OPEN ACCESS
Journal of Innovative Optical Health Sciences
Vol. 16, No. 3 (2023) 2330003 (19 pages)
© The Author(s)
DOI: 10.1142/S1793545823300033

Recent advances in Raman spectroscopy for skin diagnosis

Min Wu
Department of Plastic and Reconstructive Surgery,
Shanghai Ninth People's Hospital
Shanghai Jiao Tong University, School of Medicine
Shanghai 200011, P. R. China
sjtu_wm@126.com

Beibei Gao
School of Biomedical Engineering, Shanghai Jiao Tong University
Shanghai 200240, P. R. China
Gaobeibei919@sjtu.edu.cn

Xunbin Wei
Institute of Medical Technology, Peking University
Beijing 100181, P. R. China

Biomedical Engineering Department, Peking University
Beijing 100081, P. R. China

International Cancer Institute, Peking University
Beijing 100191, P. R. China
xwei@bjmu.edu.cn

Received 23 July 2022
Revised 11 January 2023
Accepted 11 January 2023
Published 4 March 2023

The skin is the largest organ in humans. It comprises about 16% of our body. Many diseases originate from the skin, including acne vulgaris, skin cancer, fungal skin disease, etc. As a common skin cancer in China, melanoma alone grows at year rate of nearly 4%. Therefore, it is crucial to develop an objective, reliable, accurate, non-invasive, and easy-to-use diagnostic method for skin diseases to support clinical decision-making. Raman spectroscopy is a highly specific imaging technique, which is sensitive, even to the single-cell level in skin diagnosis. Raman spectroscopy provides a pattern of signals with narrow bandwidths, making it a common and essential tool for researching individual characteristics of skin cells. Raman spectroscopy already has a number of clinical applications, including in thyroid, cervical and colorectal cancer. This review will introduce the advantages and recent developments in Raman spectroscopy, before focusing on the

This is an Open Access article. It is distributed under the terms of the Creative Commons Attribution 4.0 (CC-BY) License. Further distribution of this work is permitted, provided the original work is properly cited.

advances in skin diagnosis, including the advantages, methods, results, analysis, and notifications. Finally, we discuss the current limitations and future progress of Raman spectroscopy in the context of skin diagnosis.

Keywords: Raman spectroscopy; skin diagnosis; Raman imaging; melanoma.

1. Introduction

Skin is the largest organ in our body. It can be divided into epidermis and dermis. The skin consists of epithelial, mesenchymal, glandular, and neurovascular components.[1–3] Many diseases originate in the skin, including squamous cell carcinoma (SCC). Indeed, there are more than one million patients diagnosed with common malignant skin diseases annually.[4] As the most important skin disease, skin cancer accounts for approximately 15,000 deaths and more than three billion dollars in medical costs in the US alone annually.[5] Therefore, early diagnosis of skin diseases is crucial. However, clinical and histopathological evaluation of problematic skin lesions remains challenging, even for experienced dermatologists.[6] The gold standard for the diagnosis of skin lesions, such as vascular diseases, is histopathological evaluation of skin biopsy specimens. It is highly invasive. Moreover, for some malignant skin diseases, such as melanoma, diagnosis requires histopathological examination of a skin biopsy specimen, and patients who are at increased risk often need to undergo multiple biopsies. This is associated with morbidity and increased cost.[7–9] Therefore, it is necessary to explore objective, reliable, accurate, noninvasive and easy-to-use diagnostic methods to assist with clinical decision-making.[10,11] Many traditional spectroscopic techniques can be used to capture biological information about the structure and function of specific cellular components, such as RNA and proteins, which are rich in skin. As for conventional spectroscopic techniques, the lack of specificity remains a major problem, which limits the ability to detect individual components of different layers of the skin. Therefore, the ideal diagnosis technology should be highly specific and sensitive, with real-time detection.

Skin diseases can be classified into many categories, such as infectious diseases, skin tumors, inflammatory diseases and cosmetic problems. Skin tumors are common skin diseases. There are various types, such as basal cell carcinoma (BCC), SCC, lipoma and melanoma (Fig. 1). Taking melanoma

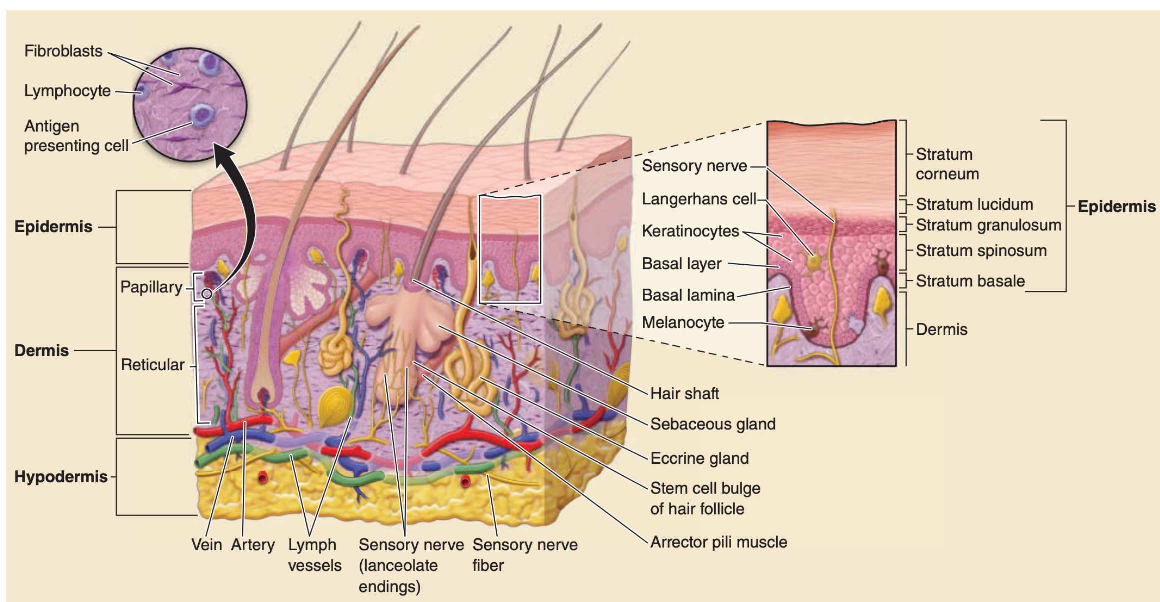

Fig. 1. Structures of the skin. In this schematic, the general architecture of the skin is depicted, clearly showing the high complexity of the formation of all skin elements (taken from *Fitzpatrick's Dermatology*).

as an example, the staging system used for it was first established in 2017 and is known as the tumor size, node status, metastasis classification (TNM) system. It can be clinically distinguished into different subtypes, including superficial spreading, nodular, acral lentiginous, lentigo malignant and desmoplastic melanoma. The expression of many genes, including BRAF, NRAS, CDK4 and c-KIT, is associated with melanoma. The combination of genotype and phenotype is achieved through different classifications, which can help dermatologists to better understand the characteristics and development direction of melanoma. Relying on various classification systems allows dermatologists to understand the homogeneity of clinical outcomes of patients with different molecular characteristics. This is a method to help diagnose different kinds of diseases. Currently, several treatment options exist for melanoma, including chemotherapy, targeted drug therapy, radiotherapy, oncolytic therapy, surgery, and local resection combined with cryotherapy and phototherapy. Among these treatment options, surgery is still the most effective and common treatment. The stage of melanoma should be considered comprehensively when determining the resection range. For early melanoma, the resection range can be appropriately reduced as the risk of metastasis increases with increasing depth of the primary tumor. However, in addition to the classic pathological examination of excision tissues, the selection of a surgical plan largely relies on surgeons' experience. In terms of imaging, different diagnostic algorithms using dermoscopic findings have been developed for melanoma. Pattern analysis, which provides an overall impression of multiple dermoscopic patterns without rigid rules, is based primarily on a subjective, simultaneous evaluation of many criteria and is most widely used among experienced users of dermoscopy to evaluate pigmented lesions. Routine imaging and hematologic tests in asymptomatic patients are rarely able to identify occult systemic disease. These methods all have significant shortcomings, including high cost, slow speed of detection and poor single-cell sensitivity. Imaging with CT, MRI or PET is indicated only in high-risk metastatic melanoma with the previously confirmed diagnosis. Therefore, rapid skin diagnostic techniques will serve to improve guidance for doctors to select therapeutic options with greater confidence, such as surgery or rare-invasive therapy.

As mentioned before, many spectroscopy techniques have been used for skin diagnosis in the field the author majored in, including electrical impedance spectroscopy (EIS), high-frequency ultrasonography (HFUS), multispectral imaging, and Raman spectroscopy. However, these techniques have certain limitations. For example, EIS has a trade-off of higher sensitivity for lower specificity. This is also common in many other diagnostic tools and clinical evaluations by dermatologists.[10] Histology is the gold standard. Compared to histology, HFUS, as the only imaging method for characterization and diagnosis of skin at present, cannot identify cell types and has insufficient resolution to provide definitive diagnosis[12] (Table 1).

Raman spectroscopy is an emerging tool,[13,14] to provide a noninvasive and label-free analysis of biological cells. Due to its high biological specificity, Raman spectroscopy can be used to acquire spectral fingerprints that allow the characterization of skin cell types and states. We can use a confocal Raman spectrometer to collect these characterizations. The spectral signature of the cell component can be

Table 1. Current methods for skin diagnosis.

Method	Advantages	Disadvantages	References
EIS	High sensitivity	Low specificity	10
HFUS	Noninvasive, easy-to-use, not too expensive	Cannot be used to identify cell types, insufficient resolution to provide definitive diagnosis	12
CT	Noninvasive, convenient, not too expensive	Indicated only in high-risk metastatic diseases with the previously confirmed diagnosis	10
Dermoscopy	High sensitivity, noninvasive, fast	High cost, low specificity	11
Histopathological examination	Gold standard, reliable	Highly invasive, cause of transfer	6–9
Raman spectroscopy	Label-free, noninvasive, high specificity,	Experienced user	13, 14

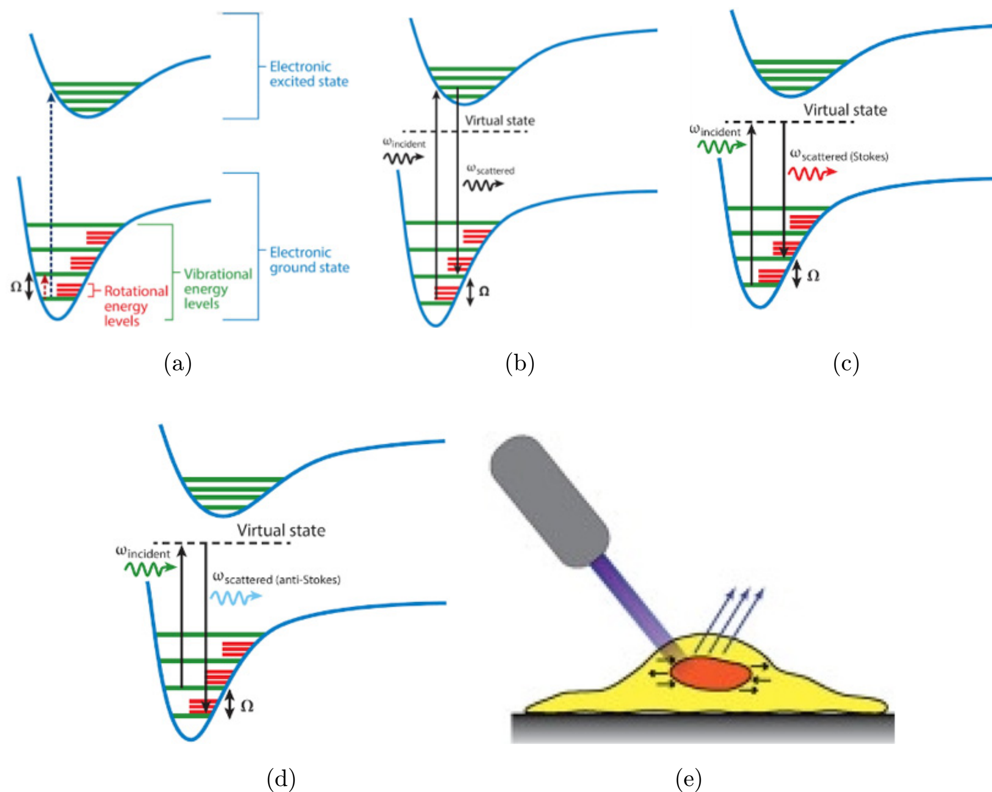

Fig. 2. Principle of Raman spectroscopy. (a) Rotational, vibrational, and electronic states in a molecule. The process of energy transfer in resonance Raman scattering. (b) Stokes, and (c) anti-Stokes. (d) Raman scattering and Ω represent the energy difference between the fundamental and excited ($\upsilon = 1$) and fundamental vibrational states of mid-infrared (from Ref. 142). (e) Raman spectroscopy involves the extraction of information through the monitoring of vibrational energy transitions (from Ref. 144).

obtained through the Raman scattering effect, which occurs when a photon is excited in a low vibrational state, i.e., a higher virtual state (Fig. 2). As there is no electronic state shift, the system cannot exist in the virtual state. The system returns to a low vibration state. The energy difference between these two states is expressed by the wavelength of the emitted Raman photons. The scattered light and incident light wavelength changes vary with the group. We can obtain the same data as the molecular fingerprint.[15] Fibroblasts, corneocytes, and keratinocytes differentiate into the skin without prior knowledge of cells. Therefore, in the context of skin diagnosis, such as skin cancer, it is important to clarify how to maintain sensitive single-cell Raman spectroscopy to the skin. Separation and identification of a single cell often require defined protocols.[16] It is common to observe multiple cell types or cell subpopulations that cause spatially complex multifunctional tissues. Furthermore, the cells dynamically change depending on local microenvironmental cues.[17] Taking melanoma as an example, a lack of NRAS signaling or the mutation of BRAF kinase may facilitate the onset of this disease,[18] which may be optimally targeted and treated when detected at an early start stage before the occurrence of extensive proliferation. The morphology of mammalian cells can be easily determined by visible light microscopy.[19,20] However, when cells are subjected to ultraviolet light, the energy exposure may break the chemical bond and cause apoptosis,[21,22] collagen crosslinking,[23] and even cancer. Therefore, not all spectroscopy tools are suitable for studying cellular characteristics, and careful consideration of the trade-offs and benefits is necessary. As mentioned before, melanoma is one of the most important malignant skin diseases, and most mutations in melanoma are BRAF kinase mutations.[24] In 2020, heath and his colleagues employed Raman spectroscopy to study the intracellular drug metabolome and identified the phenotypic specific drug sensitivity by using a series of BRAF mutant melanoma cell lines, including A375 cell lines, as a

model system. These results prove that single cell sensitivity can be used for skin detection with Raman spectroscopy.[25]

In this review, we highlight recent advances in Raman spectroscopy and its application to skin diagnosis. We first introduce the advantages and disadvantages of Raman spectroscopy, before focusing on a methodological approach for implementing Raman spectroscopy in skin diseases diagnosis, including methods, analysis of results and notifications. Finally, we will summarize the current limitations of this technology and progress in Raman spectroscopy.

2. Raman Spectroscopy: Advantages and Disadvantages

Skin is composed of many different cells as the largest organ of the human body, including fibroblasts, corneocytes, and keratinocytes. Raman spectroscopy is noninvasive, label-free, molecular-specific, high resolution, and suitable for different kinds of analysis. It is considered better than conventional measurements for the detection of skin diseases. As mentioned previously, the energy exposure to ultraviolet light can cause damage to living cells within apoptosis, collagen crosslinking, and cancer. Raman spectroscopy commonly uses 532 nm as an excitation wavelength, which is not ultraviolet light and leads to resonance enhancement following interaction with cytochromes in cells.[26] In contrast, laser irradiation in the 514.5 nm range may still induce photodamage to living cells.[27] Alternatively, 785 nm excitation seems to be more suitable for living cell research, showing lower phototoxic effects.[28] Secondly, although low numbers of cells are used in most Raman imaging studies,[29–31] widefield and line mapping or global imaging have been proposed to improve the speed and lateral resolution.[32] Except the nucleus, lipid droplets can be decomposed by single cell Raman images, whose metabolism and subsequent storage patterns are critical in cell processes like atherogenesis, in which lipids adhere to plaque in the arterial wall. Another advantage of Raman spectroscopy is the ability to distinguish molecules from other natural lipids spectroscopically, such as palmitic acid, oleic acid, arachidonic acid and cholesterol, which may be contained in skin lotions. Raman spectroscopy has been reported to provide an information-rich and unique fingerprint from biomolecules in cells.[33] Raman spectroscopy is also an ideal choice to detect the cell environment at the molecular level and is unaffected by factors such as oxygen, temperature and water. Furthermore, compared to fluorescence microscopy, Raman spectroscopy does not require the molecules to be labeled and the spectrum emitted is entirely dependent on the biochemical composition of the observed voxels, which is performed without damaging the cell environment or labeling other samples.[34,35]

Raman spectroscopic investigation also has some limitations, including the requirement to acquire sufficient spectra per skin sample to accommodate different cells' variations. Currently, consented approaches are lacking to sampling size evaluation required to capture the targeted level of diversity and complexity in environmental systems. In addition, the background fluorescence in the sample matrices sometimes interferes with the inherently weak Raman scattering, which will result in low spectral quality or longer acquisition time.

3. Current Applications of Raman Spectroscopy

Numerous studies have shown that Raman spectroscopy is a powerful tool in applications such as identification of cells, discrimination of cells, biomedical imaging, bioprocessing, and analysis of biopolymer.[36–39] In 2019, Zhang and his colleagues used the multivariate curve analysis rate to analyze three components in water, protein, and lipid with a high wave number in skin confocal Raman data. By analyzing the relative quality of these three substances, they demonstrated a statistical difference in the Raman spectra of non-lesion and dermatitis lesion tissues.[40] Raman spectroscopy is also widely used in the analysis of nonbiological materials. Indeed, for more than 40 years, Raman spectroscopy has been at the forefront of blood research. It can be used to observe relative changes in plasma tryptophan, tyrosine, phenylalanine, carotenoids, protein, lipids and other biomolecules.[41] Parlatan and his colleagues combined Raman spectroscopy and statistical classification models to diagnose serum endometriosis, which can reduce the necessity of laparoscopy.[42]

Importantly, Raman spectroscopy can be applied to tumors. Currently, tumors or established tumor

cell lines are organized into hierarchies of heterogeneous groups. The ability to sustain the growth of entire cancer cell lines depends on a small subset of cancer stem cells. Raman spectroscopy has been used to characterize and distinguish metabolic changes associated with malignant transformation in thyroid,[43,44] lymphoma[45] and leukemia,[46] cervical,[47] and colorectal cancers.[48] Moreover, simultaneous quantification of multiple functionally relevant intracellular polymers, including glycogen and polyphosphate, has provided new insight into the metabolic state-based distribution of the polymers in microbial cells, the specific population-level dynamics of carbon and energy sources and the corresponding use of diverse metabolic pathways in an enhanced biological phosphorous removal (EBPR) bioprocess.[49,50] Furthermore, the presence of intracellular and extracellular biosulfur granules[51] and the structural dynamics of sulfur species in the biosystem[52] have been recently reported using a type of Raman spectroscopy technique called SCRS; This has contributed to a better understanding of the functional roles of biogenic elements in natural and engineered ecosystems. Krishna and colleagues used Fourier transform infrared and Raman spectroscopy simultaneously to identify multidrug-resistant phenotype mutations in malignant breast disease cells, as well as three drug resistant derivatives of this cell line.[53] Recent studies emphasized the potential of this technology in breast cancer diagnostics.[54] Surmacki and colleagues showed that Raman markers based on lipids and antioxidants could be measured easily and reproducibly. They exhibited high sensitivity and specificity in human invasive lobular cancer and breast invasive ductal cancer.[55] They also used principal component analysis (PCA) to analyze the vibrational signatures obtained by Raman spectroscopy or micro-spectroscopy, the results of which demonstrated the technique could accurately distinguish between normal and malignant breast tissues. Some proteins involved in fatty acid metabolism are altered in prostate tumor cells and although pathological examination remains the gold standard of diagnosis, this metabolic feature has contributed to the development of new therapeutic approaches for this disease. Indeed, Crow demonstrated the ability to discriminate three grades of prostatic adenocarcinoma *in vitro* with sensitivity and specificity above 96%.[56] Moreover, Taleb distinguished between normal and metastatic malignant prostate cell lines by identifying specific amino acids, DNA, and proteins.[57]

4. Raman Spectroscopy in Skin Diagnosis

As mentioned in section one, skin diseases can be classified into many categories, such as infectious diseases, skin tumors, inflammatory diseases and cosmetic problems. We can also divide skin diseases into pigmented lesions, vascular diseases and so on [Figs. 3(a)–4(d)]. Raman spectroscopy can help detect and diagnose these kinds of skin diseases.[58–60] For example, eczema, a common skin condition that causes dry, itchy and cracked skin, which is common in children can be diagnosed with the help of Raman spectroscopy.[61] In 2018, Zakhrov performed *in vivo* studies of Raman scattering and autofluorescence of skin tumors with 785 nm excitation laser in the near-infrared region such as malignant melanoma [Fig. 3(b)], BCC and many kinds of benign tumors [Fig. 3(a)]. He pointed that only combining analysis of Raman and autofluorescence signals, 100% accuracy of skin tumors differentiation

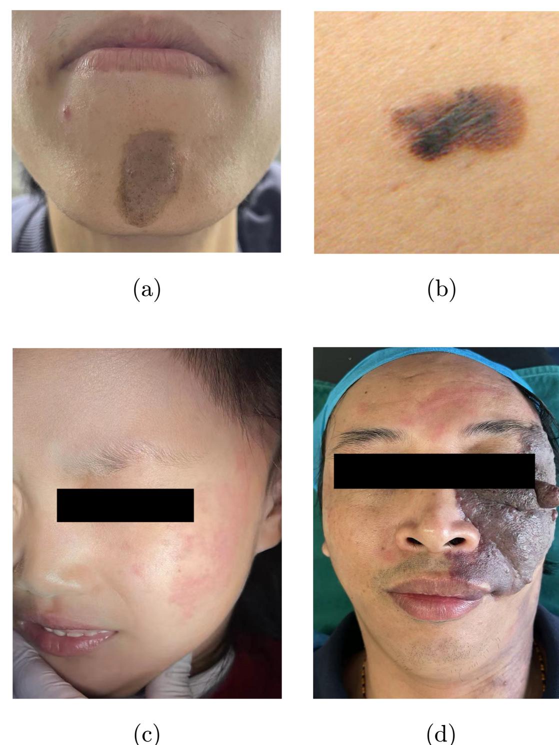

Fig. 3. Examples of typical skin diseases can be detected by Raman spectroscopy. (a) Congenital melanocytic nevi. (b) Skin melanoma. (c) Port wine stain on face. (d) Encephalotrigeminal angiomatosis.

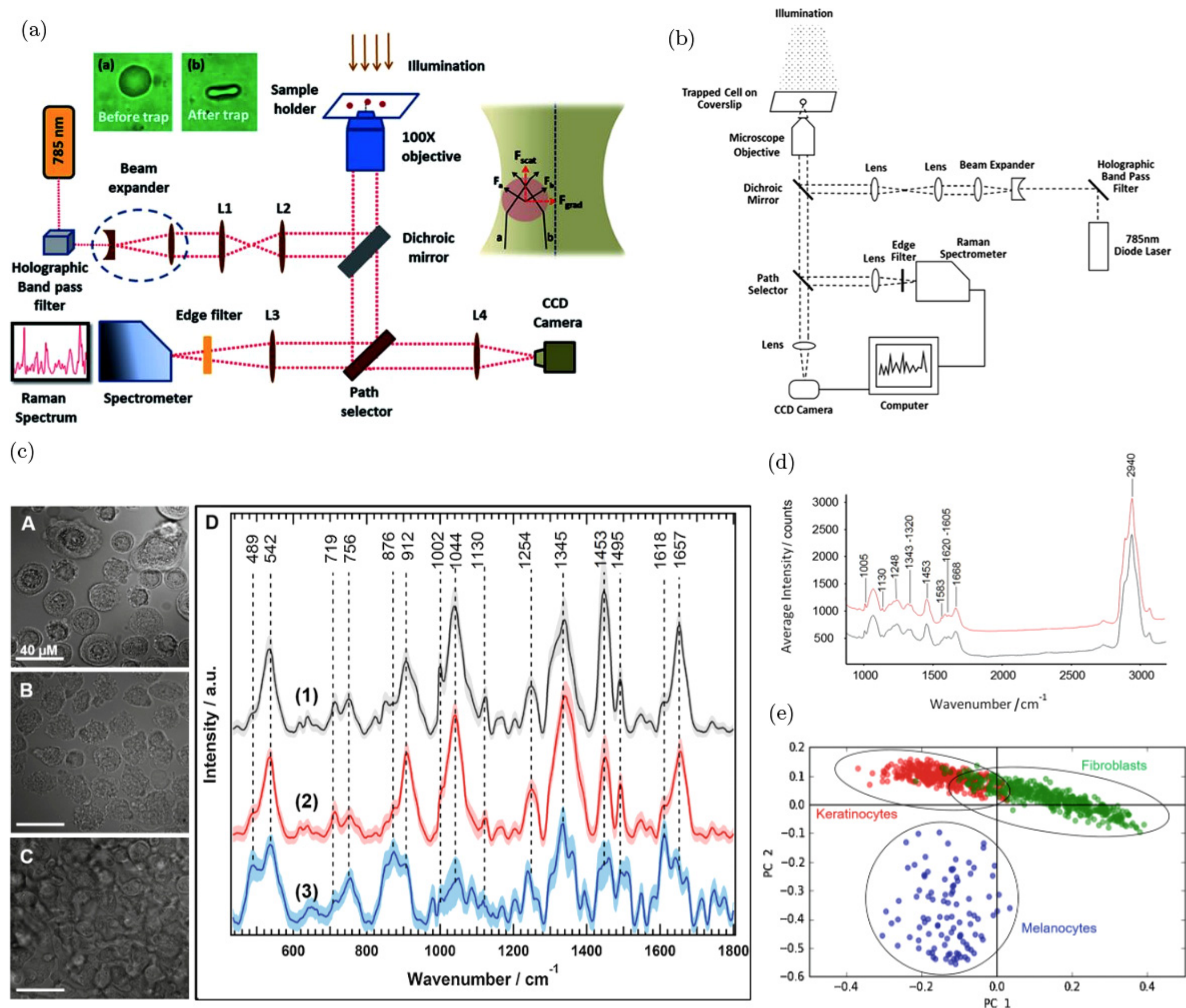

Fig. 4. Principle of Raman spectroscopy. (a) Schematic of the indigenously built Raman Tweezers instrument (from Ref. 145). (b) Schematic of the micro-Raman tweezer setup (from Ref. 146). (c) Bright field micrograph of keratinocytes (A–C), fibroblasts (B–C), and melanocytes (C). The Raman mean spectra (D) of keratinocytes (1), fibroblasts (2), and melanocytes (3) were collected. Single Cell Raman Spectroscopy was collected using a 785 nm laser (from Ref. 147). (d) An example of preliminary band-shape analysis, showing the average Raman spectra in the 800–3200 cm^{-1} range of normal cells with black trace and tumor cells with red trace (from Ref. 148). (e) PCA score plot of skin keratinocytes, fibroblasts, and melanocytes (from Ref. 147).

can be achieved.[62] What is more, in the field of skin diagnosis, diabetes can be quickly screened in a non-invasive way by using Raman spectroscopy together with artificial neural networks. Guevara and his colleagues proved this conclusion. Their work demonstrated an overall better performance of artificial neural network in combination with Raman spectroscopy than the capillary blood glucose measurement.[63] According to Pro. Zakharov's research, conventional Raman spectroscopy can provide the basis for cost-effective and accurate detection of kidney failure associated *in vivo* metabolic skin changes.[64]

4.1. *Excitation laser*

The wavelength of the excitation laser, which is focused onto the sample solution via a high numerical aperture microscope objective, should be considered carefully according to the characteristics of the sample. The scattered Raman signals are dispersed using a spectrograph and a detector is

employed to acquire the Raman spectra of optically trapped cells. Various wavelengths, including 532, 632,[65] 647, 785, and 1064 nm can be used depending on different applications. The selection of the optimal excitation wavelength should be carefully weighted. As mentioned in Sec. 2, excitation at a wavelength of 532 nm is most suitable for single-cell measurements. However, it may cause rapid cell damage and has high autofluorescence properties. Thus, it cannot be used for tissue characterization. With the development of lasers, modern cameras, high-quality optical filters, and detectors, Raman spectra can be easily acquired directly from almost any kind of biological sample now.[66,67]

All of these excitation wavelengths can be used in the diagnosis of skin diseases. Because of the limited penetration depth within the wavelengths discussed above, the diagnosis of skin diseases as superficial lesions may be the most suitable application of Raman spectroscopy.

4.2. *Methods and data analysis*

Before Raman spectroscopy measurements, the obtained cells should be carefully diluted and suspended in specific solutions to avoid capturing multiple cells from the laser spot. For example, erythrocytes should be diluted and suspended in plasma or hydroxyethyl starch solutions. Moreover, all experiments should be completed within 1 h of sample collection. Taking an example from an original research article, the laser wavelength was 785 nm, and was tightly focused onto the sample solution via a high numerical aperture. An oil immersion microscope objective and a spectrograph equipped with 1200 grooves per millimeter holographic grating were used to disperse the scattered Raman signals. Finally, a liquid nitrogen-cooled CCD detector was employed to acquire the Raman spectra of optically trapped cells.[68]

Progress in Raman spectroscopy technologies certainly has yielded large spectroscopic datasets that require advanced data analysis approaches rapidly.[69,70] The identification of intracellular biomolecules has been performed in reference to the standard of the material of interest or to Raman spectral libraries by employing univariate analysis based on peak position, height criteria or multivariate tools, such as the multiplicative curve resolution, by unmixing the contributing components.[71,72] In addition, the use of tiered clustering analysis to differentiate cell groups using combined dimension reduction, clustering and classification before cell identification may be helpful to improve the accuracy of complex and diverse environmental samples.[73]

There are many machine learning methods, such as projection on latent structures (PLS), principal component analysis (PCA), the support vector machines (SVM),[74] the nearest neighbor classifiers (KNN) and so on.[75] PLS can help analyze the experimental dataset. For multicollinear spectral data, PLS is one of the most common approaches to solve these problems because the projection analysis methods can provide a statistically reliable result.[64] Moreover, PCA, a widely used statistical analysis tool in Raman spectroscopy to obtain spectral data differences, is also used for data visualization and compression.[76]

In the diagnosis of skin disease, PCA can be employed to reduce the dimensionality of the features and select the optimal set. CT-RamSES can be used to process Raman spectra as well as for PCA multivariate analysis and data visualization, while the MATLAB calculation platform can be used during further processing. Many other specific analyses also exist, including preliminary band-shape analysis, multivariate analysis, and univariate analysis, which is classical least-squares curve fitting [Figs. 4(c) and 4(d)].[77–79]

4.3. *Components of skin detected by Raman spectroscopy*

In the field of dermatology, Raman spectroscopy is widely used for laser treatment, as well as to detect the relative contents of the major components in skin to monitor health conditions, identify skin diseases, and evaluate therapeutic effects.[80] Raman spectroscopy is an efficient and easy way to investigate the infiltration process of active components, as well as to assess the formulation components of locally applied substances penetrating into human skin.[81]

Raman spectral bands can reveal important chemical information about the macromolecules in biological cells. Each spectral band can be assigned to a specific molecular vibration in the cell. Numerous cell types are found in skin, including fibroblasts, melanin, corneocytes, and keratinocytes.

In 2019, Feng and his colleagues sued Raman micro-imaging to identify cellular components of

Table 2. Relative contribution from various cellular components of different skin diseases.

Skin diseases	Components and relative contribution tendency
Normal	18%Collagen, 12%Elastin, 41%Triolein, 9%Nucleus, 1%Keratin, 9%Ceramide, 5%Melanin, 5%Water
BCC	15%Collagen↓, 23%Elastin↑, 28%Triolein↓, 20%Nucleus↑, 1%Keratin, 4%Ceramide, 3%Melanin, 6%Water
AK	15%Collagen↓, 16%Elastin, 32%Triolein↓, 14%Nucleus↑, 3%Keratin, 10%Ceramide, 7%Melanin, 3%Water
SCC	9%Collagen↓, 12%Elastin, 32%Triolein↓, 6%Nucleus, 11%Keratin↑, 12%Ceramide, 6%Melanin, 12%Water↑
PL	30%Collagen↑, 5%Elastin↓, 23%Triolein↓, 6%Nucleus, 2%Keratin, 14%Ceramide, 19%Melanin↑, 1%Water
MM	18%Collagen, 14%Elastin, 11%Triolein↓, 6%Nucleus, 2%Keratin, 17%Ceramide↑, 29%Melanin↑, 3%Water

different skin lesions, including normal skin, BCC, SCC, actinic keratosis (AK), lichen planus (PL) and malignant melanoma (MM). Different kinds of skin lesions contain various cellular components with different ratios (Table 2). They found that their Raman images had similar characteristic peaks with the known spectra measured from pure chemicals. For example, the spectra of cytoplasm and synthetic actin are highly similar, while major differences can be seen at 1003 cm^{-1} phenylalanine peak, 1081 and 1092 cm^{-1} lipid band.[82]

Melanin is one of the major bio-aggregates, and consists subunits of different pigment species formed by the oxidation and cyclization of the amino acid tyrosine.[83] Melanin plays a significant regulatory role in skin.[84] The Fitzpatrick Scale is a commonly used semiquantitative scale, consisting of six optical types describing skin color, which depend on the melanin level, inflammatory response to ultraviolet light, basal complexion, and risk of cancer.[85] The detection of melanin is important for the diagnosis of skin diseases, especially pigmented lesions. It can be proven by the Raman mean spectra collected from suspended melanocytes, fibroblasts, and keratinocytes, where Raman bands show the difference in spectral positions and intensities between different cell types (Table 3).[86,87]

Table 3. Different Raman bands at spectral intensities and positions among different cells in skin.

Factors	Wave Number (cm^{-1})
Glycogen	489
Protein	542, 756, 1254, 1345, 1453, 1495, 1618, 1657
Cysteine	542
DNA/RNA	719, 912, 1254
Lipid	719, 876, 1130, 1254, 1453
Collagen	876, 1044
Phenylalanine	1002, 1618
Carbohydrates	1345
Tryptophan	1618

Many studies have reported using Raman spectroscopy to detect skin components. In 2018, Santos *et al.* analyzed the Raman spectra of various high waves and short peak positions of newly resected suspected melanoma to objectively diagnose all types.[88] In 2019, Darvin and his colleagues proposed a calculation method for the determination of the water profiles in oil-treated skin, which is based on the calculation of the ratio between the Raman band intensities of water at 2250–3550 cm^{-1} and keratin amide at 1650 cm^{-1}.[89] In 2022, Zakharov and his colleagues investigated the Raman spectra of human skin recorded using a portable conventional Raman spectroscopy setup. They analyzed Raman spectra of normal skin, BCC, MM and nevus with MCR-ALS, finding that multivariate curve resolution alternating least squares analysis can provide novel information on skin tissues' biomedical profiles.[90] Moreover, in 2020, Kourbaj and his colleagues detected samples from different individuals by Raman spectroscopy, and found that the percentage of water in the dermal layer could be used to evaluate the degree of photoaging.[91] This is useful in the field of dermatology as means to assess methods of rejuvenation. Puppels and colleagues also demonstrated the utility of confocal Raman spectroscopy to determine the water levels of the skin *in vivo*.[92]

4.4. *Surface-enhanced Raman spectroscopy (SERS)*

SERS is a possible solution to improve the low sensitivity of Raman spectroscopy, because it cannot be used to analyze samples with low concentration samples. SERS was first discovered from the enhanced Raman spectra of pyridine molecules on a roughened silver surface in 1974.[93] SERS is based on the phenomenon that, under good conditions, molecules are adsorbed onto a precious metal surface to generate a million or more enhancement

signals.[94,95] Furthermore, the surface plasmon resonance of a particular metal nanoparticle can enhance the relatively weak Raman signals in normal Raman to improve sensitivity and accuracy. As an advantage, SERS allows for the analysis of samples at minimum analytical concentrations,[96] with considerably greater sensitivity than conventional Raman.[97] The enhancement of the Raman effect is highly dependent on structures of SERS substrates where metal colloids and their aggregates are most commonly used, although gold and silver nanoparticles are also used.[98–100] In 2007, Kahraman and colleagues showed that it was possible to employ SERS to analyze several bacterial cells.[101] Moreover, Kubryk and colleagues introduced a reproducible SERS analysis of bacteria and demonstrated that SERS can help to detect stable isotope uptake.[102,103]

Recent studies on SERS for bioanalysis have focused on the characterization of biomolecules such as protein, DNA, pathogens, and even cancer cell detection.[104–107] Therefore, SERS is expected to be a valuable and promising tool for the diagnosis of inflammatory diseases such as acne, as well as skin cancer.[108]

4.5. *Tip-enhanced Raman spectroscopy (TERS)*

TERS is a nano-spectroscopic method. It always combines Raman spectroscopy with scanning probe microscopy techniques, such as atomic force microscopy. TERS enhances the localized Raman signals from a tiny sample volume in the vicinity of the tip apex using a sharp metallic active tip.[109] The spatial resolution of conventional optical techniques is governed by the diffraction limit. Compared to them, TERS captures signals with a pronounced increase in resolution, thus facilitating bioanalysis at the nanoscale.[110,111] Various biological samples can be analyzed by TERS, including microbial cells,[112,113] viruses,[114] and biomolecules, such as nucleic acids, lipids, and proteins.[115,116] Recent advances in TERS investigations of DNA or RNA with single nucleobase resolution indicate that it is an attractive application for direct nucleic acid sequencing without the need for fluorescent labeling and amplification.[117] Abnormal subcellular structures are present in many skin diseases including melasma, a common hypermelanosis skin disease and also known as a dermatologic skin disease. Melasma usually occurs on the areas of the skin that are exposed to sun. Besides traditional methods, a novel microscopy technique is needed to observe the ultrastructural changes of melanosomes in melanocytes after laser treatment and distinguish it from other pigmented diseases of skin. TERS may meet these acquisitions as a nano-spectroscopic method.

While, if we want to ensure the reliability and reproducibility of TERS measurements, we must consider optical properties, tip design, the operating environment and conditions, and the far-field scattering background. In the field of clinical skin detection, this might translate to uncertainty and high cost for patients.

4.6. *Resonance Raman spectroscopy (RRS)*

Enhanced Raman signal is achieved by excitation at frequencies close to the region of the electron absorption band of a certain molecule. This technique is called RRS, which can result in a six digit increase in the signal-to-noise ratio. RRS selectively enhances cell-specific Raman active molecules. A typical RR-active biological compound contains various pigments and metal proteins. RRS has many applications including in the study of erythrocytes, which are rich in blood vessels, including veins and arteries. Many skin diseases, including port wine stain and hemangioma, are related to abnormal vessels. RRS is helpful to diagnose these skin diseases. The dermal tissue[118] of skin is rich and selective excitation can be obtained by adjusting the excitation wavelength. The UV excitation ranges from 200 to 260 nm, mainly eliciting signals from aromatic amino and nucleic acids. This excitation spectrum has another advantage. The advantage of high excitation wavelengths eliminated the background signal by native cell fluorescence.[119]

4.7. *Coherent Raman scattering*

Coherent anti-Stokes Raman scattering (CARS) and SRS are two widely used CRS techniques. CRS drastically amplifies the Raman signal by using multiple lasers to coherently excite the molecular vibration associated with the selected chemical bond.[120–123] Compared to spontaneous Raman

scattering, these nonlinear optical processes allow for video-rate imaging of living cells.[124] Raman labeling techniques can be used to selectively record specific biomolecules and metabolic activities, which provide functional information for metabolism studies.[125–127] However, other than a full spectrum, most coherent Raman scattering techniques work at a single frequency matching sole vibrational transition. Therefore, the obtained information may not be sufficient to distinguish spectrally overlapped chemical components in heterogeneous samples.[128]

Regarding skin detection, fluorescent probes are inappropriate for investigating properties such as transport or absorption of skin or hair due to the small size of molecules of interest.

Stimulated Raman scattering (SRS) microcopy is another powerful modality. It is a modality for imaging sensitive chemical bonds, resolution, and speed.[129] SRS is a nonlinear optical process that has two synchronized pulsed lasers called the pump beam and Stokes beam. The selective molecular vibrations are coherently excited. When the frequency difference between the pump and the Stokes laser matches the vibration (ω_ν) of the chemical bond of interest, the vibrational activation rates can be greatly amplified through quantum stimulation of photons in the Stokes beam, rather than inherently weak spontaneous Raman scattering. Thus, studying skin tumor heterogeneity will allow for a better understanding of the cause and progression of these tumors for more effective diagnosis and treatment. SRS imaging has been applied to the early features of skin tumors. Visualization of tumor boundaries is especially useful for excision surgery,[130] which may increase the five-year survival rate of patients.

CARS is a nonlinear optical imaging modality that is used at the label-free and chemically determined frontiers of skin diagnosis. With CARS, the tuned frequency of the laser pump and Stokes scattering is matched to the Raman frequency of the molecule and leads to amplification of the anti-Stokes signals. This method was previously used to study the transport and motility of lipid droplets in living cells,[131] and proved its value as a noninvasive tool for the study of dynamic processes in single cells. CARS also provides 3D imaging capabilities, which were reported by Xie and his colleagues.[132]

4.8. *Confocal Raman spectroscopy (CRS) and laser tweezer Raman spectroscopy*

CRS is one of the most advanced Raman spectroscopic techniques. In 2022, Kocsis and his colleagues used CRS to study *in vivo* skin diffusion. They pointed that it was recently developed for skin composition test and epidermal penetration of different molecules at a specific time point. In their experiment, CRS was used to analyze skin spectra, natural moisturizing factor (NMF), keratin, ceramide, cholesterol, urea and water content.[133] According to another paper from Kocsis and his colleagues, they used CRS to study the chemical composition of excised rat and human skins. They also used it to monitor treatment-induced changes in skin parameters. Mainly components including ceramides, fatty acids, lactate and cholesterol are analyzed. First wavelength of 785 nm was used to analyze the skin fingerprint region (FP) and second laser of 671 nm was used for analyzing the high wave number (HWN) region. In their experiment, confocal Raman spectroscopy was performed with a GEN-2 SCA skin composition Analyser, which is River-D system.[134]

Many biological samples, including leukocytes and erythrocytes, require suspension in an aqueous solution to maintain their functionality. This condition limits the ability of conventional Raman spectroscopy to conduct single-cell measurements because living cells in a liquid may be displaced away from the laser excitation region due to Brownian motion.[135] This problem can be resolved by incorporating optical trapping in combination with a Raman spectrometer (Raman tweezers), in which Raman measurements are performed on a single cell that is optically immobilized in suspension away from the substrate [Figs. 2(a) and 2(b)].[136] The net trapping force resulting from the laser beam on a dielectric particle (e.g., a blood cell) is the cumulative effect of the scattering force, which acts in the light propagation direction, and the gradient force, which acts along the spatial light gradient.[137] Single-cell spectroscopy using the Raman tweezer is based on the principle of Raman spectroscopy. The Raman tweezer is an indispensable spectroscopic tool, which provides valuable insights into erythrocyte dynamics and cells under the influence of exogenous agents.

Raman tweezers are indispensable for investigating cellular behavior because of their label-free analysis and capability to characterize live cells.[138,139] This technique also allows for single-cell analysis of microbes, which can be applied to inflammatory skin diseases. Moreover, using an optical trap, living cells suspended in a liquid cultivation medium can be immobilized in a solution using the forces generated by a tightly focused laser beam, which has led to the recent use of Raman tweezers in cellular and molecular biology. Optical tweezers (OT) have already been used to evaluate hematologic diseases, such as erythrocyte deformation in sickle-cell anemia and thalassemia. Indeed, a study based on OT suggested that erythrocytes increase in patients with iron-deficient anemia. OT may also be used in the field of dermatology. Raman spectroscopy with OT has been proven as a reliable way to examine biochemical changes at the single-cell level of skin. For example, Raman tweezers can be used as an effective spectroscopic tool to study individual living erythrocytes given their ability to provide a micro-Raman spectrum. Meanwhile, the laser power used for capture can itself act as an external stimulus that can transmit pressure. Researchers have performed blood grouping, where Raman tweezer analysis combined with statistical models has been shown to be able to discriminate the AB group from other blood groups with absolute accuracy.[140]

Many other methods are available to help diagnose skin diseases. Wang and colleagues presented an elegant way to sort single cells by Raman spectroscopy along with laser-induced forward transfer, which was termed Raman-activated cell ejection.[141] In addition, Raman-FISH is a combination of fluorescence and Raman technology,[142] while Raman-activated cell sorting can be used to separate skin cells according to their Raman spectra. Moreover, some of these methods, such as surface-enhanced resonance Raman scattering, can be combined during skin disease diagnosis to combine the strengths of both SERS and RRS.

Although Raman spectroscopy has been widely used in various professional fields, significant limitations remain. In particular, two major challenges need to be addressed here. Firstly, the Raman signal is weak. Secondly, the interpretation of Raman spectral data is challenging. Indeed, a long acquisition time is always necessary to obtain a reliable Raman spectrum from a single skin cell. Furthermore, a high signal-to-noise ratio has been reported, with only one in 10 photons undergoing spontaneous Raman scattering.[143] In addition, when fixed to the matrix using physical or chemical methods such as freezing and drying, the inherent function of the cell will be disturbed. Other problems include the relatively high input costs and the need for complicated instrumentation and specialized operators.

Table 4. Raman spectroscopy techniques used for skin diagnosis.

Technique	Advantages	Limitations	Application of Raman spectroscopy in skin diagnosis	References
SERS	High sensitivity, high spectral resolution	Unsatisfactory molecular generality, relatively poor reproducibility	Skin cancer cells detection (e.g. melanoma, basal cell carcinoma, squamous-cell carcinoma)	92–99
TERS	High sensitivity, spatial resolution high (nanometer-scale)	Sample heating effect, far-field scattering background, low reproducibility	Virus discrimination of skin diseases such as vulgaris verruca, chicken pox	109–116
RRS	Chromophore selectivity, high sensitivity	Expensive, the intensity of RRS becomes weak or cannot be recorded with increasing wave number	Skin vascular anomalies diagnosis	117, 118
CARS, SRS	No signal overlap with one-photon excited fluorescence, natural confocality, Significant signal enhancement	Fiber optic probes for coupling with endscopes and automatic algorithms for data processing and evaluation, lack of easy-to-use and dedicated instruments	Skin tumor identification, surgical margin detection for different kinds of skin lesions, ability to investigate different properties of cream, shampoos and other cosmetic products, skin rejuvenation effect	119–127

5. Conclusions and Outlook

Raman spectroscopy is a non-invasive analysis of skin tissues that may be utilized in clinical practice. The growth in biological knowledge, the evolution of the instrument, and the development of a common Raman database will enhance the mutual collaboration and advancement in Raman spectroscopy technology, with increasing clinical applications expected in the future. Limitation such as the relationship between levels of natural moisturizing factors or the protein structure with hydration levels in single cells necessitates further study.

Although the application of Raman spectroscopy has many limitations, it holds potential value for detection, sorting and characterization in the field of skin diagnosis.

Acknowledgments

The authors gratefully acknowledge the financial support received from the Medicine and Engineering Cross Research Foundation of Shanghai Jiao Tong University (Project No. YG2022QN047), as well as the National Nature Science Foundation of China (Grant No. 62027824), and the National Key Research and Development Program of China (2019YFC1604604).

Conflicts of Interest

The authors declare that there are no conflicts of interest relevant to this paper.

References

1. N. Yadav, S. Parveen, S. Chakravarty, M. Banerjee, Skin anatomy and morphology, *Skin Aging & Cancer*, pp. 1–10, Springer, New York (2019).
2. S. Lawton, "Skin 1: the structure and functions of the skin," *Nurs. Times* **115**, 30–33 (2019).
3. E. S. Chambers, M. Vukmanovic-Stejic, "Skin barrier immunity and ageing," *Immunology* **160** (2), 116–125 (2020).
4. J. K. Cullen, J. L. Simmons, P. G. Parsons, G. M. Boyle, "Topical treatments for skin cancer," *Adv. Drug Delivery Rev.* **153**, 54–64 (2020).
5. D. Lee, S. N. Yoon, "Application of artificial intelligence-based technologies in the healthcare industry: Opportunities and challenges," *Int. J. Environ. Res. Public Health* **18**(1), 271 (2021).
6. P. Gerami *et al.*, "Development and validation of a noninvasive 2-gene molecular assay for cutaneous melanoma," *J. Am. Acad. Dermatol.* **76**, 114–120 (2017).
7. J. March, M. Hand, D. Grossman, "Practical application of new technologies for melanoma diagnosis," *J. Am. Acad. Dermatol.* **72**, 929–941 (2015).
8. P. Gerami, J. P. Alsobrook, T. J. Palmer, H. S. Robin, "Development of a novel noninvasive adhesive patch test for the evaluation of pigmented lesions of the skin," *J. Am. Acad. Dermatol.* **71**, 237–244 (2014).
9. C. J. Cockerell, J. Tschen, S. D. Billings, C. Rock, B. Evans, L. Clarke, "A retrospective study of the influence of a gene expression signature on the treatment of melanocytic tumors by dermatologists," *J. Am. Acad. Dermatol.* **72**, AB3 (2015).
10. U. B. Ansari, T. Sarode, "Skin cancer detection using image processing," *Int. Res. J. Eng. Technol.* **4**(4), 2875–2881 (2017).
11. L.-S. Wei, Q. Gan, T. Ji, "Skin disease recognition method based on image color and texture features," *Comput. Math. Method. M.* **2018**, 8145713 (2018).
12. M. Crisan, D. Crisan, G. Sannino, M. Lupsor, R. Badea, F. Amzica, "Ultrasonographic staging of cutaneous malignant tumors: an ultrasonographic depth index," *Arch Dermatol Res.* **305**, 305–313 (2013).
13. Y. Liu, X. Li, J. Cheng, N. Zhou, L. Zhang, H. Mao, C. Huang, "Sers devices with "hedgehog-like" nanosphere arrays for detection of trace pesticides," *J. Innovative Opt. Health Sci.* **14**(4), 2141005 (2021).
14. P. Yin, Q. Lin, Y. Duan, "Applications of Raman spectroscopy in two-dimensional materials," *J. Innovative Opt. Health Sci.* **13**(5), 2030010 (2020).
15. D. Wang, P. He, Z. Wang, G. Li, N. Majed, A. Z. Gu, "Advances in single cell Raman spectroscopy technologies for biological and environ mental applications," *Curr. Opin. Biotechnol.* **64**, 218–229 (2020).
16. D. A. Brafman, S. Chien, K. Willert, "Arrayed cellular microenvironments for identifying culture and differentiation conditions for stem, primary and rare cell populations," *Nat. Protoc.* **7**, 703–717 (2012).
17. A. J. Engler, S. Sen, H. L. Sweeney, D. E. Discher, "Matrix elasticity directs stem cell lineage specification," *Cell.* **126**, 677–689 (2006).
18. D. E. Jaalouk, J. Lammerding. "Mechanotransduction gone awry," *Nat. Rev. Mol. Cell Biol.* **10**, 63–73 (2009).

19. J. Xu, H. Ma, J. Jin, S. Uttam, R. Fu, Y. Huang, Y. Liu, "Super-resolution imaging of higher-order chromatin structures at different epigenomic states in single mammalian cells," *Cell Rep.* **24**(4), 873–882 (2018).
20. P. Fara, "A microscopic reality tale," *Nature* **459**, 642–644 (2009).
21. P. Gerami *et al.*, "Development and validation of a noninvasive 2-gene molecular assay for cutaneous melanoma," *J. Am. Acad. Dermatol.* **76**, 114–120 (2017).
22. M. Bustamante *et al.*, "Dose and time effects of solar-simulated ultraviolet radiation on the in vivo human skin transcriptome," *Brit. J. Dermatol.* **182**(6), 1458–1468 (2020).
23. A. Caporossi, S. Baiocchi, C. Mazzotta, C. Traversi, T. Caporossi, "Parasurgical therapy for keratoconus by riboflavin-ultraviolet type A rays induced cross-linking of corneal collagen: Preliminary refractive results in an Italian study," *J. Cataract. Refr. Surg.* **32**, 837–845 (2006).
24. J. Du, Y. Su, C. Qian, D. Yuan, K. Miao, D. Lee, A. H. C. Ng, R. S. Wijker, A. Ribas, R. D. Levine, J. R. Heath, L. Wei, "Raman-guided subcellular pharmaco-metabolomics for metastatic melanoma cells," *Nat. Commun.* **11**, 4830 (2020).
25. M. Kakita, M. Okuno, H. O. Hamaguchi, "Quantitative analysis of the redox states of cytochromes in a living L929 (NCTC) cell by resonance Raman microspectroscopy," *J. Biophotonics.* **6**, 256–259 (2013).
26. R. D. Snook, T. J. Harvey, E. Correia Faria, P. Gardner, "Raman tweezers and their application to the study of singly trapped eukaryotic cells," *Integr. Biol.* **1**(1), 43–52 (2009).
27. D. W. Shipp, F. Sinjab, I. Notingher, "Raman spectroscopy: techniques and applications in the life sciences," *Adv. Opt. Photonics* **9**(2), 315–428 (2017).
28. L. Xian, F. Xu, J. Liu, N. Xu, H. Ge, K. Shao, J. Fan, G. Xiao, X. Peng, "MicroRNA detection with turnover amplification via hybridization-mediated staudinger reduction for pancreatic cancer diagnosis," *J. Am. Chem. Soc.* **141**(51), 20490–20497 (2019).
29. C. Krafft, T. Knetschke, R. H. Funk, R. Salzer, "Studies on stress-induced changes at the subcellular level by Raman microspectroscopic mapping," *Anal. Chem.* **78**, 4424–4429 (2006).
30. U. Neugebauer, J. H. Clement, T. Bocklitz, C. Krafft, J. Popp, "Identification and differentiation of single cells from peripheral blood by Raman spectroscopic imaging," *J. Biophotonics.* **3**, 579–587 (2010).
31. C. Matthaus, C. Krafft, B. Dietzek, B. R. Brehm, S. Lorkowski, J. Popp, "Noninvasive imaging of intracellular lipid metabolism in macrophages by Raman microscopy in combination with stable isotopic labeling," *Anal Chem.* **84**, 8549–8556 (2012).
32. S. Schlücker, M. D. Schaeberle, S. W. Huffman, I. W. Levin, "Raman microspectroscopy: A comparison of point, line, and wide-field imaging methodologies," *Anal Chem.* **75**, 4312–4318 (2003).
33. M. M. Hlaing, M. Dunn, P. R. Stoddart, S. L. McArthur, "Raman spectroscopic identification of single bacterial cells at different stages of their lifecycle," *Vib Spectrosc.* **86**, 81–89 (2016).
34. T. Terai, T. Nagano, "Fluorescent probes for bioimaging applications," *Curr. Opin. Chem. Biol.* **12**, 515–521 (2008).
35. O. S. Wolfbeis, "An overview of nanoparticles commonly used in fluorescent bioimaging," *Chem. Soc. Rev.* **44**, 4743–4768 (2015).
36. M. Wagner, "Single-cell ecophysiology of microbes as revealed by Raman microspectroscopy or secondary ion mass spectrometry imaging," *Annu. Rev. Microbiol.* **63**, 411–429 (2009).
37. C. A. Owen, J. Selvakumaran, I. Notingher, G. Jell, L. L. Hench, M. M. Stevens, "In vitro toxicology evaluation of pharmaceuticals using Raman micro-spectroscopy," *J. Cell. Biochem.* **99**, 178–186 (2006).
38. Y. Li, Y. Guo, B. Ye, Z. Zhuang, P. Lan, Y. Zhang, H. Zhong, H. Liu, Z. Guo, Z. Liu, "Rapid label-free sers detection of foodborne pathogenic bacteria based on hafnium ditelluride-au nanocomposites," *J. Innovative Opt. Health Sci.* **13**(5), 2041004 (2020).
39. Y. Zhang, L. Lin, J. He, J. Ye, "Optical penetration of surface-enhanced micro-scale spatial offset raman spectroscopy in turbid gel and biological tissue," *J. Innovative Opt. Health Sci.* **14**(4), 2141001 (2021).
40. L. Zhang, T. Cambron, Y. Niu, Z. Xu, N. Su, H. Zheng, K. Wei, P. Ray, "MCR approach revealing protein, water, and lipid depth profile in atopic dermatitis patients' stratum corneum via in vivo confocal raman spectroscopy," *Anal. Chem.* **91**, 2784–2790 (2019).
41. C. Krishnamoorthy, A. Prakasarao, V. Srinivasan, G. N. S. Prasad, G. Singaravelu, "Monitoring of breast cancer patients under pre and post treated conditions using Raman spectroscopic analysis of blood plasma," *Vib. Spectrosc.* **105**, 102982 (2019).
42. U. Parlatan, M. T. Inanc, B. Y. Ozgor, E. Oral, E. Bastu, M. B. Unlu, G. Basar, "Raman spectroscopy as a non-invasive diagnostic technique for endometriosis," *Sci Rep.* **9** (2019).

43. A. T. Harris, M. Garg, X. B. Yang, S. E. Fisher, J. Kirkham, D. A. Smith, D. P. Martin-Hirsch, A. S. High, "Raman spectroscopy and advanced mathematical modelling in the discrimination of human thyroid cell lines," *Head Neck Oncol.* **38** (2009).
44. A. T. Harris, A. Lungari, C. J. Needham, S. L. Smith, M. A. Lones, S. E. Fisher, X. B. Yang, N. Cooper, J. Kirkham, D. A. Smith, D. P. Martin-Hirsch, A. S. High, "Potential for Raman spectroscopy to provide cancer screening using a peripheral blood sample," *Head Neck Oncol.* **1**, 134 (2009).
45. J. W. Chan, D. S. Taylor, T. Zwerdling, S. M. Lane, K. Ihara, T. Huser, "Micro-Raman spectroscopy detects individual neoplastic and normal hematopoietic cells," *Biophys. J.* **90**, 648–656 (2006).
46. J. W. Chan, D. S. Taylor, S. M. Lane, T. Zwerdling, J. Tuscano, T. Huser, "Nondestructive identification of individual leukemia cells by laser trapping Raman spectroscopy," *Anal. Chem.* **80**, 2180–2187 (2008).
47. P. R. Jess, D. D. Smith, M. Mazilu, K. Dholakia, A. C. Riches, C. S. Herrington, "Early detection of cervical neoplasia by Raman spectroscopy," *Int. J. Cancer.* **121**, 2723–2728 (2007).
48. F. Zheng, Y. Qin, K. Chen, "Sensitivity map of laser tweezers Raman spectroscopy for single-cell analysis of colorectal cancer," *J. Biomed. Opt.* **12**, 034002 (2007).
49. N. Majed, T. Chernenko, M. Diem, A. Z. Gu, "Identification of functionally relevant populations in enhanced biological phosphorus removal processes based on intracellular polymers profiles and insights into the metabolic diversity and heterogeneity," *Environ Sci Technol.* **46**, 5010–5017 (2012).
50. N. Majed, A. Z. Gu, "Impact of influent carbon to phosphorus ratio on performance and phenotypic dynamics in enhanced biological phosphorus removal (EBPR) system-insights into carbon distribution, intracellular polymer stoichiometry and pathways shifts," *bioRxiv.* (2019).
51. C. Nims, B. Cron, M. Wetherington, J. Macalady, J. Cosmidis. "Low frequency Raman spectroscopy for micron-scale and in vivo characterization of elemental sulfur in microbial samples," *Sci Rep.* **9**, 7971 (2019).
52. G. Guo, D. Wu, G. A. Ekama, N. P. Ivleva, X. Hao, J. Dai, Y. Cui, B. K. Biswal, G. Chen, "Investigation of multiple polymers in a denitrifying sulfur conversion-EBPR system: The structural dynamics and storage states," *Water Res.* **156**, 179–187 (2019).
53. C. M. Krishna, G. Kegelaer, I. Adt, S. Rubin, V. B. Kartha, M. Manfait, G. D. Sockalingum, "Combined Fourier transform infrared and Raman spectroscopic approach for identification of multidrug resistance phenotype in cancer cell lines," *Biopolymers.* **82**, 462–470 (2006).
54. H. Abramczyk, B. Brozek-Pluska, J. Surmacki, J. Jablonska-Gajewicz, R. Kordek, "Raman 'optical biopsy' of human breast cancer," *Prog. Biophys. Mol. Biol.* **108**, 74–81 (2012).
55. J. Surmacki, B. Brozek-Pluska, R. Kordek, H. Abramczyk, "The lipid-reactive oxygen species phenotype of breast cancer. Raman spectroscopy and mapping, PCA and PLSDA for invasive ductal carcinoma and invasive lobular carcinoma. Molecular tumorigenic mechanisms beyond Warburg effect," *Analyst.* **140**, 2121–2133 (2015).
56. P. Crow, B. Barrass, C. Kendall, M. Hart-Prieto, M. Wright, R. Persad, N. Stone, "The use of Raman spectroscopy to differentiate between different prostatic adenocarcinoma cell lines," *Br. J. Cancer.* **92**, 2166–2170 (2005).
57. A. Taleb, J. Diamond, J. J. McGarvey, J. R. Beattie, C. Toland, P. W. Hamilton, "Raman microscopy for the chemometric analysis of tumor cells," *J. Phys. Chem. B.* **110**, 19625–19631 (2006).
58. H. Lui, J. Zhao, D. McLean, H. Zeng, "Real-time Raman spectroscopy for in vivo skin cancer diagnosis," *Cancer Res.* **15**, 2491–2500 (2012).
59. I. A. Bratchenko *et al.*, "In vivo diagnosis if skin cancer with a portable Raman spectroscopic device," *Exp. Dermatol.* **30**, 652–663 (2021).
60. J. Zhao, H. Zeng, S. Kalia, H. Lui, "Using Raman spectroscopy to detect and diagnosis skin cancer in vivo," *Dermatologic Clinics.* **35**, 495–504 (2017).
61. M. M. Kelleher *et al.*, "Skin care interventions in infants for preventing eczema and food allergy," *Cochrane Database Syst. Rev.* **11**, CD013534 (2022).
62. Y. A. Khristoforova *et al.*, "Portable spectroscopic system for in vivo skin neoplasms diagnostics by Raman and autofluorescence analysis," *J. Biophotonics.* **12**, e201800400 (2018).
63. E. Guevara, J. C. Torres-Galvan, M. G. Ramirez-Elias, C. Luevano-Contreras, F. J. Gonzalez, "Use of Raman spectroscopy to screen diabetes mellitus with machine learning tools," *Biomed. Opt. Express.* **9**, 4998–5010 (2018).
64. L. A. Bratchenko *et al.*, "Raman spectroscopy of human skin for kidney failure detection," *J. Biophotonics.* **14**, e202000360 (2020).
65. M. Pannico, A. Calarco, G. Peluso, P. Musto, "Functionalized gold nanoparticles as biosensors for monitoring cellular uptake and localization in normal and tumor prostatic cells," *Biosensors (Basel).* **8**, 87 (2018).

66. K. Czamara, K. Majzner, M. Z. Pacia, K. Kochan, A. Kaczor, M. Baranska, "Raman spectroscopy of lipids: a review," *J. Raman Spectrosc.* **46**(1), 4–20 (2015).
67. A. C. S. Talari, Z. Movasaghi, S. Rehman, I. U. Rehman, "Raman spectroscopy of biological tissues," *Appl. Spectrosc. Rev.* **50**(1), 46–111 (2015).
68. S. Barkur, J. Lukose, S. Chidangil, "Probing nanoparticle–cell interaction using micro-Raman spectroscopy: silver and gold nanoparticle-induced stress effects on optically trapped live red blood cells," *ACS Omega.* **5**, 1439–1447 (2020).
69. S. E. Ament, H. S. Stein, D. Guevarra, L. Zhou, J. A. Haber, D. A. Boyd, M. Umehara, J. M. Gregoire, C. P. Gomes, "Multi-component background learning automates signal detection for spectroscopic data," *NPJ Comput. Mater.* **5**, 1–7 (2019).
70. Z. Li, X. Zhang, C. Xiao, D. Chen, S. Huang, P. Zhang, G. Wang, "Combination of multi-focus Raman spectroscopy and compressive sensing for parallel monitoring of single-cell dynamics," *J. Innovative Opt. Health Sci.* **14**(6), 2150021 (2021).
71. H. A. Cope, "Raman spectroscopy as a tool to improve enhanced biological phosphorus removal," (2016).
72. J. De Gelder, K. De Gussem, P. Vandenabeele, L. Moens, "Reference database of Raman spectra of biological molecules," *J. Raman Spectrosc.* **38**, 1133–1147 (2007).
73. S. Meisel, S. Stöckel, M. Elschner, F. Melzer, P. Rösch, J. Popp, "Raman spectroscopy as a potential tool for detection of *Brucella* spp. in milk," *Appl. Environ. Microbiol.* **78**, 5575–5583 (2012).
74. C. B. Grosan, D. A. Magdas, "Application of Raman spectroscopy and machine learning algorithms for fruit distillates discrimination," *Sci. Rep.* **10** (2020).
75. D. Lunter *et al.*, "Novel aspects of Raman spectroscopy in skin research," *Exp. Dermatol.* **31**, 1311–1329 (2022).
76. K. Varmuza, P. Filzmoser, *Introduction to Multivariate Statistical Analysis in Chemometrics*, CRC Press, Boca Raton, Florida (2009).
77. R. C. Spiker Jr., I. W. Levin, "Raman spectra and vibrational assignments for dipalmitoyl phosphatidylcholine and structurally related molecules," *Biochim. Biophys. Acta.* **388**, 361–373 (1975).
78. C. B. Fox, R. H. Uibel, J. M. Harris, "Detecting phase transitions in phosphatidylcholine vesicles by Raman microscopy and self-modeling curve resolution," *J. Phys. Chem. B.* **111**, 11428–11436 (2007).
79. C. J. Orendorff, M. W. Ducey, J. E. Pemberton, "Quantitative correlation of Raman spectral indicators in determining conformational order in alkyl chains," *J. Phys. Chem. A.* **106**, 6991–6998 (2002).
80. S. Heuke, N. Vogler, T. Meyer, D. Akimov, F. Kluschke, H. J. Rowert-Huber, J. Lademann, B. Dietzek, J. Popp, "Multimodal mapping of human skin," *Br. J. Dermatol.* **169**, 794–803 (2013).
81. B. G. Saar, L. R. Contreras-Rojas, X. S. Xie, R. H. Guy, "Imaging drug delivery to skin with stimulated Raman scattering microscopy," *Mol. Pharm.* **8**, 969–975 (2011).
82. X. Feng *et al.*, "Raman active components of skin cancer," *Biomed. Opt. Express.* **8**, 2835–2850 (2017).
83. C. J. Vavricka, B. M. Christensen, J. Li, "Melanization in living organisms: a perspective of species evolution," *Protein Cell.* **1**(9), 830–841 (2010).
84. D. Scherer, R. Kumar, "Genetics of pigmentation in skin cancer-A review," *Mutat. Res.* **705**, 141–153 (2010).
85. S. Benito-Martí nez, L. Salavessa, G. Raposo, M. S. Marks, C. Delevoye, "Melanin transfer and fate within keratinocytes in human skin pigmentation," *Integr. Comp. Biol.* **61**(4), 1546–1555 (2021).
86. F. M. Lyng *et al.*, "Raman spectroscopy for screening and diagnosis of cervical cancer," *Anal. Bioanal. Chem.* **407**, 8279–8289 (2015).
87. A. C. S. Talari, Z. Movasaghi, S. Rehman, I. Rehman, "Raman spectroscopy of biological tissues," *Appl. Spectrosc. Rev.* **50**, 46–111 (2015).
88. I. P. Santos, R. V. Doorn, P. J. Caspers, T. C. B. Schut, E. M. Barroso, T. E. C. Nijsten, V. N. Hegt, S. Koljenovic, G. J. Puppels, "Improving clinical diagnosis of early-stage cutaneous melanoma based on Raman spectroscopy," *Br. J. Cancer.* **119**, 1339–1346 (2018).
89. C. Choe, J. Schleusener, S. Choe, J. Lademann, M. E. Darvin, "A modification for the calculation of water depth profiles in oil-treated skin by in vivo confocal Raman microscopy," *J. Biophotonics.* **13** (2019).
90. I. Matveeva *et al.*, "Multivariate curve resolution alternating least squares analysis of in vivo skin Raman spectra," *Sensors.* **22**, 9588 (2022).
91. G. Kourbaj, S. Bielfeldt, M. Seise, K.-P. Wilhelm, "Measurement of dermal water content by confocal RAMAN spectroscopy to investigate intrinsic aging and photoaging of human skin in vivo," *Skin Res. Technol.* **27**, 404–413 (2020).
92. P. J. Caspers, G. W. Lucassen, E. A. Carter, H. A. Bruining, G. J. Puppels, "In vivo confocal Raman microspectroscopy of the skin: noninvasive

determination of molecular concentration profiles," *J. Invest. Dermatol.* **116**, 434–441 (2001).
93. J. Ye, H. Yang, N. Khlebtsov, "Introduction to the special issue on surface-enhanced Raman spectroscopy and functionalized plasmonic nanoparticles for biomedical applications," *J. Innovative Optical Health Sciences.* **14**, 2102002 (2021).
94. K. Kneipp, M. Moskovits, H. Kneipp, *Surface Enhanced Raman Scattering: Physics and Applications, Topics in Applied Physics*, Vol. 103, Springer, Berlin, Germany (2006).
95. P. L. Stiles, J. A. Dieringer, N. C. Shah, R. P. Van Duyne, "Surface-enhanced Raman spectroscopy," *Annu. Rev. Anal. Chem.* **1**, 601–626 (2008).
96. J. Popp, T. Mayerhofer, "Surface-enhanced Raman spectroscopy," *Anal. Bioanal. Chem.* **394**, 1717–1718 (2009).
97. C. Zong, M. Xu, L.-J. Xu, T. Wei, X. Ma, X.-S. Zheng, R. Hu, B. Ren, "Surface-enhanced Raman spectroscopy for bioanalysis: Reliability and challenges," *Chem Rev.* **118**, 4946–4980 (2018).
98. Y. Jiang, Y. Cai, S. Hu, X. Guo, Y. Ying, Y. Wen, Y. Wu, H. Yang, "Construction of au@ metal-organic framework for sensitive determination of creatinine in urine," *J. Innovative Opt. Health Sci.* **14**(4), 2141003 (2021).
99. T. Pylaev, E. Avdeeva, N. Khlebtsov, "Plasmonic nanoparticles and nucleic acids hybrids for targeted gene delivery, bioimaging, and molecular recognition," *J. Innovative Opt. Health Sci.* **14**(4), 2130003 (2021).
100. C. Duffield, N. Lyu, Y. Wang, "Synthesis and characterization of reporter molecules embedded core-shell nanoparticles as sers nanotags," *J. Innovative Opt. Health Sci.* **14**(4), 2141007 (2021).
101. M. Kahraman, M. M. Yazici, F. Şahİn, O.–"hskip 3pt "vskip 2pt..″ F. Bayrak, M. Çulha, "Reproducible surface-enhanced Raman scattering spectra of bacteria on aggregated silver nanoparticles," *Appl. Spectrosc.* **61**, 479–485 (2007).
102. P. Kubryk, J. S. Kolschbach, S. Marozava, T. Lueders, R. U. Meckenstock, R. Niessner, N. P. Ivleva, "Exploring the potential of stable isotope (resonance) Raman microspectroscopy and surface-enhanced raman scattering for the analysis of microorganisms at single cell level," *Anal. Chem.* **87**, 6622–6630 (2015).
103. R. Aroca, *Surface-Enhanced Vibrational Spectroscopy*, John Wiley & Sons, Chichester, UK (2006).
104. S. Harmsen, M. A. Wall, R. Huang, M. F. Kircher, "Cancer imaging using surface-enhanced resonance Raman scattering nanoparticles," *Nat. Protoc.* **12**, 1400 (2017).
105. J. Zhang, Y. Liu, H. Li, S. Cao, X. Li, H. Yin, Y. Li, X. Dong, X. Zhang, "Discrimination of periodontal pathogens using Raman spectroscopy combined with machine learning algorithms," *J. Innovative Opt. Health Sci.* **15**(3), 2240001 (2022).
106. J. Hu, X. Shao, C. Chi, Y. Zhu, Z. Xin, J. Sha, B. Dong, J. Pan, W. Xue, "Surface-enhanced Raman spectroscopy of serum predicts sensitivity to docetaxel-based chemotherapy in patients with metastatic castration-resistant prostate cancer," *J. Innovative Opt. Health Sci.* **14**(4), 2141006 (2021).
107. H. Li, Y. Cao, F. Lu, "Differentiation of different antifungals with various mechanisms using dynamic surface-enhanced Raman spectroscopy combined with machine learning," *J. Innovative Opt. Health Sci.* **14**(4), 2141002 (2021).
108. A. Jaafar, M. H. Mahmood, R. Holomb, L. Himics, T. Váczi, A. Y. Sdobnov, V. V. Tuchin, M. Veres, "Ex-vivo confocal Raman microspectroscopy of porcine skin with 633/785-nm laser excitation and optical clearing with glycerol/water/dmso solution," *J. Innovative Opt. Health Sci.* **14**(5), 2142003 (2021).
109. P. Verma, "Tip-enhanced Raman spectroscopy: Technique and recent advances," *Chem. Rev.* **117**, 6447–6466 (2017).
110. R. Meyer, X. Yao, V. Deckert, "Latest instrumental developments and bioanalytical applications in tip-enhanced Raman spectroscopy," *TrAC Trends Anal. Chem.* **102**, 250–258 (2018).
111. G. Kolhatkar, J. Plathier, A. Ruediger, "Nanoscale investigation of materials, chemical reactions, and biological systems by tip enhanced Raman spectroscopy–a review," *J. Mater. Chem. C.* **6**, 1307–1319 (2018).
112. N. Kumar, B. M. Weckhuysen, A. J. Wain, A. J. Pollard, "Nanoscale chemical imaging using tip-enhanced Raman spectroscopy," *Nat. Protoc.* **14**, 1169–1193 (2019).
113. L. Gao, H. Zhao, T. Li, P. Huo, D. Chen, B. Liu, "Atomic force microscopy based tip-enhanced Raman spectroscopy in biology," *Int. J. Mol. Sci.* **19**, 1193 (2018).
114. K. Olschewski, E. Kämmer, S. Stöckel, T. Bocklitz, T. Deckert-Gaudig, R. Zell, D. Cialla-May, K. Weber, V. Deckert, J. Popp, "A manual and an automatic TERS based virus discrimination," *Nanoscale.* **7**, 4545–4552 (2015).
115. S. Bonhommeau, S. Lecomte, "Tip-enhanced Raman spectroscopy: a tool for nanoscale chemical and structural characterization of biomolecules," *Chem. Phys. Chem.* **19**, 8–18 (2018).
116. E. Bailo, V. Deckert, "Tip-enhanced Raman spectroscopy of single RNA strands: Towards a novel direct-sequencing method," *Angew. Chem. Int. Ed.* **47**, 1658–1661 (2008).

117. Z. He, Z. Han, M. Kizer, R. J. Linhardt, X. Wang, A. M. Sinyukov, J. Wang, V. Deckert, A. V. Sokolov, J. Hu, "Tip-enhanced Raman imaging of single-stranded DNA with single base resolution," *J. Am. Chem. Soc.* **141**, 753–757 (2018).
118. C.-H. Liu *et al.*, "A pilot study for distinguishing basal cell carcinoma from normal human skin tissues using visible resonance raman spectroscopy," *J. Cancer Metastasis Treat.* **5**, 4 (2019).
119. E. V. Efremov, F. Ariese, C. Gooijer, "Achievements in resonance raman spectroscopy: Review of a technique with a distinct analytical chemistry potential," *Anal. Chim. Acta* **606**(2), 119–134 (2008).
120. F. Hu, L. Shi, W. Min, "Biological imaging of chemical bonds by stimulated Raman scattering microscopy," *Nat. Methods.* **16**, 830–842 (2019).
121. Y. Shen, F. Hu, W. Min, "Raman imaging of small biomolecules," *Annu. Rev. Biophys.* **48**, 347–369 (2019).
122. S. Li, Y. Li, R. Yi, L. Liu, J. Qu, "Coherent anti-stokes raman scattering microscopy and its applications," *Front. Phys.* **8**, 598420 (2020).
123. L. Gong, W. Zheng, Y. Ma, Z. Huang, "Higher-order coherent anti-Stokes Raman scattering microscopy realizes label-free super-resolution vibrational imaging," *Nat. Photon.* **14**, 115–122 (2020).
124. H. J. Lee, J.-X. Cheng, "Imaging chemistry inside living cells by stimulated Raman scattering microscopy," *Methods.* **128**, 119–128 (2017).
125. K. T. Schiessl, F. Hu, J. Jo, S. Z. Nazia, B. Wang, A. Price-Whelan, W. Min, L. E. Dietrich, "Phenazine production promotes antibiotic tolerance and metabolic heterogeneity in Pseudomonas aeruginosa biofilms," *Nat. Commun.* **10**, 762 (2019).
126. Q. Jin, X. Fan, C. Chen, L. Huang, J. Wang, X. Tang, "Multicolor Raman beads for multiplexed tumor cell and tissue imaging and in vivo tumor spectral detection," *Anal. Chem.* **91**, 3784–3789 (2019).
127. L. Zhang, L. Shi, Y. Shen, Y. Miao, M. Wei, N. Qian, Y. Liu, W. Min, "Spectral tracing of deuterium for imaging glucose metabolism," *Nat. Biomed. Eng.* **3**, 402 (2019).
128. D. Polli, V. Kumar, C. M. Valensise, M. Marangoni, G. Cerullo, "Broadband coherent Raman scattering microscopy," *Laser Photon. Rev.* **12**, 1800020 (2018).
129. R. C. Prince, R. R. Frontiera, E. O. Potma, "Stimulated Raman scattering: From bulk to nano," *Chem. Rev.* **117**(7), 5070–5094 (2017).
130. C. W. Freudiger *et al.*, "Multicolored stain-free histopathology with coherent Raman imaging," *Lab. Invest.* **92**, 1492–1502 (2012).
131. X. Nan, E. O. Potma, X. S. Xie, "Nonperturbative chemical imaging of organelle transport in living cells with coherent anti-stokes Raman scattering microscopy," *Biophys. J.* **91**, 728–735 (2006).
132. C. L. Evans, X. S. Xie, "Coherent anti-stokes Raman scattering microscopy: Chemical imaging for biology and medicine," *Annu. Rev. Anal. Chem.* **1**, 883–909 (2008).
133. D. Kocsis *et al.*, "Characterization and ex vivo evaluation of excised skin samples as substitutes for human dermal barrier in pharmaceutical and dermatological studies," *Skin Res. Technol.* **28**, 664–676 (2022).
134. D. Kocsis *et al.*, "Structural and functional analysis of excised skins and human reconstructed epidermis with confocal Raman spectroscopy and in microfluidic diffusion chambers," *Pharmaceutics.* **14**, 1689 (2022).
135. H. Ma, Y. Zhang, A. Ye, "Single-cell discrimination based on optical tweezers Raman spectroscopy," *Chin. Sci. Bull.* **58**, 2594–2600 (2013).
136. A. Bankapur, E. Zachariah, S. Chidangil, M. Valiathan, D. Mathur, "Raman tweezers spectroscopy of live, single red and white blood cells," *PLoS ONE.* **5**, e10427 (2010).
137. M. Asghari-Khiavi, A. Mechler, K. R. Bambery, D. McNaughton, B. R. Wood, "A resonance Raman spectroscopic investigation into the effects of fixation and dehydration on heme environment of hemoglobin," *J. Raman Spectrosc.* **40**, 1668–1674 (2009).
138. E. Zachariah, A. Bankapur, C. Santhosh, M. Valiathan, D. Mathur, "Probing oxidative stress in single erythrocytes with Raman Tweezers," *J. Photochem. Photobiol. B.* **100**, 113–116 (2010).
139. S. Barkur, A. Bankapur, S. Chidangil, D. Mathur, "Effect of infrared light on live blood cells: Role of β-carotene," *J. Photochem. Photobiol. B.* **171**, 104–116 (2017).
140. D. Lin, Z. Zheng, Q. Wang, H. Huang, Z. Huang, Y. Yu, S. Qiu, C. Wen, M. Cheng, S. Feng, "Label-free optical sensor based on red blood cells laser tweezers Raman spectroscopy analysis for ABO blood typing," *Opt. Express.* **24**, 24750–24759 (2016).
141. Y. Wang, Y. Ji, E. S. Wharfe, R. S. Meadows, P. March, R. Goodacre, J. Xu, W. E. Huang, "Raman activated cell ejection for isolation of single cells," *Anal. Chem.* **85**, 10697–10701 (2013).
142. M. Wagner, "Single-cell ecophysiology of microbes as revealed by Raman microspectroscopy or secondary ion mass spectrometry imaging," *Annu. Rev. Microbiol.* **63**, 411–429 (2009).

143. W. E. Huang, M. Li, R. M. Jarvis, R. Goodacre, S. A. Banwart, "Shining light on the microbial world: the application of Raman microspectroscopy," *Adv. Appl. Microbiol.* **70**, 153–186 (2010).
144. C. Lima, H. Muhamadali, R. Goodacre, "The role of raman spectroscopy within quantitative metabolomics," *Annu. Rev. Anal. Chem.* **14**, 323–345 (2021).
145. C. Mousoulis, X. Xu, D. A. Reiter, C. P. Neu, "Single cell spectroscopy: Noninvasive measures of small-scale structure and function," *Methods.* **64**, 119–128 (2013).
146. N. Mithun, J. Lukose, S. Shastry, G. Mohan, S. Chidangil, "Human red blood cell behaviour in hydroxyethyl starch: Probed by single cell spectroscopy," *RSC Adv.* **10**, 31453–31462 (2020).
147. N. Mithun, J. Lukose, G. Mohan, S. Shastry, S. Chidangil, "Single cell spectroscopy of red blood cells in intravenous crystalloid fluids," *Spectrochim. Acta. A Mol. Biomol. Spectrosc.* **257**, 119726 (2021).
148. H. K. Hesham, K. Schutze, "Raman trapping microscopy for non-invasive analysis of biological samples," *Anim. Cell Biotechnol.* **2095**, 303–317 (2019).