pharmacological functionality. Skin Pharmacol Physiol 25:47–55 . doi: 10.1159/000330904

172. Miyamoto Y, Ikeuchi M, Noguchi H, et al (2017) Enhanced Adipogenic Differentiation of Human Adipose-Derived Stem Cells in an in vitro Microenvironment: The Preparation of Adipose-Like Microtissues Using a Three-Dimensional Culture. Cell Med 9:35–44 . doi: 10.3727/215517916X693096

173. Turner PA, Gurumurthy B, Bailey JL, et al (2017) Adipogenic differentiation of human adipose-derived stem cells grown as spheroids. Process Biochem 59:312–320 . doi: 10.1016/j.procbio.2017.02.003

174. Furuhata Y, Kikuchi Y, Tomita S, Yoshimoto K (2016) Small spheroids of adipose-derived stem

cells with time-dependent enhancement of IL-8 and VEGF-A secretion. Genes to Cells 21:1380–1386 . doi: 10.1111/gtc.12448

175. Feng J, Mineda K, Wu S-H, et al (2017) An injectable non-cross-linked hyaluronic-acid gel containing therapeutic spheroids of human adipose-derived stem cells. Sci Rep 7:1548 . doi: 10.1038/s41598-017-01528-3

176. Heid H, Rickelt S, Zimbelmann R, et al (2014) On the formation of lipid droplets in human adipocytes: The organization of the perilipin-vimentin cortex. PLoS One 9: . doi: 10.1371/journal.pone.0090386

177. Paino F, La Noce M, Di Nucci D, et al (2017) Human adipose stem cell differentiation is highly affected by cancer cells both in vitro and in vivo: implication for autologous fat grafting. Cell Death Dis 8:e2568 . doi: 10.1038/cddis.2016.308

178. Poulos SP, Dodson M V, Hausman GJ (2010) Cell line models for differentiation: preadipocytes and adipocytes. Exp Biol Med 235:1185–1193 . doi: 10.1258/ebm.2010.010063

179. Todorčević M, Hilton C, McNeil C, et al (2017) A cellular model for the investigation of depot specific human adipocyte biology. Adipocyte 6:40–55 . doi: 10.1080/21623945.2016.1277052

180. Torii I, Morikawa S, Nakano A, Morikawa K (2003) Establishment of a Human Preadipose Cell Line, HPB-AML-I: Refractory to PPAR??-Mediated Adipogenic Stimulation. J Cell Physiol 197:42–52 . doi: 10.1002/jcp.10326

181. Wabitsch M, Brenner RE, Melzner I, et al (2001) Characterization of a human preadipocyte cell strain with high capacity for adipose differentiation. Int J Obes 25:8–15 . doi: 10.1038/sj.ijo.0801520

182. Iwata T, Kuribayashi K, Nakasono M, et al (2017) The AMPK/mTOR pathway is involved in D-dopachrome tautomerase gene transcription in adipocytes differentiated from SGBS cells, a human preadipocyte cell line. Cytokine 96:195–202 . doi: 10.1016/j.cyto.2017.04.017

183. Guennoun A, Kazantzis M, Thomas R, et al (2015) Comprehensive molecular characterization of human adipocytes reveals a transient brown phenotype. J Transl Med 13:135 . doi: 10.1186/s12967-015-0480-6

184. Gibellini L, De Biasi S, Pinti M, et al (2017) The protease inhibitor atazanavir triggers autophagy and mitophagy in human preadipocytes. Aids 26:2017–2026 . doi: 10.1097/QAD.0b013e328359b8be

185. Fischer-Posovszky P, Newell FS, Wabitsch M, Tornqvist HE (2008) Human SGBS cells - A unique tool for studies of human fat cell biology. Obes. Facts 1:184–189

186. Zhou Y, Robciuc MR, Wabitsch M, et al (2012) OSBP-Related Proteins (ORPs) in Human Adipose Depots and Cultured Adipocytes: Evidence for Impacts on the Adipocyte Phenotype. PLoS One 7: . doi: 10.1371/journal.pone.0045352

187. Do M-S, Jeong H-S, Choi B-H, et al (2006) Inflammatory gene expression patterns revealed by DNA microarray analysis in TNF-alpha-treated SGBS human adipocytes. Yonsei Med J 47:729–736 . doi: 10.3349/ymj.2006.47.5.729

188. Lee Y-K, Cowan C a (2014) Differentiation of white and brown adipocytes from human pluripotent stem cells.

189. Chen J-H, Goh KJ, Rocha N, et al (2017) Evaluation of human dermal fibroblasts directly reprogrammed to adipocyte-like cells as a metabolic disease model. Dis Model Mech dmm.030981 . doi: 10.1242/dmm.030981

190. Choi S, Ryoo S-B, Park KJ, et al (2017) Autologous adipose tissue-derived stem cells for the treatment of complex perianal fistulas not associated with Crohn's disease: a phase II clinical trial for safety and efficacy. Tech Coloproctol 21:345–353 . doi: 10.1007/s10151-017-1630-z

191. Dranse HJ, Muruganandan S, Fawcett JP, Sinal CJ (2016) Adipocyte-secreted chemerin is processed to a variety of isoforms and influences MMP3 and chemokine secretion through an NFkB-dependent mechanism. Mol Cell Endocrinol 436:114–129 . doi: 10.1016/j.mce.2016.07.017

192. Forni MF, Peloggia J, Braga TT, et al (2017) Caloric Restriction Promotes Structural and Metabolic Changes in the Skin. Cell Rep 20:2678–2692 . doi: 10.1016/j.celrep.2017.08.052

193. Wang YY, Attané C, Milhas D, et al (2017) Mammary adipocytes stimulate breast cancer invasion through metabolic remodeling of tumor cells. JCI Insight 2: . doi: 10.1172/jci.insight.87489

194. Berry R, Church CD, Gericke MT, et al (2014) Chapter Four – Imaging of Adipose Tissue. In: Methods in Enzymology. pp 47–73

195. Zhang L -j., Guerrero-Juarez CF, Hata T, et al (2015) Dermal adipocytes protect against invasive Staphylococcus aureus skin infection. Science (80-) 347:67–71 . doi: 10.1126/science.1260972

196. Shook B, Rivera Gonzalez G, Ebmeier S, et al (2016) The Role of Adipocytes in Tissue Regeneration and Stem Cell Niches. Annu Rev Cell Dev Biol. doi: 10.1146/annurev-cellbio-111315-125426

197. Kang S, Akerblad P, Kiviranta R, et al (2012) Regulation of Early Adipose Commitment by Zfp521. PLoS Biol 10: . doi: 10.1371/journal.pbio.1001433

198. Murano I, Barbatelli G, Parisani V, et al (2008) Dead adipocytes, detected as crown-like structures, are prevalent in visceral fat depots of genetically obese mice. J Lipid Res 49:1562–8 . doi: 10.1194/jlr.M800019-JLR200

199. Klapper M, Ehmke M, Palgunow D, et al (2011) Fluorescence-based fixative and vital staining of lipid droplets in Caenorhabditis elegans reveal fat stores using microscopy and flow cytometry approaches. J Lipid Res 52:1281–93 . doi: 10.1194/jlr.D011940

200. Jia Z, Moulson CL, Pei Z, et al (2007) Fatty Acid Transport Protein 4 Is the Principal Very Long Chain Fatty Acyl-CoA Synthetase in Skin Fibroblasts *. doi: 10.1074/jbc.M700568200

201. McDonough PM, Maciejewski-Lenoir D, Hartig SM, et al (2013) Differential Phosphorylation of Perilipin 1A at the Initiation of Lipolysis Revealed by Novel Monoclonal Antibodies and High Content Analysis. PLoS One 8:e55511 . doi: 10.1371/journal.pone.0055511

202. Wang H, Hu L, Dalen K, et al (2009) Activation of hormone-sensitive lipase requires two steps, protein phosphorylation and binding to the PAT-1 domain of lipid droplet coat proteins. J Biol Chem 284:32116–25 . doi: 10.1074/jbc.M109.006726

203. Mirancea N, Mirancea D (2011) THE ULTRASTRUCTURE OF THE WHITE SUBCUTANEOUS ADIPOSE TISSUE. Proc Rom Acad, Ser B 3:206–211

204. Kolokol'chikova EG, Pal'tsyn AA, Shchegolev AI, et al (2005) Proliferative activity of adipocytes in adipose tissue tumors. Bull Exp Biol Med 140:122–126 . doi: doi [pii]

205. Plikus M V, Mayer JA, de la Cruz D, et al (2008) Cyclic dermal BMP signalling regulates stem cell activation during hair regeneration. Nature 451:340–4 . doi: 10.1038/nature06457

206. Khan T, Muise ES, Iyengar P, et al (2009) Metabolic dysregulation and adipose tissue fibrosis: role of collagen VI. Mol Cell Biol 29:1575–91 . doi: 10.1128/MCB.01300-08

207. Sun Z, Gong J, Wu H, et al (2013) Perilipin1 promotes unilocular lipid droplet formation through the activation of Fsp27 in adipocytes. Nat Commun 4:1594 . doi:

10.1038/ncomms2581

208. Tansey JT, Sztalryd C, Hlavin EM, et al (2004) The central role of perilipin A in lipid metabolism and adipocyte lipolysis. IUBMB Life 56:379–385

209. Sun Z, Gong J, Wu H, et al (2013) Perilipin1 promotes unilocular lipid droplet formation through the activation of Fsp27 in adipocytes. Nat Commun. doi: 10.1038/ncomms2581

210. Stenson BM, Rydén M, Venteclef N, et al (2011) Liver X Receptor (LXR) regulates human adipocyte lipolysis. J Biol Chem 286:370–379 . doi: 10.1074/jbc.M110.179499

211. Mastrogiannaki M, Lichtenberger BM, Reimer A, et al (2016) β-Catenin Stabilization in Skin Fibroblasts Causes Fibrotic Lesions by Preventing Adipocyte Differentiation of the Reticular Dermis. J Invest Dermatol 136:1130–1142 . doi: 10.1016/j.jid.2016.01.036

212. Öst A, Örtegren U, Gustavsson J, et al (2005) Triacylglycerol is synthesized in a specific subclass of caveolae in primary adipocytes. J Biol Chem 280:5–8 . doi: 10.1074/jbc.C400429200

213. Briand N, Prado C, Mabilleau G, et al (2014) Caveolin-1 expression and cavin stability regulate caveolae dynamics in adipocyte lipid store fluctuation. Diabetes 63:4032–4044 . doi: 10.2337/db13-1961

214. Catalán V, Gómez-Ambrosi J, Rodríguez A, et al (2008) Expression of caveolin-1 in human adipose tissue is upregulated in obesity and obesity-associated type 2 diabetes mellitus and related to inflammation. Clin Endocrinol (Oxf) 68:213–219 . doi: 10.1111/j.1365-2265.2007.03021.x

215. Giusti V, Verdumo C, Suter M, et al (2003) Expression of peroxisome proliferator-activated receptor-gamma1 and peroxisome proliferator-activated receptor-gamma2 in visceral and subcutaneous adipose tissue of obese women. Diabetes 52:1673–1676

216. Tanaka T, Yoshida N, Kishimoto T, Akira S (1997) Defective adipocyte differentiation in mice lacking the C/EBPbeta and/or C/EBPdelta gene. EMBO J 16:7432–43 . doi: 10.1093/emboj/16.24.7432

217. Muruganandan S, Roman AA, Sinal CJ (2009) Adipocyte differentiation of bone marrow-derived mesenchymal stem cells: Cross talk with the osteoblastogenic program. Cell. Mol. Life Sci. 66:236–253

218. Yang HN, Park JS, Woo DG, et al (2011) C/EBP-?? and C/EBP-??-mediated adipogenesis of human mesenchymal stem cells (hMSCs) using PLGA nanoparticles complexed with poly(ethyleneimmine). Biomaterials 32:5924–5933 . doi: 10.1016/j.biomaterials.2011.04.072

219. Garcés C, Ruiz-Hidalgo MJ, Bonvini E, et al (1999) Adipocyte differentiation is modulated by secreted delta-like (dlk) variants and requires the expression of membrane-associated dlk. Differentiation 64:103–114 . doi: 10.1007/s002580050265

220. Mei B, Zhao L, Chen L, Sul HS (2002) Only the large soluble form of preadipocyte factor-1 (Pref-1), but not the small soluble and membrane forms, inhibits adipocyte differentiation: role of alternative splicing. Biochem J 364:137–44

221. Mortensen SB, Jensen CH, Schneider M, et al (2012) Membrane-Tethered Delta-Like 1 Homolog (DLK1) Restricts Adipose Tissue Size by Inhibiting Preadipocyte Proliferation. Diabetes 61:

222. Sul HS (2009) Minireview: Pref-1: Role in Adipogenesis and Mesenchymal Cell Fate. Mol Endocrinol 23:1717–1725 . doi: 10.1210/me.2009-0160

223. Wang Y, Kim K-A, Kim J-H, Sul HS (2006) Pref-1, a Preadipocyte Secreted Factor That Inhibits Adipogenesis. Jounal Nutr 136:2953–2956 . doi: 136/12/2953 [pii]

224. Gupta RK, Arany Z, Seale P, et al (2010) Transcriptional control of preadipocyte determination by Zfp423. Nature 464:619–623 . doi: 10.1038/nature08816

225. Gupta RK, Mepani RJ, Kleiner S, et al (2012) Zfp423 expression identifies committed preadipocytes and localizes to adipose endothelial and perivascular cells. Cell Metab 15:230–239 . doi: 10.1016/j.cmet.2012.01.010

226. Longo M, Raciti GA, Zatterale F, et al (2017) Epigenetic modifications of the Zfp / ZNF423 gene control murine adipogenic commitment and are dysregulated in human hypertrophic obesity. 4:

227. Meegalla RL, Billheimer JT, Cheng D (2002) Concerted elevation of acyl-coenzyme A:diacylglycerol acyltransferase (DGAT) activity through independent stimulation of mRNA expression of DGAT1 and DGAT2 by carbohydrate and insulin. Biochem Biophys Res Commun 298:317–323 . doi: 10.1016/S0006-291X(02)02466-X

228. Lopes JLS, Nobre TM, Cilli EM, et al (2014) Deconstructing the DGAT1 enzyme: Binding sites and substrate interactions. Biochim Biophys Acta - Biomembr 1838:3145–3152 . doi:

10.1016/j.bbamem.2014.08.017

229. Loftus TM (2000) Reduced Food Intake and Body Weight in Mice Treated with Fatty Acid Synthase Inhibitors. Science (80-) 288:2379–2381 . doi: 10.1126/science.288.5475.2379

230. Padilla-Benavides T, Velez-delValle C, Marsch-Moreno M, et al (2016) Lipogenic Enzymes Complexes and Cytoplasmic Lipid Droplet Formation During Adipogenesis. J Cell Biochem 2315–2326 . doi: 10.1002/jcb.25529

231. Mayas MD, Ortega FJ, Macías-González M, et al (2010) Inverse relation between FASN expression in human adipose tissue and the insulin resistance level. Nutr Metab (Lond) 7:3 . doi: 10.1186/1743-7075-7-3

232. Kusakabe T, Maeda M, Hoshi N, et al (2000) Fatty acid synthase is expressed mainly in adult hormone-sensitive cells or cells with high lipid metabolism and in proliferating fetal cells. J Histochem Cytochem 48:613–622 . doi: 10.1177/002215540004800505

233. Merkel M, Eckel RH, Goldberg IJ (2002) Lipoprotein lipase: genetics, lipid uptake, and regulation. J Lipid Res 43:1997–2006 . doi: 10.1194/jlr.R200015-JLR200

234. Mead JR, Irvine SA, Ramji DP (2002) Lipoprotein lipase: structure, function, regulation, and role in disease. J Mol Med (Berl) 80:753–69 . doi: 10.1007/s00109-002-0384-9

235. Zimmermann R (2004) Fat Mobilization in Adipose Tissue Is Promoted by Adipose Triglyceride Lipase. Science (80-) 306:1383–1386 . doi: 10.1126/science.1100747

236. Bézaire V, Langin D (2009) Regulation of adipose tissue lipolysis revisited. Proc Nutr Soc 68:350–60 . doi: 10.1017/S0029665109990279

237. Brasaemle DL (2007) *Thematic review series: Adipocyte Biology* . The perilipin family of structural lipid droplet proteins: stabilization of lipid droplets and control of lipolysis. J Lipid Res 48:2547–2559 . doi: 10.1194/jlr.R700014-JLR200

238. Haemmerle G, Zimmermann R, Hayn M, et al (2002) Hormone-sensitive lipase deficiency in mice causes diglyceride accumulation in adipose tissue, muscle, and testis. J Biol Chem 277:4806–4815 . doi: 10.1074/jbc.M110355200

239. Haemmerle G, Lass A, Zimmermann R, et al (2006) Defective lipolysis and altered energy metabolism in mice lacking adipose triglyceride lipase. Science 312:734–7 . doi: 10.1126/science.1123965

240. Bezaire V, Mairal A, Ribet C, et al (2009) Contribution of adipose triglyceride lipase and hormone-sensitive lipase to lipolysis in hMADS adipocytes. J Biol Chem 284:18282–91 . doi: 10.1074/jbc.M109.008631

241. Bezaire V, Langin D (2009) Regulation of adipose tissue lipolysis revisited. Proc Nutr Soc 68:350–360 . doi: S0029665109990279 [pii]\r10.1017/S0029665109990279
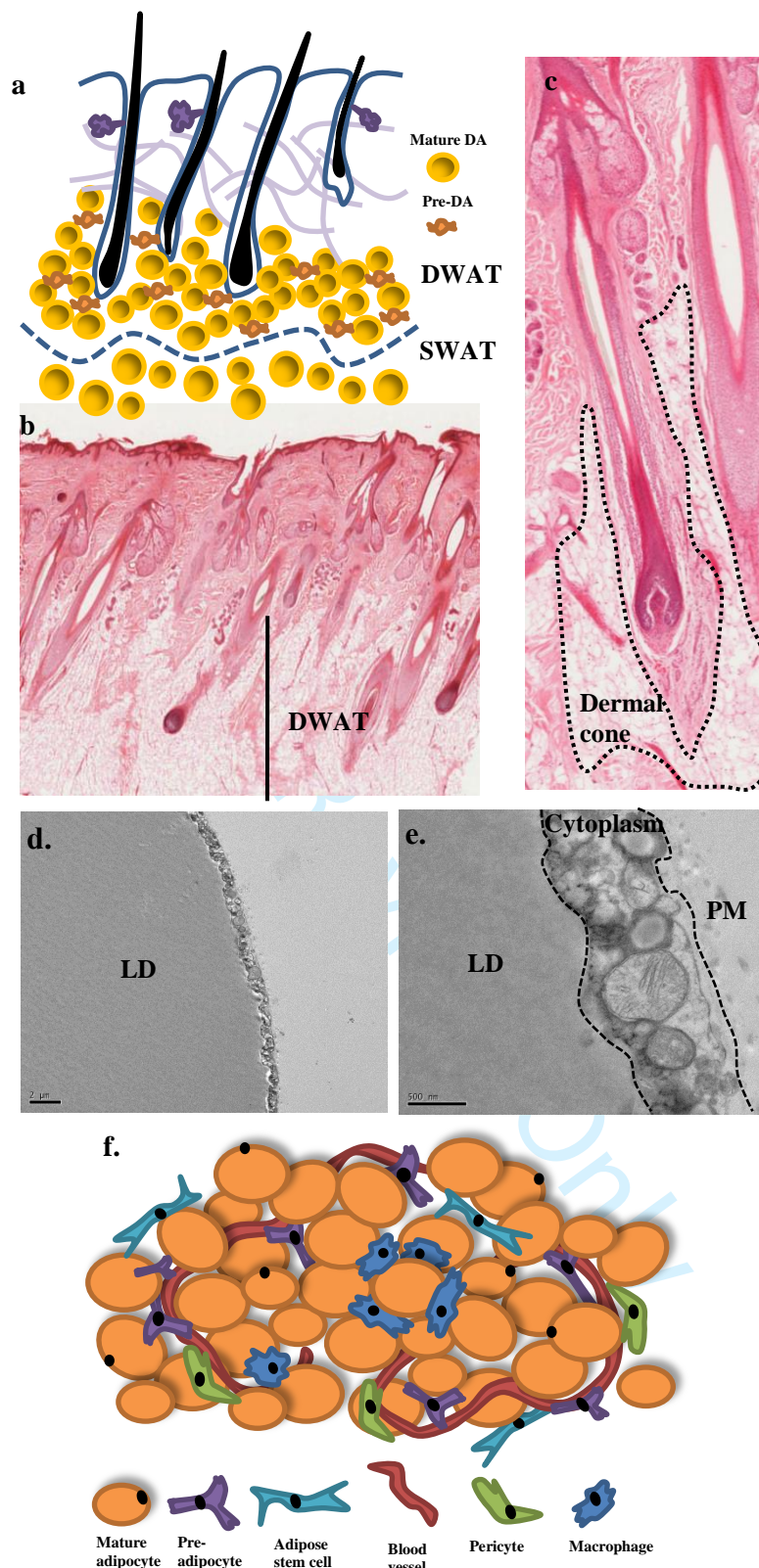
Figure 1. Structure of dermal white adipose tissue and its components. Whilst murine DWAT is divided from subcutaneous adipocytes by the *panniculus carnosus*, human DWAT is found as a continuous layer in the human dermis (a) with no equivalent muscle structure separating it from subcutaneous white adipose 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).
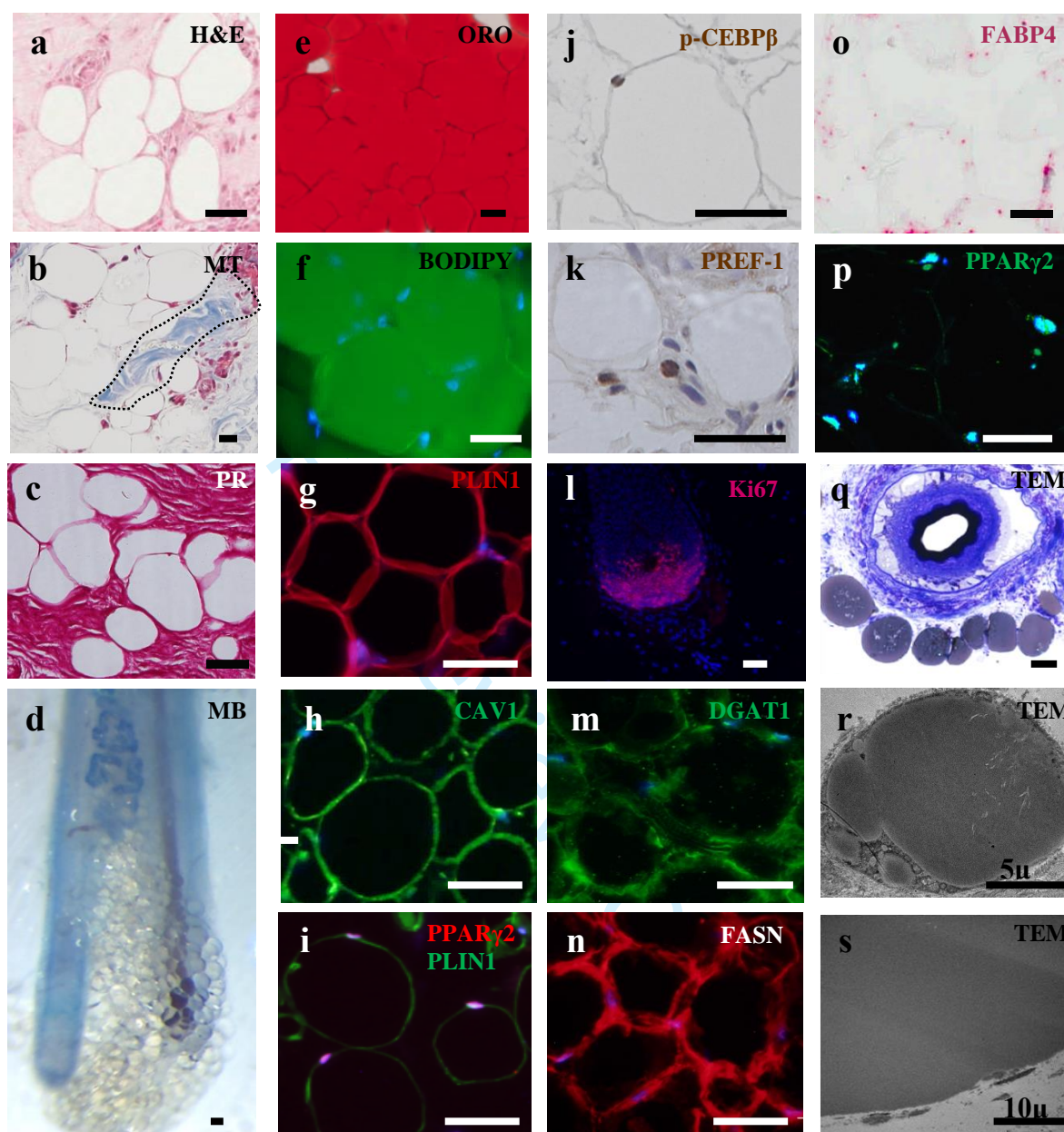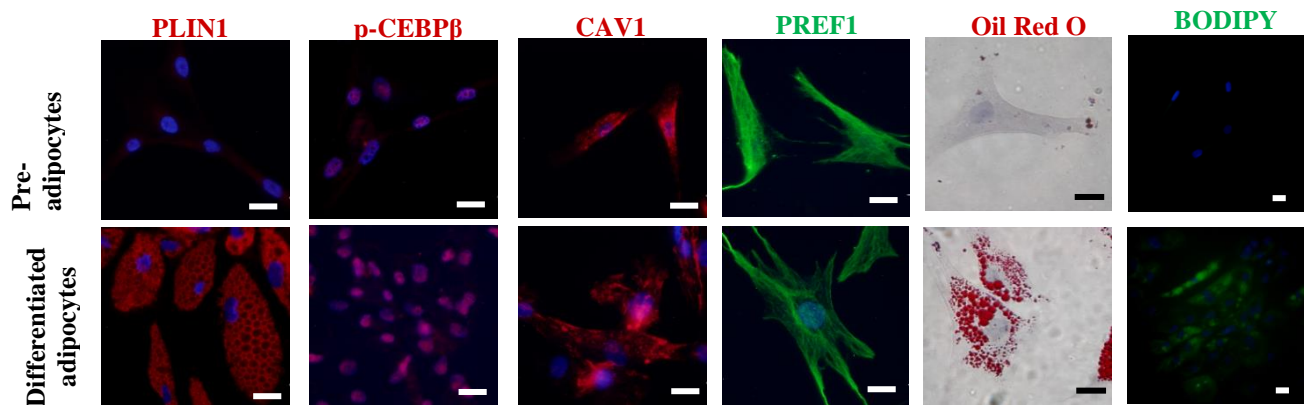
Figure 2. Stains for observing and quantifying human dermal white adipose tissue *in situ*. Adipocytes can be examined using histological stains such as (a) Haematoxylin and Eosin (H&E), (b) Masson's Trichrome, (b) Picrosirius Red and (d) Methylene blue (MB). The lipid within DAs can be measured using (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) diacyglycerol 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-CEPBβ, 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 1. A guide to choosing the right processing route and stain for analysing dermal adipocytes.
LD – lipid droplet; BC – breast carcinoma; BF – brightfield; CFM –confocal microscopy; DA – dermal adipocyte; FB – fibroblasts; FFPE – formalin fixed paraffin embedded; H&E – haematoxylin and eosin; IFM – immunofluorescence microscopy; KC - keratinocyte; LD – lipid droplet; MP – macrophage; PC- prostate carcinoma; SC – stratum corneum; SG – sebaceous gland; TEM – transmission electron microscopy

What should I choose when studying…?	How to process the tissue	Stains/ Markers	Target / Antigen Localization	Positive Control Tissue	Imaging and Analysis	References
DWAT Morphology	FFPE Cryo	H&E	n/a	n/a	BF	[68, 69, 194]
Lipid droplet content and Morphology	Cryo	Oil Red O, Nile Red	LD	SG, SC, Breast	BF	[3, 7, 105, 115, 140, 148, 183, 194–196]
		BODIPY, LipidTOX	LD	SG, SC, Breast	IFM CFM	
Mature Adipocyte Adipocyte size and number	FFPE Cryo	PLIN1	LD Membrane	SC, BC	BF/IFM	[7, 101, 108, 163-166, 169-173]
		CAV1	Cell membrane	KCs, FBs, HeLa Cells		
		FABP4	Cytoplasm	KCs, Lymph node		
		CPM	Cell membrane	All cells, Any tissue	IFM	
		H&E	n/a	n/a	BF	
		DGAT1/2	Cytoplasm - ER	KCs, Colon	BF/IFM	
		FASN	Cytoplasm	KCs, PC, BC		
		LPL	Membrane	Intestine		
Adipocyte differentiation	FFPE Cryo	pC/EBPβ	Nucleus	SG, BC	BF/IFM	[7, 195]
		PPARγ	Nucleus	SG, BC		
Pre-adipocytes	FFPE Cryo	Pref1	Membrane / Nucleus	Placenta	BF/IFM	[195, 197]
		Zfp521	Nucleus	Bone		
Adipocyte apoptosis	FFPE Cryo	PLIN1	LD Membrane	SC, BC	Loss of Perilipin1	[27, 194, 198]
		CAV1	Cell membrane	KCs, FBs, HeLa Cells	Loss of Caveolin1	
Pre-adipocyte proliferation	FFPE Cryo	Pref1 + Ki67/ BrdU/ EdU	Nucleus	Placenta	BF/IFM	[7, 115, 195]
Lipid synthesis (lipogenesis)	Unfixed tissue	BODIPY C1/C12	Cytoplasm - ER	Adipocytes	IFM	[132, 134, 199, 200]
	FFPE Cryo	Mature DA stain to meaure change in size (see above)	Various	Dependent on stain chosen	BF/IFM	See section above

| Lipid metabolism (lipolysis)	FFPE Cryo	phospho-Perilipin1	Cytoplasm	Adipocyte cell line stimulated to undergo lipolysis	BF/IFM	[127, 201, 202]
		phospho-HSL	Cytoplasm			
Adipocyte Ultrastructure	EM-specific	OsO4 Uranyl Acetate	Only for immuno-EM	n/a	TEM	[203] [109, 144, 148, 193, 204] [176]
3D Volume and Thickness	As per MRI/μCT protocol	Various	n/a	n/a	MRI μCT	[150–157, 160–164]
mRNA	FFPE Cryo	Fluorescent or chromogenic ISH	Cytoplasm	Dependent upon gene of interest PPIB; HeLa cells	ISH	[139] [205]
Fibrosis	FFPE	Masson's Trichrome/ Picrosirius Red	Fibrillary Collagens I and III	n/a	BF	[70, 206, 207]
		Methylene Blue	Dermal components other than DAs	n/a		

Table 2. Markers of human dermal adipocytes and their functional importance.

| Marker	Target Localization	Function	Murine References	Human References
Perilipin1	LD membrane ER	Master regulator of lipolysis Upon phosphorylation by PKA, Perilipin1 allows HSL to translocate to the adipocyte lipid droplet and commence lipolysis	[7, 69, 140, 195, 196, 207, 208] [209]	[102, 170, 176, 210, 211]
Caveolin1	Plasma membrane	Endo- and exocytosis Component of caveolae which are sites of triacylglycerol synthesis	[212, 213]	[214]
PPARγ2	Nucleus	Master regulator of adipogenesis	[95]	[102, 215]
pC/EBPβ	Nucleus	Promotes terminal adipocyte differentiation together with C/EBPα	[216]	[217, 218]
Pref1/Dlk1	Plasma Membrane	Inhibits pre-adipocyte proliferation in its membrane-bound form Prevents adipocyte differentiation (adipogenesis)	[99, 219–223]	[100–102]
Zfp423/ Znf521	Nucleus	Induces PPARγ expression thus initiating preadipocyte commitment	[224–226]	[226]
DGAT 1/2	Cytoplasm - ER	Catalyses addition of third fatty acid to diacylglycerol	[119, 227, 228]	[120]
FASN	Cytoplasm	Uses acetylCoA, malonyl-CoA, and NADPH for *de novo* synthesis of long chain saturated fatty acids.	[229, 230]	[120, 231, 232]
LPL	Adipocyte plasma membrane	Hydrolyses lipoproteins at capillary endothelium that are triglyceride-rich to generate NEFA	[233, 234]	[120, 234]
ATGL	LD membrane	Hydrolyses TAG to DAG	[235–237]	[236, 237]
HSL	Cytosol, LD membrane	Hydrolyses TAG to DAG, and DAG to MAG	[237–240]	[237, 241]
MGL	Cytosol	Hydrolyses MAG to glycerol and and a fatty acid.	[236, 237]	[237]

Box 1. Pitfalls and tricks when working with dermal adipose tissue.

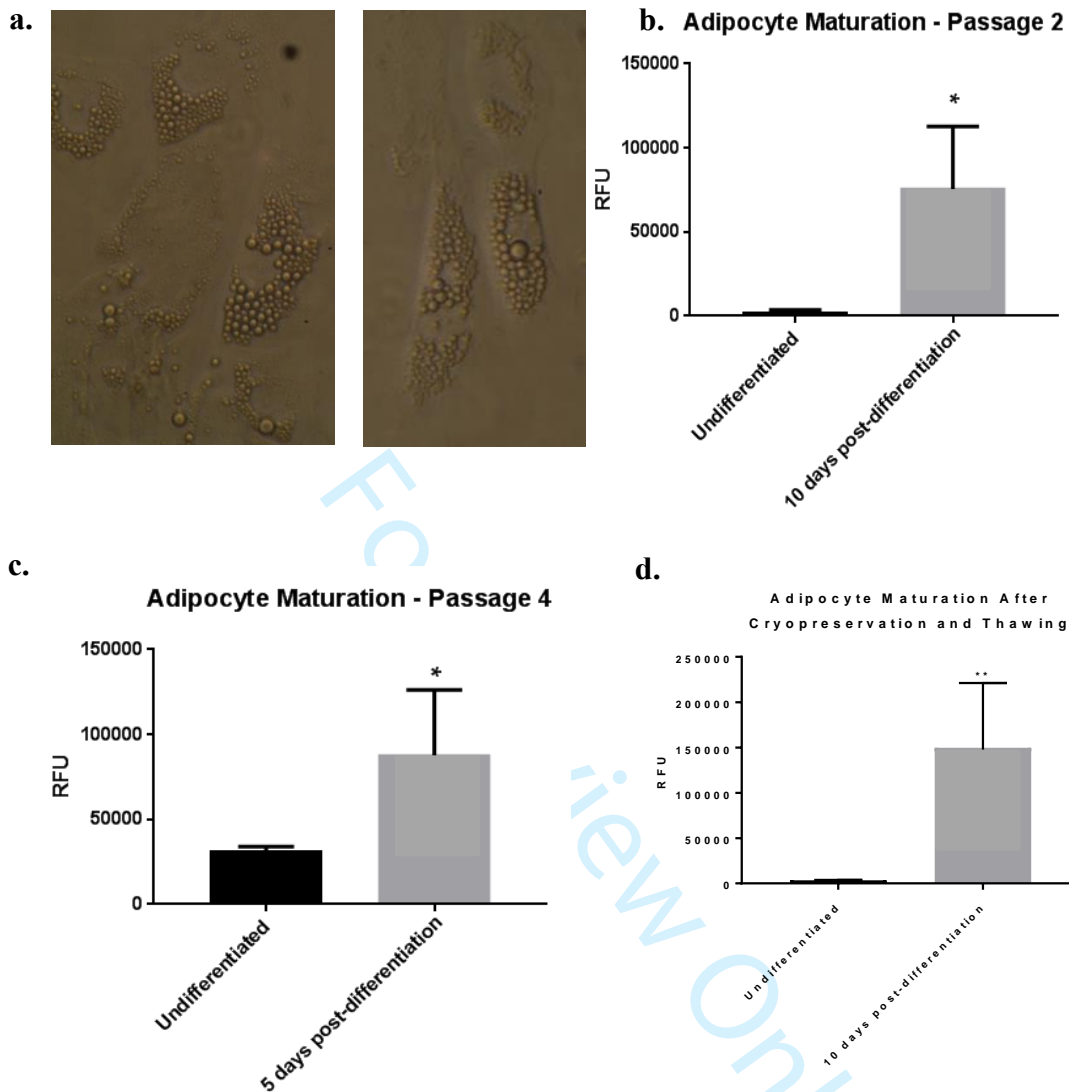| Issue	Solution(s)
Poor quality DWAT in cryosections	• **Cut thicker sections** • **Lower the cryostat temperature to -35 degrees** • **Gently apply coverslips upon tissue sections**
Lipid is displaced from adipocytes in cryosections	• **Lower the cryostat temperature to -35 degrees** • **Store slides 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
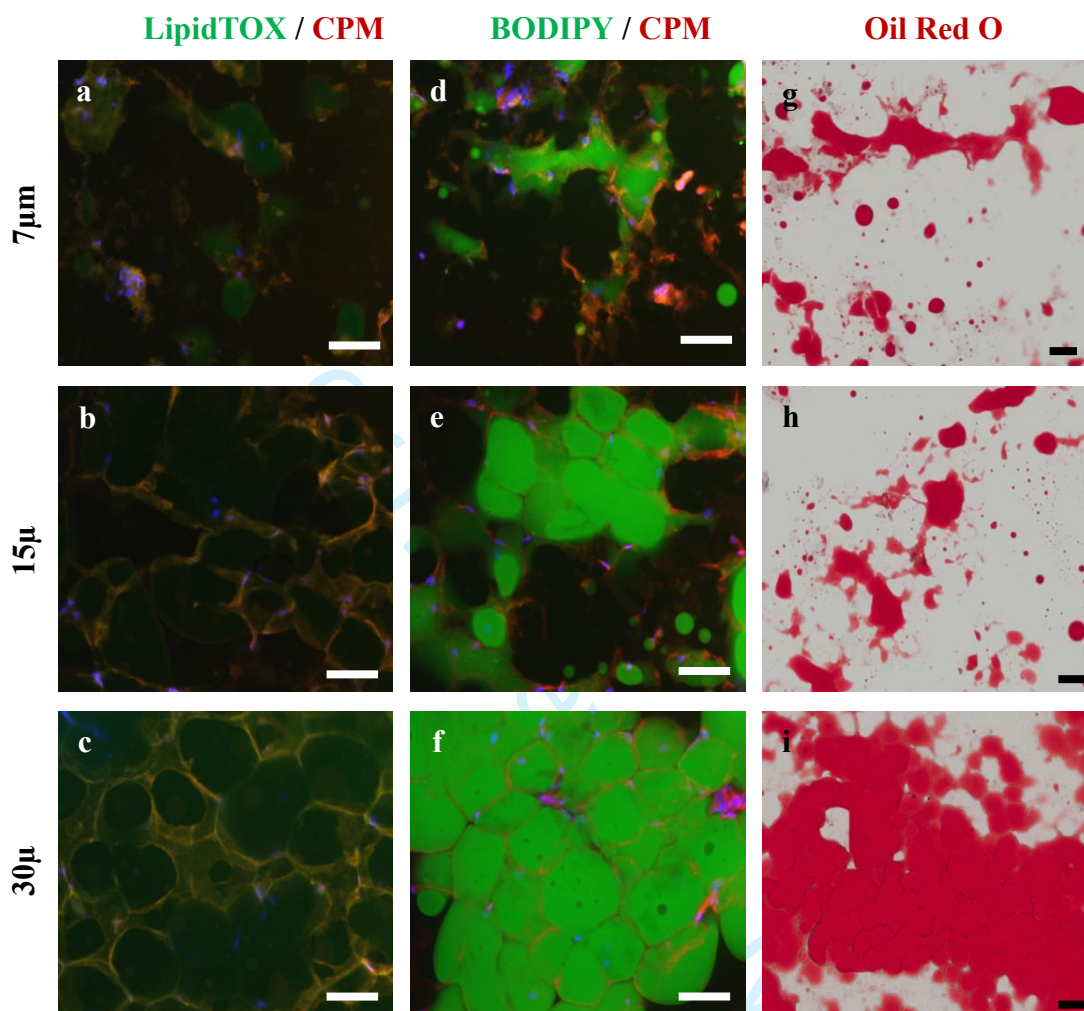
Suppl. Fig. 1. Section thickness determines lipid droplet content and morphology in dermal adipocytes. Although integrity of adipocyte cytoplasm and plasma membrane is maintained in samples sectioned at 7um, most of the lipid droplet remains intact only when cutting the tissue at a thickness of 15-30μm 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.
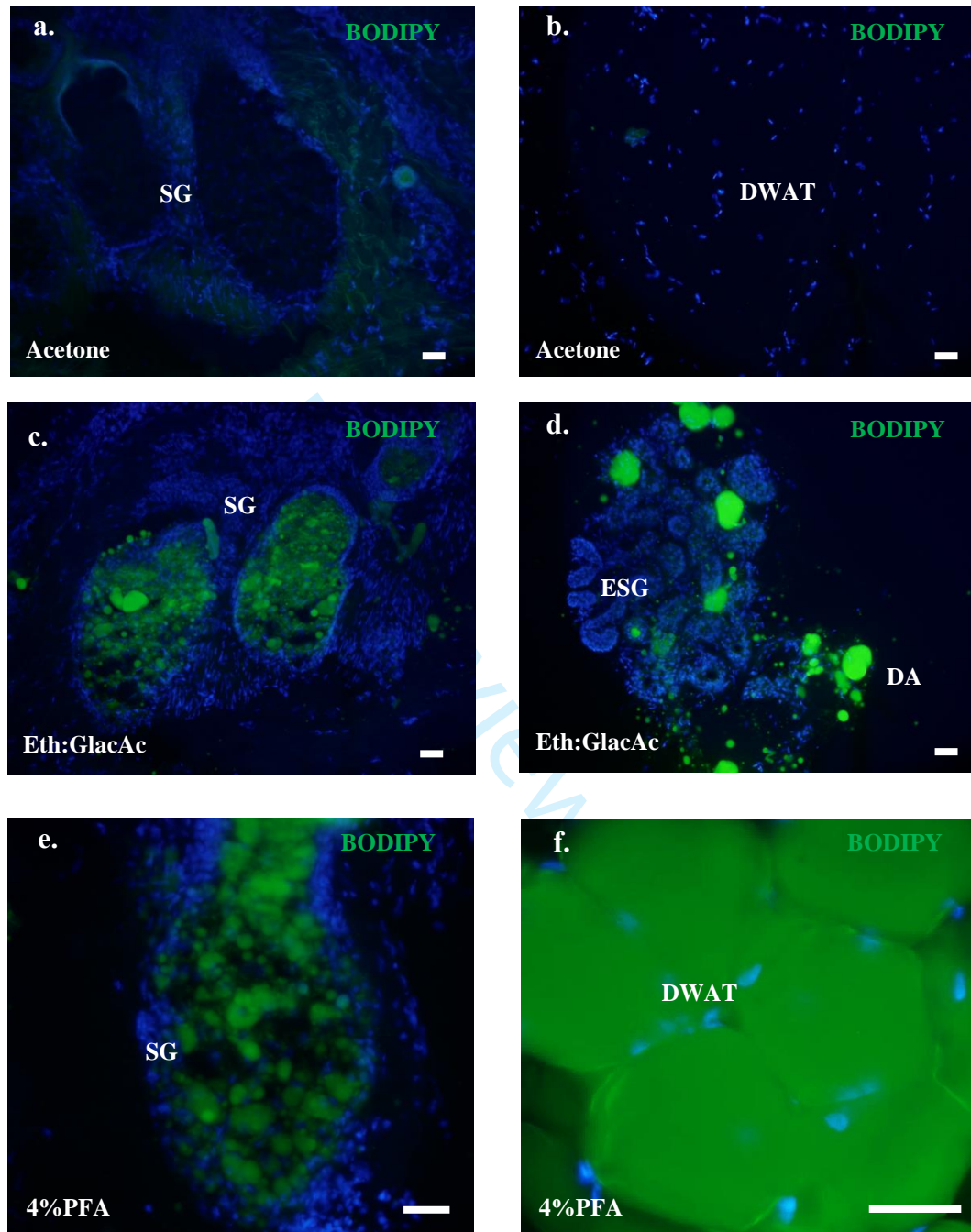
Suppl. Fig. 2. Effect of fixation upon lipid content of human dermal adipocytes. The choice of fixative has a great impact upon the lipid content of human dermal adipocytes. Whilst acetone displaces the lipid in the sebaceous gland (SG) and DWAT (a, b) thus making it an unsuitable choice for fixation 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.

a

b

c
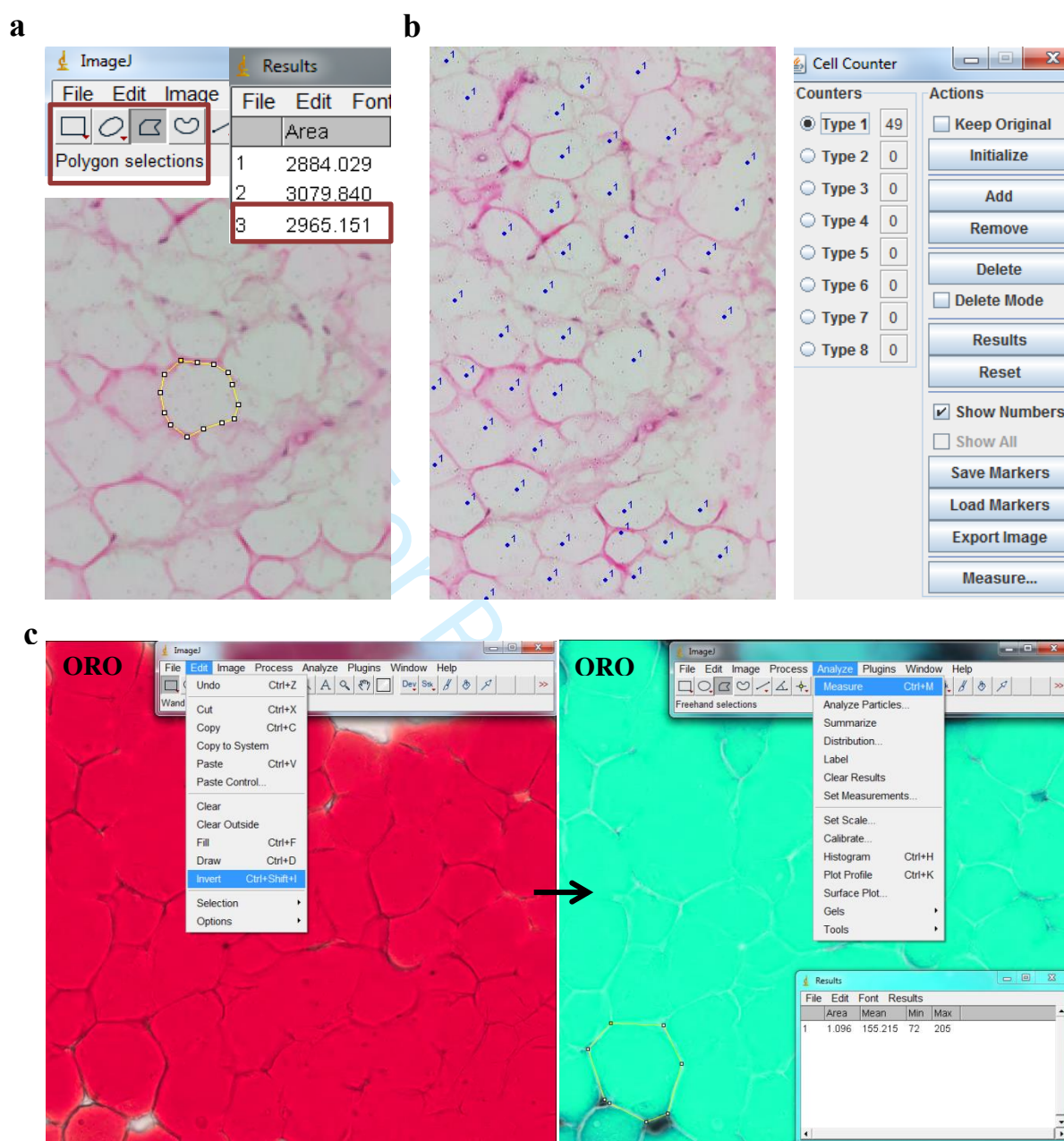
Suppl. Fig. 3. Quantifying adipocyte size/volume and lipid content in ImageJ. Different tools can be employed to measure adipocyte size/volume in ImageJ (a) such as freehand selection, elliptical tool and polygon selection. We recommend utilizing the polygon selection tool (a) to measure adipocyte size/volume, 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.

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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).
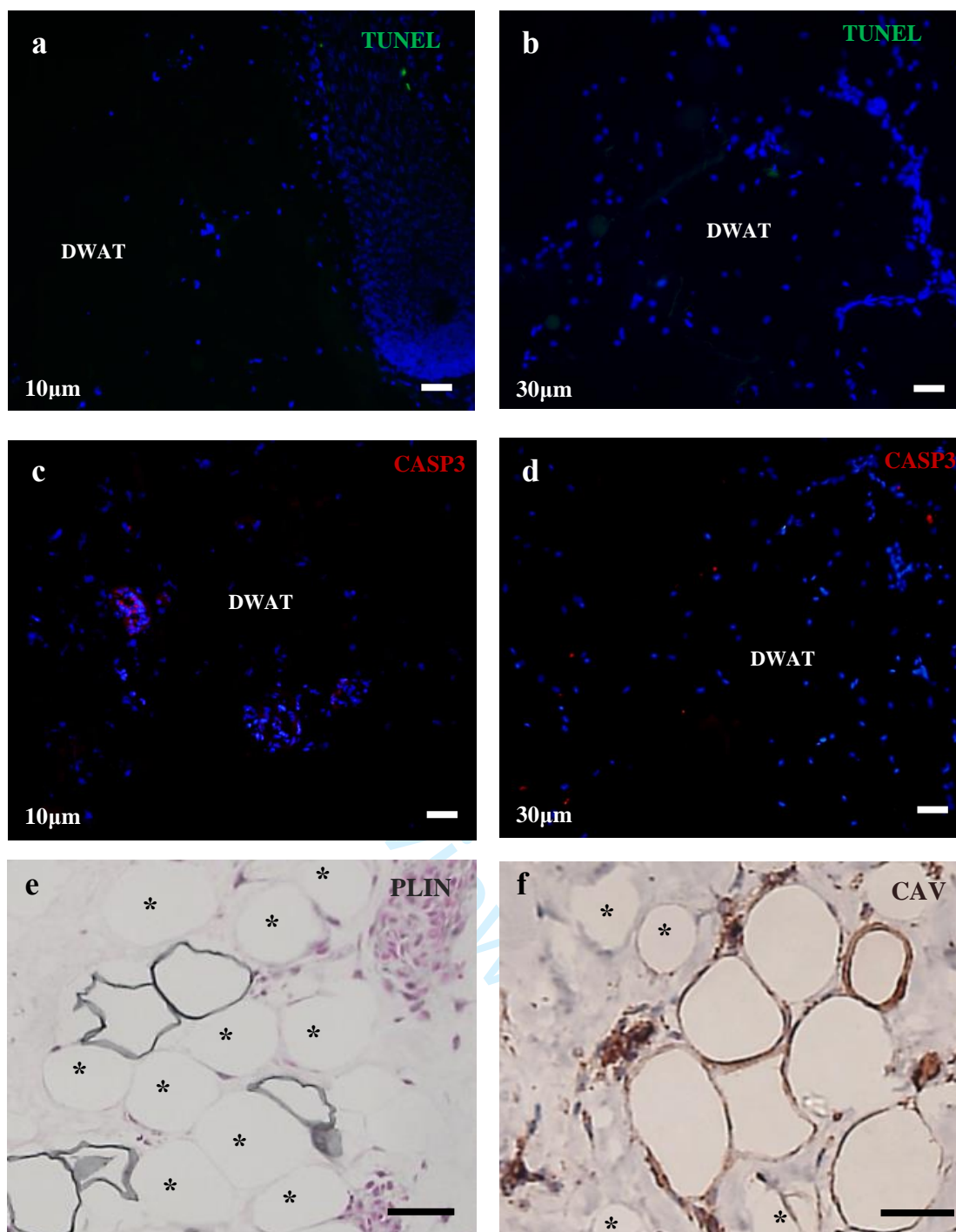
Suppl. Fig. 5. Cell death of skin adipocytes. Conventional methods for detecting apoptosis such as TUNEL (a,b) and Cleaved Caspase 3 (CASP3) (c,d) show no cell death within human DAs. Instead, apoptosis can be measured via different approaches, for example by measuring loss of Perilipin1 (c) or Caveolin1 (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.
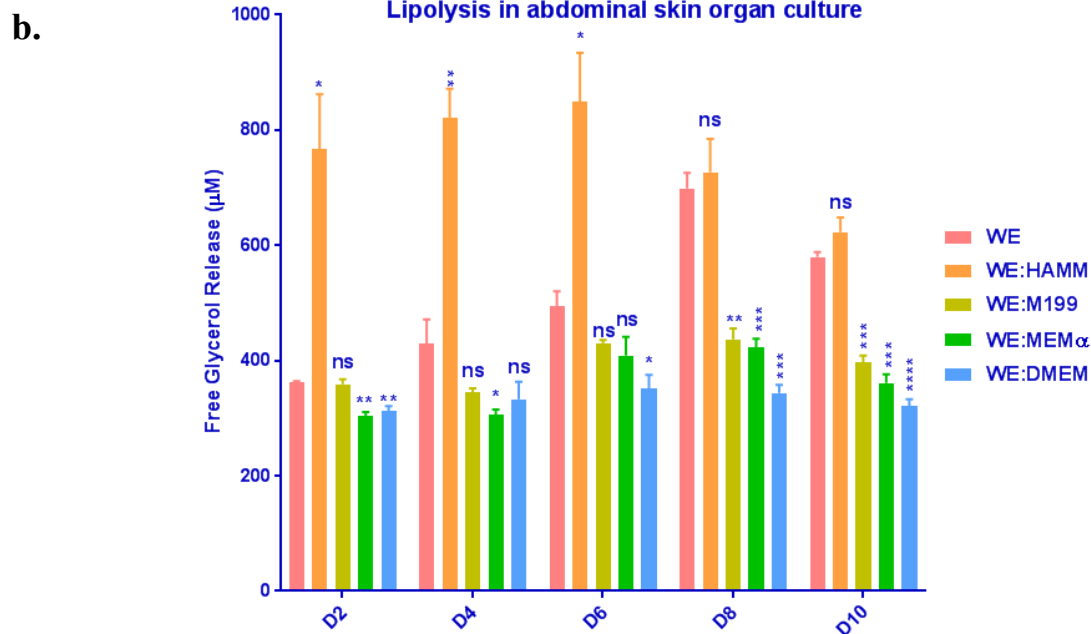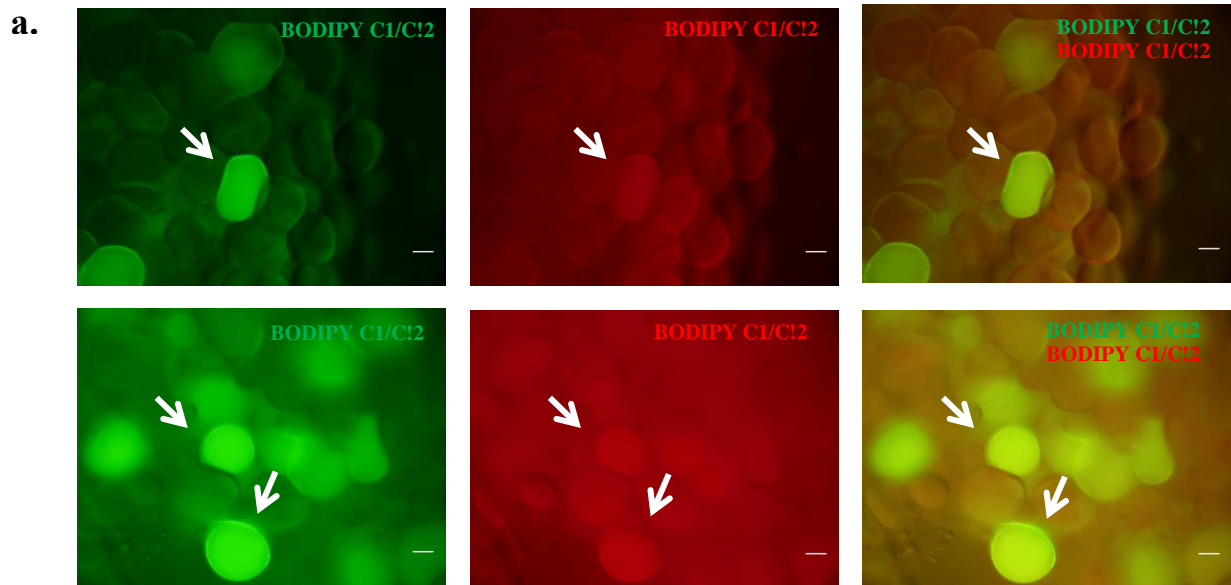
Suppl. Fig. 6. Measuring lipogenesis and lipolysis in human DWAT *ex vivo*. One of the methods that can be used to assess fatty acid uptake *ex vivo* is by adding BODIPY C1/C12 (green or red) to the media of abdominal skin organ culture (a). For instance, BODIPY C1/C12 can be added to adipocyte culture 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.
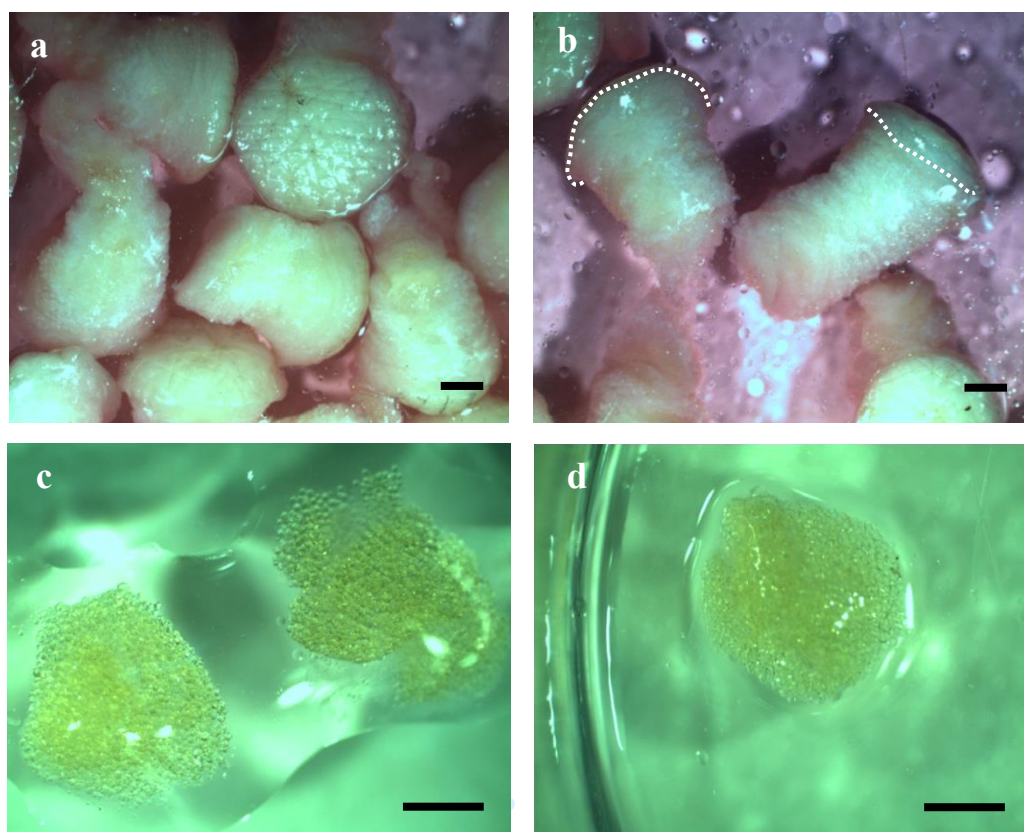
Suppl. Fig. 7 Culturing human DWAT *ex vivo*. Several methods can be used to culture human DWAT *ex vivo* such as 4mm punch biopsies collected from human abdominal skin (a,b) or human DWAT carefully dissected from scalp skin. Depending on the depth of the punch biopsy, significant amounts of subcutaneous 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