A practical guide for the study of human and murine sebaceous glands *in situ*

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Abstract: The skin of most mammals is characterised by the presence of sebaceous glands (SGs), whose predominant constituent cell population is sebocytes, that is, lipid-producing epithelial cells, which develop from the hair follicle. Besides holocrine sebum production (which contributes 90% of skin surface lipids), multiple additional SG functions have emerged. These range from antimicrobial peptide production and immunomodulation, via lipid and hormone synthesis/metabolism, to the provision of an epithelial progenitor cell reservoir. Therefore, in addition to its involvement in common skin diseases (e.g. acne vulgaris), the unfolding diversity of SG functions, both in skin health and disease, has raised interest in this integral component of the pilosebaceous unit. This practical guide provides an introduction to SG biology and to relevant SG histochemical and immunohistochemical techniques, with emphasis placed on *in situ* evaluation methods that can be easily employed. We propose a range of simple, established markers, which are particularly instructive when addressing specific SG research questions in the two most commonly investigated species in SG research, humans and mice. To facilitate the development of reproducible analysis techniques for the *in situ* evaluation of SGs, this methods review concludes by suggesting quantitative (immuno-)histomorphometric methods for standardised SG evaluation.

Key words: human – immunohistochemistry – mouse – quantification – sebaceous gland

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Introduction

Interest in the sebaceous gland (SG) has long been overshadowed by the prominent clinical role of this important skin appendage in acne vulgaris (1–3). However, beyond the mere production of sebum, the SG also possesses complex metabolic, (neuro)endocrine and immunological functions (4–9) and acts in the context of the skin neuroendocrine system (10–16) to maintain and regulate local homoeostasis (14,17). The SG is one of the most intriguing mammalian skin structures as it can serve as a model for studying numerous central life sciences issues, for example in cell biology and in skin, lipid or hormone research (18–22). Moreover, the SG is unique in at least two ways: its main cellular component, sebocytes, is of epithelial origin, yet engage in lipid production and metabolism, a feature usually associated with adipocytes; and sebum is produced by holocrine secretion, that is, via an unusual mode of cell suicide that sebocytes undergo (21,23–25).

Whilst there are many reviews on SG biology and function (8,18,20,22,26–29), there are no recent methodological reviews, targeted to newcomers, which explain how to professionally examine the SG *in situ*. Therefore, this review provides a succinct introduction into essentials of SG biology and offers hands-on guidance for beginners in SG research on relevant *in situ* evaluation methods, focussing on histochemistry and immunohistochemistry of human and murine SGs; the two most commonly investigated species in SG research.

Just as concerted efforts are being made to develop a universal grading scale for the clinical presentation of the main SG disorder,

acne vulgaris (30), it is equally important to develop standardised techniques for the histological evaluation of SG parameters in human and murine skin. Therefore, we also encourage the standardisation of histomorphometric techniques to evaluate changes in SG size/activities, thus hoping to improve the inter-observer reproducibility of published histological data, for example in trials of SG disorders (31). The techniques described here are transferable to isolated/specialised SGs and are relevant to other SG research models, for example hamster SGs and cultured sebocytes (24,25,32).

Overview of SG biology

Sebaceous glands are prominent in the skin of most mammals, including humans and rodents. Most SGs are located in the mid-dermis in association with a hair follicle, into whose (follicular) canal the sebaceous duct inserts, thus forming an integral part of the pilosebaceous unit. Just as other skin appendages, most notably the hair follicle, the SG is supported and surrounded by a dense, collagen-rich stroma (18), for which we propose, in analogy to the hair follicle stroma, the term 'SG connective tissue sheath' (SG-CTS). In some regions, the pilosebaceous unit is complemented by the apocrine gland (18,29,33), whose duct inserts into the follicular canal above that of the SG (Fig. 1). In mice, the first sebocytes of the SG begin to appear at stage 5 of hair follicle morphogenesis (approximately days 3–4 post-partum in non-guard pelage hair follicles) and begin to form the SG at morphogenesis stage 6 around one day later. By stage 7, the SG is enlarged and begins to produce sebum (usually, this is achieved
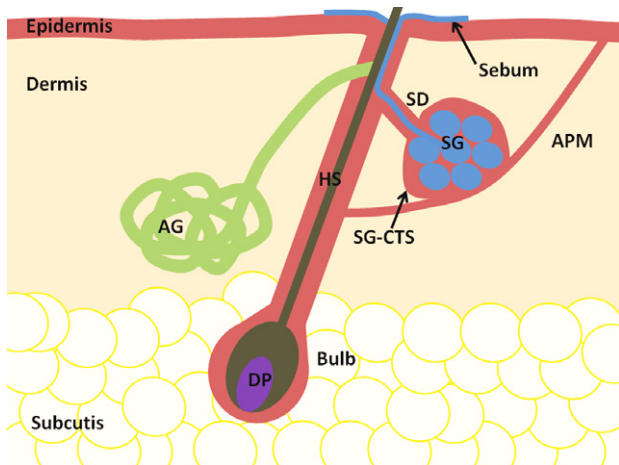
Figure 1. The Pilosebaceous Unit. The pilosebaceous unit consists of the hair follicle, the sebaceous gland, and in some regions, the apocrine gland. When in association with a hair follicle, sebum is secreted from the sebaceous gland to the cell surface via the sebaceous duct and follicular canal. Key: AG, Apocrine Gland; APM, Arector Pili Muscle; DP, Dermal Papilla; HS, Hair Shaft; SD, Sebaceous Duct; SG, Sebaceous Gland; SG-CTS, Sebaceous Gland Connective Tissue Sheath. To view the full-page figure, please see the Supporting Information.

Figure 2. Histochemical Stains for Analysis of the Sebaceous Glands. Histochemical staining of the SG in humans and mice. Oil Red O staining and Sudan Black B staining require the use of cryosections – paraffin sections are not appropriate for these staining techniques. Note that with the Oil Red O and Sudan stains, distinguishing individual cells is difficult, and that staining intensity of sebum within the sebaceous duct is often stronger than the sebocytes within the gland (image E). Key: HF, Hair follicle; SC, Subcutis; SD, Sebaceous duct; SG, Sebaceous gland. (A) Haematoxylin & Eosin, human; (B) Haematoxylin & Eosin, mouse; (C) Periodic Acid-Schiff, human; (D) Periodic Acid-Schiff, mouse; (E) Oil Red O, human; (F) Oil Red O, mouse; (G) Sudan Black B, human; (H) Sudan Black B, mouse; (a) sebocyte membrane highlighted with PAS; (b) SG basement membrane highlighted with PAS; (c) SG-CTS highlighted with PAS. Scale bars: A, C–D, F–H = 50 μm; B = 10 μm; E = 200 μm. To view the full-page figure, please see the Supporting Information.
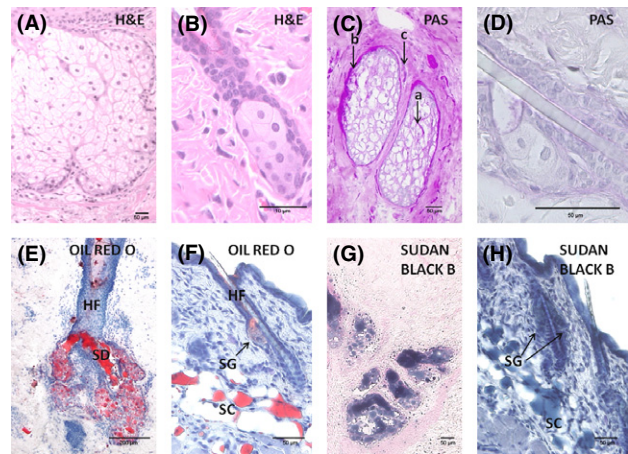
by day 5 post-partum) (34). Only in selected regions of human skin, SGs are connected directly to the epidermal surface (18,21,28) (see the Supporting Information for further details). Free SGs are also found in pheromone-producing rodent preputial glands (29).

Although previously presumed to be an 'atavistic' structure with 'absent, unimportant or unrecognisable' functions in man (35) or 'a living fossil with a past but no future' (36), there is now compelling evidence to the contrary. The SG plays important roles in skin barrier function, immunomodulation, antimicrobial peptide production, lipid metabolism and various signalling pathways within the skin, including steroid and peptide hormone synthesis and metabolism (4–8,14,17,20,28,29,31,37–51).
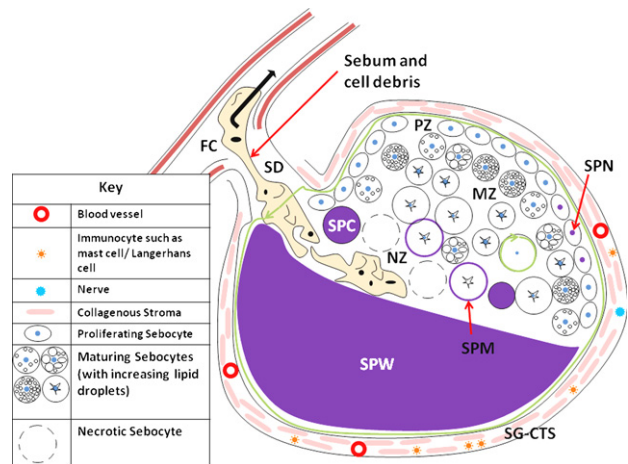
Just as the hair follicle epithelium (52), the SG may even have established a functional hypothalamus-pituitary-adrenal (HPA) axis equivalent (29), which mimics aspects of the cutaneous HPA axis equivalent (53). For example, evidence of proopiomelanocortin, β-endorphin and corticotrophin-releasing-hormone signalling pathways has been reported in both the hair follicle and SG (54–57). These examples substantiate the hypothesis of a functional skin HPA axis equivalent proposed in 1996 (58). Furthermore, the SG and its SG-CTS harbour important immunocytes, for example Langerhans cells, macrophages and T cells (18,59) as well as different progenitor cell populations (23,60). Contrary to the popular belief that SGs are scarcely innervated (18), more recent research has shown that SGs can show rich sensory innervation, namely in facial skin and acne patients (61).

Sebocytes

The predominant constituent cell population of the SG is the sebocyte, whose most recognised role is the production of sebum. Within skin epithelium, sebocytes are easily identified by their large size and 'foamy' appearance (Fig. 2) resulting from the production of intracellular lipid droplets (18,62). Having developed early during hair follicle morphogenesis, from intrafollicular epithelial progenitor cells (22,34), relatively undifferentiated sebocytes

Figure 3. Schematic Representation of Sebaceous Gland Structure and of the Various Staining Patterns Achieved by (Immuno)histochemistry. The sebaceous gland contains sebocytes of varying degrees of differentiation, with the outermost cell layer comprised of proliferating sebocytes, followed by maturing, differentiating sebocytes that acquire increasing numbers of lipid droplets; the central SG lobule then contains the fully differentiated/mature sebocytes undergoing holocrine secretion. Various staining patterns may be obtained when staining the SG by (immuno)histochemistry, namely nuclear, membranous, cytoplasmic or whole gland staining patterns (shown in purple). Green arrows represent the area to be included when measurements of gland area/sebocyte area are performed. Key: FC, Follicular Canal; MZ, Maturation Zone; NZ, Necrosis Zone; PZ, Peripheral Zone; SD, Sebaceous Duct; SG-CTS, Sebaceous Gland Connective Tissue Sheath; SPC, Staining Pattern Cytoplasmic; SPM, Staining Pattern Membranous; SPN, Staining Pattern Nuclear; SPW, Staining Pattern Whole gland; black arrow- direction of sebum flow from sebaceous duct to skin surface. To view the full-page figure, please see the Supporting Information.

in mature SGs are located in the outermost layer (peripheral zone), which shows the highest proliferative activity (Fig. 3). Moving towards the centre of the SG, the maturation zone contains

differentiating sebocytes, as evidenced by increasing numbers of intracellular lipid droplets (63). Sebocyte proliferation and differentiation are under tight hormonal control (e.g. stimulation by androgens and prolactin; inhibition by oestrogens and some vanilloids (7,27,38)) and are governed by selected protein kinase C isoforms (64) and peroxisome proliferator-activated receptor-mediated signalling (65–67). The centre of the gland forms the necrosis zone, containing terminally differentiated (mature) sebocytes. These are lipid-laden and feature pyknotic nuclei before cell rupture and dispersion of the cell contents, completing the process of holocrine secretion (20–22), a unique method of secretion employed by the SG (21,27). The mechanisms of holocrine secretion are still hypothetical, but include the lysis of sebocytes by hydrolytic enzymes within lysosomes (18), apoptosis-induced cell lysis (68) and/or the release of sebum via an ABCB1 transporter-dependent mechanism (24,25).

Sebum

The constituents of sebum are varied and include cell debris, various lipids (44,69) (Table S1), antimicrobial peptides, free fatty acids and histone H4 (43,46). In patients with acne vulgaris, it also contains matrix metalloproteinases (70).

Sebum is deposited onto the skin surface via the sebaceous duct and when associated with a hair follicle, the outwardly moving hair shaft serves as a wick that facilitates sebum dispersion (71) (Fig. 1). Once secreted, sebum forms the skin surface lipid film, providing a protective barrier against external insults (18,20,21,28,39).

Relevance in clinical dermatology

Acne vulgaris is the classic disease associated with SG dysfunction (1–3,27,31,72–75). The SG may also be involved in other disorders, for example the pathogenesis of cicatricial alopecia (76–78) and of chemotherapy-induced alopecia (79). Whilst they rarely give rise to benign/malignant tumors (a distinctive feature of Muir-Torre syndrome) (20,80–83), SG hyperplasia is a frequently encountered cosmetic nuisance (84).

An ever-increasing number of mouse mutants is being described that display a SG phenotype (22,39,76,85–87), suggesting that the range of skin disorders associated with SG dysfunction is wider than currently appreciated, possibly including androgenetic alopecia (20,88–90). Many of these conditions can be instructively studied using histological/immunohistological techniques. Thus, there are multiple biological and clinical reasons to develop a standardised methodology for assessing SGs *in situ* in humans and mice.

Practical considerations: Choosing the correct tissue processing and SG read-out parameters

When beginning any analysis of the SG, one must consider appropriate tissue processing, that is, cryo- versus paraffin embedding. Although paraffin-embedded tissue provides excellent morphological detail, the subsequent de-paraffinisation process removes SG lipids, and consequently, crucial lipid histochemical stains, for example Oil Red O, can no longer be performed. Therefore, cryosections are more versatile for SG analysis.

One particularly instructive analysis method that is now commonly employed in murine SG research is the epidermal whole mount technique (23,91,92). Used in conjunction with confocal microscopy, it provides excellent morphological detail of the SG, whilst maintaining tissue integrity and allows three-dimensional visualisation and measurement of the SG *in situ* in a large cutaneous field (this can be complemented with immunohistochemistry, with the number of markers which can be analysed within one sample being limited by the number of colour channels available).

Another major consideration is the selection of appropriate read-out parameters. In Table 1, we describe some of the frequently employed immunohistochemical (IHC) and immunofluorescent (IF) markers of differentiating sebocytes/sebocyte progenitor cells. In Table 2, we suggest some of the *in situ* techniques, which may be employed to address frequently asked, specific SG research questions. Used together, these tables provide detailed information on which commonly used markers of sebocytes and SGs are available, and on the conditions under which these may best be utilised.

Visualising the SG by routine histochemistry

Routine haematoxylin and eosin (H&E) histochemistry remains the mainstay of SG visualisation. In murine and human skin, sebocytes stand out as the largest intradermal cells with a 'foamy' phenotype (Fig. 2). H&E clearly outlines all components of the SG, its position within the skin, and the insertion of its duct into the hair follicle epithelium. This can be complemented by periodic acid-schiff (PAS) histochemistry, highlighting a) sebocyte membranes, b) the SG basement membrane zone, and c) the surrounding SG-CTS (Figs. 2C, D).

Giemsa and/or toluidine blue histochemistry offer a straightforward assessment of the inflammatory cell infiltrate within/around the SG. Where needed, this can then be followed up by immunohistology for the appropriate cluster of differentiation antigens (see e.g. 59). Due to space constraints, the current guide only covers sebocyte-related IHC/IF markers.

Basic quantitative morphometry of the SG

Because the SG is a multi-lobulated structure comprising sebocytes in several distinct stages of differentiation (18,22,63,93), there are limits to how well the SG can be visualised and quantified by two-dimensional morphometric techniques. Where confocal microscopy or three-dimensional ultrasound microscopy (94,95) are unavailable, standard morphometric techniques can only yield approximations of SG architecture. With this important caveat in mind, routine H&E histology can still provide instructive, quantitative data (Figure S1).

Cell number, proliferation, apoptosis

Quantifying the average sebocyte number in H&E-stained SG sections provides invaluable baseline data for subsequent *in situ* analyses, which permit insights into altered sebocyte proliferation/apoptosis. Cell numbers can also be very easily quantified by counting 4′,6-diamidino-2-phenylindole (DAPI)+ nuclei within the SG (Figure S1). These data can then be followed up by, for example Ki67 or terminal deoxynucleotidyl transferase dUTP nick end labelling (TUNEL) immunohistochemistry (see below). If alterations in SG lipid/antigen staining intensity are found without corresponding changes in cell numbers, this can indicate an altered sebocyte differentiation pattern.

SG volume/size

The overall size of the SG is a useful measure of gland stability, that is, whether the test condition has resulted in, for example, SG atrophy. If combined with cell number counts, SG hypertrophy versus SG hyperplasia can be distinguished. SG planimetry, using image analysis software, for example ImageJ (96), permits an estimate of SG volume in lieu of three-dimensional techniques, by

Table 1. IHC/IF markers of differentiating sebocytes or sebocyte progenitor cells

Marker	Alternative Names	Type of Molecule	Description	References
Mucin-1 (MUC1)	Epithelial membrane antigen (EMA)	Polymorphous epithelial sialomucin (PEM)	Highly glycosylated, high MW glycoproteins expressed at cell surface or released as secretory product. Functions include hydration, lubrication and antimicrobial defense	(22, 112–117)
MAM-6c	Not applicable	Polymorphous epithelial sialomucin (PEM)	Highly glycosylated, high MW glycoproteins expressed at cell surface or released as secretory product. Functions include hydration, lubrication andantimicrobial defense	(63, 112, 115)
Human Milk Fat Globulin 2 (HMFG2)	Not applicable	Milk Fat Globulin	Highly glycosylated, high MW glycoproteins	(63, 112, 113, 115, 118)
Ovarian-Carcinoma-Associated Sebaceous Gland Antigen (SGA)	Not applicable	Milk Fat Globulin	Highly glycosylated, high MW glycoproteins	(63, 112, 113, 115, 118)
Keratin 7 (K7)	Cytokeratin 7 (CK7)	Keratin	Present in human (but absent in mouse) SG	(22, 63, 115, 119, 120)
Perilipin 2	Adipophilin; Adipose differentiation-related protein (ADRP)	Perilipin	Lipid droplet-associated protein. First identified in adipocytes but since shown to be present in sebocytes	(21, 22, 60, 121–125)
Fatty Acid Synthase (FASN)	Not applicable	Enzyme involved in lipogenesis	Synthesises long-chain fatty acids that are present in human and murine sebum	(21, 22, 28, 44, 126)
Stearoyl-Coenzyme A desaturase 1 (SCD1)	Not applicable	Enzyme involved in fatty acid metabolism	Catalyses the Δ-9-cis desaturation of saturated fatty acids. Involved in sebocyte differentiation, lipogenesis and lipid metabolism. Essential for SG maintenance.	(22, 60, 85, 127–130)
Indian Hedgehog (IHH)	Not applicable	Hedgehog signalling protein	Important during foetal SG development. Promotes proliferation of undifferentiated sebocyte precursors	(131–135)
Peroxisome proliferator-activated receptor gamma (PPARγ)	Not applicable	Nuclear hormone receptor	Acts as transcriptional regulator of genes involved in lipid metabolism. Loss in the hair follicle bulge induces SG atrophy	(111, 136–138)
Melanocortin 5 receptor (MC5R)	Not applicable	G-protein coupled receptor	Present in SG. Inhibition causes reduction in differentiation and reduction in production of squalene and wax esters	(47, 139–141)
Leucine-rich repeats and immunoglobulin-like domain protein 1 (Lrig1)	Not applicable	Transmembrane protein (receptor antagonist)	Suppresses signalling by ErbB growth factor receptors. Marker of multipotent stem cells. Lrig1 positive cells may act as sebocyte progenitors under homoeostatic conditions	(22, 60, 142–144)
PR domain-containing protein 1 (PRDM1)*	B-lymphocyte-induced maturation protein 1 (BLIMP1)	Transcriptional repressor (transcription factor)	Possible marker of sebocyte progenitor cells*	(137, 145)

*PRDM1 as a marker of sebocyte progenitor cells is contested by some (see (23, 146, 147)). To view the full-page table, please see the Supporting Information.

measuring multiple reference areas (Figure S1), ideally, on multiple non-consecutive histological sections through the same gland (Figures S2 and S3) or by assessing many different glands in the same sample. Note that the number of glands per hair follicle and SG size differs depending upon body location and hair follicle type; therefore, analyses should compare like samples, for example human sebaceous follicle-associated SGs should not be directly compared with human terminal scalp or vellus hair associated SGs. In mice, it is particularly important to distinguish SGs associated with guard (tylotrich) hair follicles, which normally carry two SGs, from the majority of non-guard pelage hair follicles that display only a single SG (97).

Volume/size of individual sebocytes

Measuring changes in the volume/size of individual sebocytes is another useful parameter, which gauges sebocyte differentiation. An increase in cell area (and therefore likely cell volume) may suggest progression of differentiation, which is usually associated with an increase in sebum production (Fig. 3 and Figure S1).

Sebocyte size measurements should be performed on a large number of mature, differentiated sebocytes with clearly visible nuclei. Importantly, inhibition of lipogenesis does not lead to shrinkage of fully differentiated sebocytes. Rather, reduced lipogenesis inhibits the differentiation of small, basal sebocytes, increasing

the number of undifferentiated and reducing the number of differentiated sebocytes, thus eventually reducing the volume of the entire SG (63).

Whilst H&E suffices to assess sebocyte volume, PAS histochemistry and mucin-1 (MUC1)/milk fat globulin immunohistochemistry demarcate the cell boundary of sebocytes *in situ* even more sharply.

SG histochemistry

Other important histochemical stains for SG visualisation are Oil Red O (28,98), which demarcates neutral lipids, for example triglycerides, and Sudan Black B, which stains all lipids (Fig. 2). Clinically, these stains allow one to identify cancers of sebaceous origin (e.g. use of Oil Red O to identify metastatic sebaceous gland carcinoma in lymph nodes (99); Sudan Black B for diagnosing sebaceous adenoma of the palate (83)).

Nile Red staining is also employed in SG research (22,98), enabling both neutral and polar lipids to be distinguished within the same specimen using different excitation and emission filters. When dissolved in neutral lipids, for example triacylglycerol, Nile Red will fluoresce yellow-gold (485 nm excitation and 565 nm emission filters); whereas in phospholipids, for example phosphatidylcholine, Nile Red will fluoresce red (494 nm excitation and 523 nm emission filters) (5,100,101).

Table 2. Choosing SG read-out parameters

Which technique is best employed for visualising the SG when my main focus is the following...?	Appropriate staining technique(s)	Species Specificity	Antigen/Target Location	Analysis Technique(s)	References
Sebaceous gland and sebocyte microanatomy and morphometry	H&E	H M	N/A	ACN; ACS; AGA	(63, 93)
Sebum distribution using a light microscope, specifically focussing on neutral lipids such as triglycerides and cholesterol esters	Oil Red O	H M	Cytoplasm	AGA; SI	(21, 98, 115)
Sebum distribution using a light microscope, highlighting all lipids	Sudan Black B	H M	Cytoplasm	AGA; SI	(62, 83)
Sebum distribution using fluorescence microscopy, with the ability to distinguish neutral from polar lipids	Nile Red	H M	Cytoplasm	AGA; SI	(98, 100, 101)
Enzymatic activity within the SG	Alkaline Phosphatase	H M	Cytoplasm	AGA; SI	(148)
Expression of markers of differentiating/ differentiated sebocytes using immunohistochemistry	MUC1, MAM6c, HMFG2, SGA	H	Membrane	ACN; ACS, AGA; ECP; SI	(63, 112, 113, 115, 118, 149)
Expression of essential structural proteins of sebocytes using immunohistochemistry	Keratin 7	H	Cytoplasm	ACN; ACS; AGA; ECP; SI	(63, 115, 119, 150)
Lipid droplet presence and conformation in sebocytes using immunohistochemistry	Perilipin 2	H M	Lipid Droplet Membrane	ACN; ACS; AGA, ECP; SI	(21, 122, 124)
Levels of key enzymes involved in lipid metabolism of sebocytes using immunohistochemistry	Fatty Acid Synthase	H M	Cytoplasm	ACN; AGA; SI	(21, 85, 130)
	SCD1	H M	Cytoplasm	ACN; AGA; SI	
Levels of developmental proteins involved in the proliferation and differentiation of sebocytes using immunohistochemistry	Indian Hedgehog	H M	Membrane/ Excreted	ACN; ACS; AGA; ECP; SI	(132, 134)
Numbers of cells undergoing proliferation using immunohistochemistry	Ki67	H M	Nucleus	ACN; AGA; ECP	(151, 152)
	BrdU	H M	Nucleus	ACN; AGA; ECP	
	EdU	H M	Nucleus	ACN; AGA; ECP	
Numbers of cells undergoing apoptosis using immunohistochemistry	TUNEL	H M	Nucleus	ACN; AGA; ECP	(68, 153)
	Caspase 3	H M	Cytoplasm	ACN; AGA; ECP	

H, human; M, murine.
ACN, average cell number; ACS, average cell size; AGA, average gland area; ECP, expressing cell percentage; SI, staining intensity. To view the full-page table, please see the Supporting Information.

With the exception of Nile Red staining, these techniques require little in the way of specialist equipment, and thus offer particularly useful read-out parameters.

## Quantitative analysis of specific SG histochemistry

Quantitative analysis of histochemical SG stains, for example Oil Red O, routinely requires the use of image analysis software, such as ImageJ. As the SG will often be intensely stained, demarcation of specific areas/cells of the SG is difficult (Fig. 2). In these cases, the relative staining intensity of the SG area can be calculated. To reduce investigator bias, the whole SG area, using the SG-CTS as an outline and the beginning of the sebaceous duct as a cut-off point, should be measured for staining intensity (preferably on several non-consecutive sections) (Figure S1).

Immunohistochemical (IHC)/immunofluorescent (IF) SG markers

Several instructive IHC and IF techniques are available for studying SGs. These techniques not only localise the expression of defined antigens to specific cells within/around the SG, but also permit functional studies on sebocyte proliferation/apoptosis. We have selected several IHC markers of differentiating sebocytes/sebocyte progenitor cells (Table 1) that we consider to be most useful in SG research (see also Fig. 4). Note that not all of these markers are relevant to both humans and mice (see Table 2), and that species-dependent differences need to be taken into account. For guidance on which typically asked SG research questions can be best answered by employing which specific markers, consult Table 2.

Studying SG proliferation and apoptosis *in situ*

General markers for proliferation/apoptosis should be routinely incorporated into SG research *in situ* to complement the basic cell count and SG size data. Due to the distinctive compartmental lo-
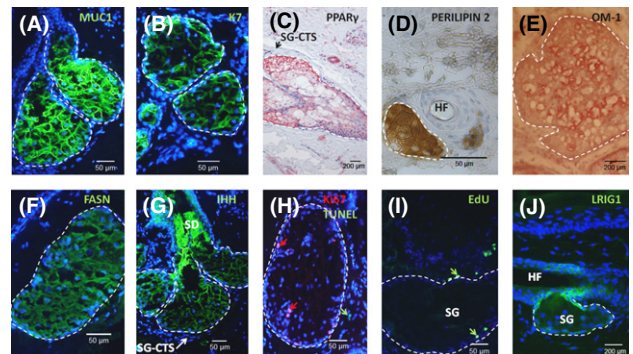
Figure 4. Immunohistochemical Stains for Analysis of the Sebaceous Glands. Various immunohistochemical stains are available for analysis of the sebaceous glands. We have highlighted the markers that we feel are the most instructive however, this list is not exhaustive. Note that image H represents healthy tissue and therefore the presence of apoptotic cells within the SG is a very infrequent event. Nevertheless, apoptotic cells can still be seen surrounding the gland, highlighting the sensitivity of the TUNEL method for detecting apoptotic cells. Dashed lines indicate the peripheral zone of the sebaceous gland. Key: HF, hair follicle; SD, sebaceous duct; SG, sebaceous gland; SG-CTS, Sebaceous Gland Connective Tissue Sheath; (A) Mucin1, human; (B) Keratin 7, human; (C) Peroxisome proliferator-activated receptor gamma, human; (D) Perilipin 2, mouse; (E) OM-1 antigen, human; (F) Fatty Acid Synthase, human; (G) Indian Hedgehog, human; (H) Ki67/TUNEL, human; (I) EdU, human; (J) Leucine-rich repeats and immunoglobulin-like domain protein 1, mouse. Scale bars: A–B, D, F–I = 50 μm; C, E, J = 200μm. To view the full-page figure, please see the Supporting Information.

calisation and highly characteristic morphology of sebocytes, it is easy to restrict the quantitative assessment of standard proliferation/apoptosis markers exclusively to sebocytes. Proliferation/apoptosis in the SG-CTS should strictly be regarded separately from that of sebocytes.

Proliferation markers: Ki67, BrdU, EdU

Ki67 is a well-established proliferation marker that highlights cells which have entered the cell cycle (102). However, Ki67 does not provide unequivocal evidence of actual proliferation or mitosis. Light microscopically, the latter can be assessed by counting the number of mitotic figures after colchicine-induction of mitotic spindle arrest (ideally, in iron haematoxylin stains (103)), or by more in-depth immunofluorescent cell cycle and DNA synthesis analyses in situ (e.g. 104,105), although these remain to be systematically applied to SG research. In human and murine skin, analysis of Ki67 immunoreactivity is best complemented by determining the number of sebocytes that actively synthesise DNA in situ, by measuring the incorporation of bromodeoxyuridine (BrdU) or 5-Ethynyl-2′-deoxyuridine (EdU) (Fig. 4I, – see Supporting Information for details).

Apoptosis markers: TUNEL, cleaved caspase 3

Apart from ultrastructural techniques, TUNEL immunohistochemistry offers a simple method for detecting changes in sebocyte apoptosis. However, the TUNEL technique has its limitations in that it can give false-positive results, for example in terminally differentiating epithelial cells (for discussion, see 106). Therefore, it is advisable to complement TUNEL data with additional methods (e.g. cleaved caspase 3 – for details, see Supporting Information) (Fig. 4).

Quantitative immunohistomorphometry

All of the IHC/IF read-out parameters listed above should be assessed by quantitative immunohistomorphometry so as to generate robust, standardised and reproducible data. The methods described above (sebaceous gland size, sebocyte size, cell number, staining intensity) can all be incorporated into analysis of IHC/IF markers, as well as the percentage of antigen-expressing cells (for further information on standardised quantitative immunohistomorphometry techniques, see Supporting Information).

Summary

Here, we have discussed the use of routine histochemistry and immunohistochemistry in the visualisation and quantitative assessment of human and murine SGs in situ. Whilst, due to space constraints, in vitro methods of sebocyte isolation, culture and analysis cannot be discussed here, many of the markers and techniques described here are also relevant for isolated primary sebocytes and sebocyte cell lines (for methodological details, see (98,107,108)). The techniques described can be complemented with, for example double-immunofluorescence stains, laser capture microdissection or lipidomics to provide an in-depth assessment of the SG and can be utilised in both clinical studies and laboratory analyses. Such standardised (immuno-) histomorphometric SG assessment criteria for evaluating therapeutic outcomes would have been useful in past studies (e.g. 63,64,109–111), where sometimes arbitrary or insufficiently defined/standardised SG read-out parameters were employed. Routine use of the assessment criteria and techniques suggested here should greatly facilitate the reproducibility and standardisation of read-out parameters and assessment techniques employed in future clinical SG-related trials, thus standardising SG evaluation methods.

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Conflict of interests

The authors state no conflict of interest.

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Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1. How to Perform Analysis using ImageJ.

Figure S2. Sebaceous gland size measurements using various planes of the same gland.

Figure S3. Area of 2 SG within the same skin sample over a number of sections.

Table S1. Normal sebum composition of humans and mice.