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RESEARCH ARTICLE

Bioethical Education and Standardization of Sample Handling Procedures in Raman Spectroscopy Research Studies Involving Human Subjects

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Abstract

Novelty: Raman spectroscopy is extensively explored for the disease diagnostics in recent decade. There is limited literature available concerning the standardization of sample handling procedures in Raman spectroscopy research studies involving human subjects. In fact, the present study provided guidance for a harmonize data to conduct Raman spectroscopy research studies involving human subjects for better outcomes based on ethical principles. **Objectives**: Globally, multi-disciplinary research is conducted for better outcomes in the welfare of humankind. The present study was aimed to provide basic guidance of bioethical education for research students conducting research involving human subjects. Further, the present study was attempted to standardize sample handling procedure in Raman spectroscopy research involving human subjects for better outcomes in disease diagnosis. **Materials and methods**: We have provided bioethical recommendations based on fundamental ethics codes. The standardization of sample handling procedure was developed using human surgical samples of breast cancer patients. **Findings**: The researcher should justify the inclusion and exclusion criteria for biological samples to conduct the scientifically valid research study. Research studies involving biological samples shall develop research protocol for the preparation and handling of biological samples. The results of present study suggest that fresh clinically unprocessed tissue samples are superior to conduct research studies involving Raman spectroscopy for disease progression. In case the research is not concerned with positive net benefit for human participants with diagnosis of communicable diseases, the researcher shall exclude these patients from the research study. Informed consent, preferably in writing, has to be obtained from participants in a language in that participant comprehend. **Conclusion**: The present study described primary bioethical education and standardization techniques for research students to plan and conduct Raman spectroscopy research studies involving human biological materials.

Keywords: Raman spectroscopy; Bioethics; Cancer; Diagnosis; Spectrum

1 Introduction

Ethics of the practice have a long history but formal documents containing information about the ethical education and practices in research involving human subjects were developed in the middle of the 20th century. Fundamental ethical standards for research involving humans were first codified into the Nuremberg Code in 1947 E$. The World Health Organization (WHO) recognized a need for guidelines or ethical standards that were broader in scope than the Nuremberg Code, and The Declaration of Helsinki: Recommendations Guiding Medical Doctors in Biomedical Research Involving Human Subjects was adopted by the World Medical Society in 1964. The concept of ethical committee was first appeared in the Declaration of Helsinki. The Declaration of Helsinki guidelines are key for ethics in medicine and these have been revised periodically [1] $. The National Commission for the Protection of Human Subjects of Biomedical and Behavioral Research, issued a comprehensive ethical document titled The Belmont Report: Ethical Principles and Guidelines for the Protection of Human Subjects of Research in 1979 [2] $. These are the best-known ethics codes and used globally to conduct research involving human subjects.

The term "Research" is defined as any systematic investigation designed to develop or contribute to generalizable knowledge. Globally, multi-disciplinary research is conducted for better outcomes in the welfare of humankind [3]. In general, the research students are not fully aware of the rules and regulations involving human subjects for biomedical research. Most of the research students are unaware of precautions related to handling biological samples and protecting patient rights. There is minimal literature available for providing guidance or formal education for planning and conducting research studies involving human subjects. In this line, the present study attempted to provide basic guidance for researchers conducting research involving human subjects.

Raman spectroscopy has wide range of utility not only in the field of science and technology but also growing research towards forensic, food, archeological and biomedicine [4]. The use of Raman spectroscopy for the analysis of biological specimens has significantly increased during the past few years. Raman spectroscopy has been extensively used in clinical diagnostics of cancer [5] including breast cancer [6–12], lung cancer [13], ovarian cancer [14,15], cervical cancer [16–18], oral cancer [19], nasopharyngeal carcinomas [20], brain tumors [21–23], Gastrointestinal Tumors [24,25], chondrogenic tumors [26], prostate cancer [27], colorectal cancer [28,29], skin cancer [30], lymphoma [31]. Moreover, Raman spectroscopy has been utilized as an emerging tool for diagnostics of various infectious diseases [32]. Therefore, it is important to discuss and standardize relevant Raman spectroscopic data acquisition procedure/ methodologies of biological samples, which will enhance understanding of this field for better outcomes. Understanding the practices of ethical foundation involve in biological samples will assist research students to enhance patient safety and protection. The present study summarizes the standardization of sample handling procedure for biological samples using Raman spectroscopy. The aim of this study is to provide professional guidance for bioethical education in Raman spectroscopy of biological samples.

2 Materials and methods

2.1 Acquisition of biological samples and experiment set up

2.1.1 Description of patients and samples

The present study uses human surgical samples of cancer patients who give consent to perform the study. The biological samples were obtained from the Modified Radical Mastectomy (MRM) surgical procedure. The breast was removed during this surgery. The normal tissue sections were obtained from 2 to 3 cm away from gross tumour volume with negative margin of the tissue removed during the surgery. The study was conducted under a protocol approved by the institutional bioethical committee at the Medical College (3095/MC/EC/12/04/2017). Five clinically unprocessed, fresh human surgical tissue samples were obtained from a patient. All excised samples in our study were normal tissue of the breast. The adoption of this methodology reduced selection bias in the standardization procedure.

2.1.2 Instrumentation and experiment details

The Raman spectra were recorded with a Confocal micro Raman spectrometer system with Photoluminescence (PL) STR 500 system (AIRIX Corp., Tokyo, Japan). In the present experimental work, confocal Spontaneous Raman Spectroscopy (SRS) in reflection mode was performed using incident excitation laser monochromatic beam of 532 nm with different combination of average laser excitation power and integration time 1mW, 5mW and 5sx3, 10sx3 respectively. It has Diode-Pumped Solid State (DPSS) Nd-YAG Green laser (DL 532, AIRIX Corp., Tokyo, Japan). The incident laser beam of the Nd:YAG laser (532 nm) was focused on the sample through a 20x dry objective, and a 4.0–2.9 mm working distance. The notch filter (Kaiser Optics, Ann Arbor, MI, USA) was used to remove Rayleigh scattered light. Further, all data processing and chemometrics analysis have been performed using the data analysis and graphing software OriginPro version 8.5 (OriginLab Corporation, Northampton, MA, United States).

Prior to the analysis, each raw spectral data was processed to auto-polynomial background correction and intensity normalization, cosmic ray removal, and spectral smoothening using OriginPro software. The removal of sharp spikes attributed to cosmic rays for spectra measurement was removed by using frequency and spatial filtering. Raman output data was processed with OriginPro software to quickly analyze and present meaningful information for visualizing, processing and managing spectroscopy data.

2.2 Bioethics in research involving human subjects

Bacterial infections related deaths are estimated among top 10 leading cause of death worldwide[33]. Healthcare-related infections prevalence in mixed patient populations of high-income countries is about 7.6% and about 10% in low-and middle-income countries[34]. Mostly, bacterial contamination is a necessary precursor to infection and commonly encountered in clinical settings. Skin bacteria are always present, despite thorough skin preparation during clinical procedures. In addition, numerous bacteria contaminate any other biological sample or body site involving a body structure/ organ ordinarily colonized by bacteria, such as the bowel[35]. The role of bioethical education in research involving human subjects is essential for protecting people and promoting trust. The present paper discusses fundamental issues and recommendations in bioethics in Raman spectroscopy research involving human subjects based on good work practices and ethics codes.

3 Results and discussion

In this section, we described our experimental setup for the acquisition of biological samples and handling protocol for Raman spectroscopy experiments and presented our results. The goal for this work was not only to create the best practices based on bioethical considerations for Raman spectroscopy, but also to provide comprehensive information for spectroscopy of biological samples involving human subjects. Finally, we report the results and experiment details for our experiments.

3.1 Protocol for handling and analysis of biological samples for Raman spectroscopy

3.1.1 Biological samples

The biological samples were immediately mounted on acetate paper. The absorption of excessive liquid around the tissue shall reduce the fluorescence background in the experiment. The biological samples were placed in an airtight sterile vial. The vial should withstand at low temperature for transportation to the Raman spectroscopy acquisition facility. The microscope slide was used as a platform for microscopic specimen observation. They are nearly transparent to visible light and chemically inert in nature. Following spectral data acquisition, formaldehyde was used to preserve tissue samples for histopathological analysis.

Hematoxylin and Eosin (H&E) was used as staining agents. Samples were prepared and stained using standard H&E-stained protocol. This allowed direct comparisons of tissue to group in normal or cancerous for spectral analysis in case multiple samples are involved.

The effect of formaldehyde on Raman spectroscopy is illustrated in Figure 1. The fluorescence was observed very high for human breast normal tissue sample after immersion in formaldehyde. In contrary to that, the spectral profile of fresh unprocessed normal tissue samples was observed pronounced. Further, notable spectroscopic differences exist with distinct peaks in the spectral profile. This is evident that fresh clinically unprocessed samples were the most suitable for conducting Raman spectroscopy.

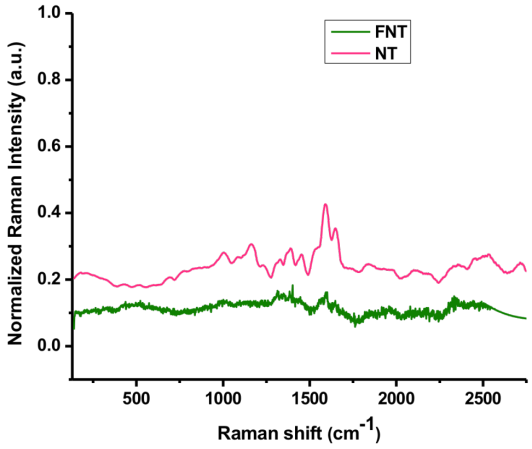

Fig 1. The comparison of typical average Raman spectra of the human breast fresh unprocessed normal tissue (red line) and breast normal tissue after immersion in formaldehyde (green line). NT – fresh unprocessed normal tissue, FNT – normal tissue after immersion in formaldehyde

3.1.2 Raman spectroscopy

The working distance between the sample and spectroscope eyepiece should be kept a minimum to reduce the fluorescence background. In the present study, the spectra of human surgical tissue samples were obtained and monitored at 22°C temperature. It is advised that the room temperature should be kept identical throughout the experiment. The spectra obtained at a high spectral resolution of 0.5 cm^{-1} and within the identical spectral range of 100 – 3000 cm^{-1} in the present study due to instrument limitation. However, the biological sample covers a spectral range of 500 – 4000 cm^{-1} for Raman spectroscopy depending on the sample type. After conformal focus, Raman spectroscopy was performed three consecutive times for each sample and average spectra were obtained to reduce uncertainty in the measurements.

Each corrected spectrum was processed and smoothened to increase the Signal-to-Noise Ratio (SNR) via spectral smoothing (Savitzky - Golay algorithm) procedure across the full wavenumber region recorded (100 – 3000 cm^{-1}). This reduces the influence of intensity changes caused by differences in cellular density and thickness of the tissue. The baseline subtraction was performed manually (using Origin) and the Raman spectral intensities were normalized. Baseline subtraction eliminates of the Rayleigh scattering. After baseline removal, the dominant remaining source of the distinction between spectra is the intensity of the Raman features, arising from the variable amount of biological material within the sample. Average Raman spectra were calculated as an arithmetic average of the recorded Raman spectra. For comparisons, Raman spectra were vector normalized in the Origin software (Raman intensity divided by the norm). The normalization allows comparing the Raman spectra from different measurements and samples. Apart from spectrometer calibration, the different combination of parameters in a spectroscope shall be tested for biological samples and analyzed the data to get maximum sensitivity and meaningful output, as shown in Figure 2. Average laser excitation power and integration time were (1mW, 5sx3), (5mW, 5sx3) and (5mW, 10sx3) and presented in the blue line, orange line and red line respectively. The results of our study indicate that the probability of Raman scattering decreases with increasing laser excitation power. However, it was observed that the amount of fluorescence also increases at low laser excitation power. Further, it was noted that increasing the integration time was contributing to enhance peak sharpness and intensity in the Raman fingerprint region. In conclusion, it is crucial to consider the parameters

for spectroscopic data acquisition for the application of spectroscopy in real-world settings. However, the researcher should critically think of the implementation of technology in routine practice before considering the parameters for spectroscopic data acquisition using human tissue samples.

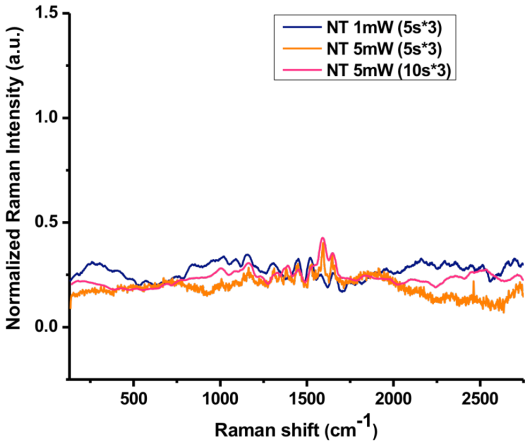

Fig 2. The comparison of typical normalized Raman spectra for various spectroscopic parameters (average laser excitation power and integration time) in human breast normal tissue. NT- Normal tissue

3.2 Bioethical practices

The investigator/researcher should specify the inclusion and exclusion criteria for biological samples to conduct the scientifically valid research study. The inclusion and exclusion criteria are devised after careful consideration as per the demand of the research design. This is performed while formulating the research question and defining the population/ group involved in conducting the research study. In general, the cross-sectional research study (such as surveys) involving healthy human participants, healthy volunteers/ controls poses no risk to the investigator/researcher. It is advised and preferable in case the research study can be performed with voluntary participation excluding the patients with pre-existing diseases. However, any prospective, experimental research and clinical trial involving humans or biological samples shall only be conducted after obtaining the ethical approval from the concerned ethical committee/ authority mandatorily.

The research study involving human participants with confirmed/ suspected diagnosis of non-communicable diseases such as diabetes, cancer, hypertension etc., pose almost no risk/ minimal risk to the researcher depending on the study or acquired sample. However, it should be noted that an infected researcher/ surveyor may transmit the infection to a cancer patient while conducting research. Since the cancer patient is immune-compressed. Hence, the research study shall be conducted only if the researcher in good health condition. Research studies involving biological samples shall develop a research protocol for the handling of biological samples. Appropriate measures shall be taken such as wearing gloves, masks/ respirators, sterile tongs, airtight, sterile chemical-free container and kept in a cool dust-free environment if they are fresh clinically unprocessed biological samples. These measures are enough to handle, transport and short duration storage for data acquisition. For long-term storage of the biological sample, samples shall be immersed into formalin solution or in the form of paraffin-embedded tissue blocks, also known as Formalin-Fixed Paraffin-Embedded (FFPE) tissue specimen. It is advisable to keep samples with tissue repositories and biobanks for long term storage of biological specimen. Several research studies conducted involving FFPE tissue blocks from tissue repositories for retrospective analysis of samples and understanding the process of carcinogenesis using spectroscopy [36,37]. They have reported deparaffinization method for spectroscopy of tissue blocks to attain more detailed results. However, they reported that the paraffin method of sample preparation can produce a background for spectroscopy. Further, they concluded that paraffin had an effect relating to the peaks intensity obtained by the Raman spectroscopy. Hence, the present study also recommends using spectroscopy with fresh unprocessed tissue samples to gain accurate results in understanding the disease progression.

After proper handling of biological samples, gloves and masks have to be disposed of as biomedical waste of the institute and washing hands with soap and water is recommended. WHO recommended 7 step washing of hand with water and soap for

hand hygiene to lowering the chances of any suspected infectious disease transmission. The use of masks to cover the mouth and nose is standard practice. The purpose is to prevent contamination and prevent exposure of the mouth and nose of the investigator/researcher from blood or other fluids from patients during a procedure involving biological samples. The use of masks significantly reduces contamination. It is well known that sterilization is the process by which an item is purged of all microorganisms and spores. Appropriate methods for sterilization of materials/ instruments are important in case they are reused during the research study involving biological samples.

Formalin is commonly used for long term storage of biological samples. Formalin contains aqueous solution of formaldehyde. Formaldehyde presents evidence for increased cancer risk in exposed individuals. WHO categorized formaldehyde in carcinogenic risks to humans. Formaldehyde presents strong evidence for increased cancer risk of Nasopharynx and Leukaemia/ lymphoma in exposed individuals. Appropriate preventive measures for inhalation and ingestion of formaldehyde shall be taken care of while handling with use of facemask and hand gloves.

The research study involving human participants with confirmed/ suspected diagnosis of communicable disease such as HIV, Hepatitis positive patients, Covid-19 and other biohazard patients, a great precaution has to be taken as they pose high risk of disease transmission. In case the research is not concerned with positive net benefit for these patients, one shall exclude these patients from the research study. Further, the researcher may seek the help from expert healthcare professional to develop research protocol to conduct study involving these patients/ biological samples. It is important to mention that the output discharge of waste generated by these patients shall strictly be disposed of as per the recent national guidelines of the Bio-Medical Waste Management Rules.

Before conducting the study for the individual, well documented informed consent, preferably in writing, must be obtained from study participants in a language that the participant comprehends. Prospective individuals (patient/participant) are independent in decision making for participation in the study and the participation is voluntary and they are free to withdraw from the study at any point. Potentially vulnerable subjects may include elderly people, prisoners, children, fetuses, cognitively impaired individuals, pregnant women, ill patients, tribals, or economically or educationally disadvantaged people. All vulnerable populations should receive specifically considered protection. Medical research with a vulnerable population is only justified in case the research is responsive to health needs or priorities of the vulnerable group and the research study is not feasible to conduct in a non-vulnerable group. Also, there is a reasonable likelihood that vulnerable population should be benefited from outcomes of the research. For vulnerable patients who are often unable to give consent, investigator/researcher shall obtain written informed consent from the approving authority, who is in close relation with the vulnerable patient and understand the condition of the patient. Moreover, it is the ethical responsibility of investigator/researcher that inclusion of vulnerable group is completely justified and these patients should not be exploited to generate clinical data in the research study.

4 Conclusion

The present study describes basic bioethical education and sample handling procedures for researchers to plan and conduct research studies involving human biological specimens obtained to develop new technologies and advance science to improve preventive, diagnostic and therapeutic potential. Fresh unprocessed tissue samples yield better spectroscopic information. Informed consent is good way and mandatory for the protection of patient rights. The researchers shall present and get approval from the concerned ethical committee/ authority prior to conduct research studies involving human subjects. The scope of this article is not limited to Raman spectroscopy and further can be used for other spectroscopic techniques involving biological samples. The standardization of procedure under bioethical considerations will provide the best outcomes in an optimized way for conducting the research studies involving human subjects.

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