Biobanking for cancer research: Preservation of tissue integrity – Some technical considerations

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ABSTRACT

Biobanking and biomarker discovery have become an integral part of neuro-oncology research. Towards achieving this end, the essential requirement is optimizing methods of tissue preservation of human tissues removed at surgery for diagnostic purposes and banking them for subserving future research. Owing to recent advances in molecular diagnostic tools, this clinical material has become a precious source for proteomic and genomic studies. The advent of biotechnological tools such as microarray, proteomics, and genomics has made it essential to preserve not just morphology but also the quality of nucleic acids and proteins, changing the traditional workflow of a pathology laboratory. It is therefore essential to develop simple technologies for tissue fixation and storage ensure that receptor and molecular integrity is reasonably maintained. Knowledge of the basic chemistry of tissue fixatives, the biochemical changes that take place in biological material by utilizing different techniques of fixation is essential while undertaking molecular, genomic, and proteomic studies on fresh and archival tissues.

Key words: Formalin, formalin-free fixatives, molecular integrity of tissue, biobanking, neuro-oncology

THE NEED FOR "BIOBANKS"

The pathologist continues to be the central figure in the diagnosis of cancer. The pathologist examines the resected specimen first and takes samples for diagnosis. Standard protocols have been evolved for tissue sampling in a systematic way for diagnosis of the tumor, with grading based on local and distant invasion. Immunohistochemistry and molecular biological techniques are now added to the diagnostic armamentarium for more accurate characterization of tumors and correlation with progression and prognosis. Studies on lymphomas, breast cancer, ovarian, and prostatic cancer are good examples wherein application of recent molecular techniques have made forays into understanding the biology and natural history of the tumors as well as to evolve newer directed therapies. Tissue microarray technology has enhanced our capacity to study and compare cancers from different individuals by high-throughput immunohistochemical and molecular biological studies, ushering in the possibility of personalized therapy. With advances in technology, demand for a well-annotated, properly preserved specimen has increased. To meet this, demand biobanks (archival tissue repositories) are established in many countries. Biobanking involves collection, processing, storage, and distribution of biological samples and their associated clinical data organized in a systematic way. A well-managed biobank is a prerequisite for biomedical research. *Time Magazine*, May 2009, has reported that "biobanking is one of the ten ideas changing the world right now."

In response to the demand, over the past 15 years, several biobanks have been established in different continents with the objective of archiving and distributing human specimens and promoting research and scientific initiatives. In view of the operational costs, many of the unregistered, "surgeon/pathologist" oriented small operations exist either as a few freezers in a research laboratory, hospital clinical laboratory, or pathology department. Some national biobanks operate in tandem with a centralized biobank that accepts samples from multiple locations. Some are project driven, meaning that specimens of cancer tissue and adjacent verified normal tissue are collected to answer specific research

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questions. "General collections" are established not to meet a specific research goal, but to be able to respond to multiple requests from researchers in an attempt to answer diverse questions. The success of biobanking models depends on a variety of factors including (a) ethical clearance from the patient to use the resected specimen for research and teaching, (b) the specimen collection procedure according to strict quality and operational controls, (c) proper diagnosis made according to international diagnostic criteria on the specimen, ensuring the representative area are banked fresh, (d) proper tissue fixation methods have been followed to ensure preservation of the antigen epitopes, receptors, and DNA/RNA in the tumor tissue, (e) proper database is maintained to retrieve the appropriate specimen and provide to the researcher with clinical/imaging/biochemical and pathological details, (f) distribution of the tissue with ensured maintenance of strict confidentiality of the donor, and (g) following mutually agreed sharing of research results and credits between the provider and the user. The biobanks require information about the research protocols, the funding received, justification for the specimen requested, and ethical handling of human material. They store fresh, formalin-fixed tissues, archival paraffin blocks, and derivatized samples such as buffy coat, purified DNA/RNA, serum samples, etc.

THE BIRTH AND REQUIREMENTS OF BIOBANKING

In the fall of 2005, Francil Collins, then Director of the National Human Genome Research Institute, told an audience of cancer patients, press reporters, and federal health officials that the technology employed by his team to map the complete genome by high-throughput DNA sequencing, lab automation, and computational biology will be utilized to fight cancers, calling the initiative "Cancer Genome Atlas." They initiated the study focusing on three of the most common and treatment-resistant forms of cancer: Glioblastoma of the brain, serous cancers of the ovary, and squamous cell carcinoma of the lung. A few months later, National Cancer Institute Researchers hit a stone wall, unable to analyze the DNA from hundreds of samples of cancer tissue received from different centers and tumor banks. The problem was not the data or technology, but the tumor tissue samples and blood provided by the biobanks. Many biobanks did not know the quality and appropriateness of the sample in their freezers. Some tumor samples were necrotic with very limited amount of viable tissue, inadequate for analysis. Some of them had been obtained without appropriate consent of the patient. This exemplifies the need for adherence to a set of best practices governing technical and ethical/legal issues, published by International Society for Biological and Environmental Repositories (ISBER, http://www.isber.org and NCI, http://biospecimens.cancer.gov).

Another important aspect realized in biobanks is to evaluate the quality of tissue for the first time when it is about to be shipped to the researcher. Checking the quality of the specimen when logged into the biobanks initially itself for storage will reduce the unpleasant surprises later to the researcher. For NCI (USA) funding, this is a mandatory guideline to cut the cost of wastage and enhance success in analysis. Biomarker research in cancer is a multibillion-dollar proposition which is heavily dependent on the availability of high quality tumor tissue in biobanks. The tumor tissue needs to be checked for the amount of viable tissue and relative absence of hemorrhage, necrosis, and calcification. This will facilitate availability of reasonable quantity of good quality tissue to initiate the research activity.

In India, neuro-oncology has emerged as a subspeciality, both in therapy and basic research. Demand for fresh control brain tissue and brain tumor tissue is increasing. High-throughput microarray analysis is attempted in some of the centers. Realizing the need of good quality archival tissue for various studies, this presentation deals with some of the important technical considerations vital for tissue banking archiving and neuro-oncology research. This material may be of "no or limited interest" to knife wielding active neurosurgeons but hopefully the "neuro-oncologist–surgeon–scientist" will realize its utility in safeguarding the interests of the future of neuroscience.

MOLECULAR QUALITY OF THE BANKED HUMAN TISSUES

For optimal extraction and feasibility of whole length sequencing of the genetic material, optimally frozen viable cancer tissues devoid of hemorrhage and necrosis are needed. In a serendipitous discovery in 1947, British scientist Christopher Polge discovered glycerol as an effective cryoprotectant.[1] Glycerol is still used in biobanks for preserving the blood cells, saliva, urine, and spermatozoa. Another decade old discovery still in use is dimethyl sulfoxide (DMSO). This technology is used to preserve small tumor tissue, cell lines, and blood cells and stem cells.[2] DMSO is, however, found to be toxic when transfused along with the cell lines for therapeutic purposes.[3,4] Hanna and Hubel patented a DMSO-free method of preserving the cells intended for stem cell

transfusion directly into patients.[5] Other technology tried is freezing the tissue at a carefully controlled rate immediately after harvesting, reducing the need of toxic antifreeze.

Following clamping the arterial supply to the tumor, within minutes the genes in the tissue start switching on and off, trying to adapt to the hypoxic/anoxic environment, yet attempting to utilize the remaining nutrients in the tissue, thus altering the gene expression pattern. The freeze–thaw cycles in the presence of cryoprotectants cannot shield the genome from the stress and the cells tend to enter apoptotic cycles. DMSO, the commonly used cryoprotectant, is found to have the potential of amplifying the genes related to metastatic potential thus reflecting the banked cancer tissue to be a "super cancer" while maintaining the morphology of cancer cells intact. Some of the cryoprotectants can lead to over estimating the molecular quality of biospecimens, thus partially corrupting the genomic data.[6] With the development of personalized medicine, these genetic alterations in the biobank environment have to be subtracted to compute genetic changes of relevance to develop therapeutic strategies. Recently, investigators have announced development of a blood test for cancer based on mutation in individual's mitochondria.[7,8] All the cryopreservatives and tissue preservatives have limitations, yet facilitate understanding the cellular and molecular biology of diseased tissue.

The most ideal tissue specimen is the one that carries a complete, unaltered representation of the tissue *in vivo*. Unlike the experiments with animal tissues, where the experimental conditions, the temporal sequence of tissue collection, and storage can be monitored and controlled, the human tissues collected at surgery and autopsy are subjected to unpredictable conditions that may contribute to partial degradation before they are processed and analyzed. With a growing number of scientists and oncologists requesting for human tissue for research and increasing use of tissue microarrays and high-throughput molecular profiling to evaluate and characterize the tissue, it is important to provide the investigators the data about the molecular quality of the tissue provided, especially from the archival collection.[9]

The general dogma about studies on archived tissue for molecular and genetic analysis are[10] (a) hypoxic conditions during the surgery, transport, and storage initiate cell death mechanisms and degradation,[11] (b) inactivating the proteolytic and degrading enzymes is important for preserving the tissue and nucleic acid integrity,[12] (c) fresh and frozen tissue are better than chemically fixed (formalin, alcohol, etc.) tissues to extract of DNA/RNA,[13] (d) short storage periods yield better quality and quantity of nucleic acid than long archival storage,[14] and (e) in the case of frozen tissue, the yield of DNA/RNA is enhanced by rapid thawing.[15] Human tissues (tumor and brain) stored frozen for variable periods are found to be useful for biochemical studies[16] and molecular studies as shown in the study by the Cooperative Human Tissue Network (CHTN).[10]

Immediate freezing of the tumor biopsy samples in liquid nitrogen and storing at -70 to -80°C or in liquid nitrogen vapor is the frequently prescribed protocol for analysis of RNA. However, in a busy operation theater it may not be logistic or practical in many centers. It is shown by Jewell *et al.*[10] using a time course study that good nucleic acid stability is maintained up to 5 hrs following resection at room temperature and mRNA is measurable by RT-PCR up to 12 hrs before fixation of tissue. It is essential to establish a database of stability of nucleic acid in various tumor tissues in view of inherent differences in the nuclease content in different tissue types. It must be kept in mind that quality control tests for molecular integrity of the tissues procured and stored in the banks need to be maintained. A protective effect on RNA quality is found when tissues had been stored at 4°C as compared to room temperature. The mean size of the mRNA fragments decrease with longer prefixation times. van Maldegem *et al.*[17] could achieve an amplified maximum PCR product length of 750 bp from fresh tissues kept at 4°C or room temperature up to 24 hrs. By RT-PCR aimed at housekeeping genes, they found formalin fixation for 8–10 hrs to be optimum and PCR products of maximum length could be obtained. For biobanking, transportation of resected tissue on ice, snap freezing by liquid nitrogen, and storage at low temperature are recommended. The use of RNA later solution prevents RNA degradation in tissues and stabilizes the expression levels of selected genes. Good quality RNA and a reliable expression pattern could be obtained even after 2 weeks from tissues stored in RNA later solution at 4°C. However, the tissue morphology is better in snap frozen tissue compared to RNA later preserved samples.

The analytical approach in histopathology has undergone significant evolution as antigenic receptors, and gene evaluation is mandatory now to firmly establish the diagnosis. This evaluation is dependent on optimal tissue fixation for the molecular preservation of human specimens in the archives of pathological departments, relying on technology of Victorian era: Formalin fixation and paraffin embedding. In a typical cancer surgery, the surgeon excises the lump of fresh tissue suspected to be malignant and it is transferred to formalin (10% formaldehyde). Formalin through aldehyde bridges the reactive groups in the proteins in the tissue arresting all

metabolic processes. The paraffin embedding prevents further oxidation. Such tried and tested preservation protocol has endured the test of time because the primary consideration for the pathologist has been preservation of cellular architecture, without realizing the importance of internal cellular biochemical and molecular markers. In long archived tissues in formalin, the reactive groups are oxidized to more stable groups which cannot be got rid off by washing in water and alcohol. For gene expression analysis of DNA and RNA, the presence of extractable nucleic acids is mandatory. Although in recent days techniques are developed for protein and nucleic acid extraction from formalin-fixed tissue, quality of phosphoproteins and nucleic acids is not satisfactory for a meaningful analysis. This may be partly because of rapid dephosphorylation of proteins in the neoplastic tissues that have quickly become oxygen deficient during the surgery.[18] If the tumor specimens are not fixed immediately, majority of the phosphoproteins are lost within 60 min. This happens usually in the central part of large specimens. Protein phosphorylation is commonly used as an indicator of cell signaling activity and for targeted therapy in controlling cell proliferation and promotion of apoptosis in cancer cells.[19] The overall rate of formalin-induced modification of DNA is dependent on the concentration, temperature, and pH of the fixative, nearly 30% of high molecular weight DNA being degraded. Some of the studies suggest that tumor tissues fixed in formalin at 4°C exhibit least amount of degradation of nucleic acids.[20] When compared to DNA isolated from frozen tissues, formalin-fixed tissues exhibit a high frequency of nonreproducible sequence alterations. As a result in PCR, the Taq-DNA polymerase fails to recognize the cytosine and incorporates adenine in the place of guanine, creating an artificial C-T or G-T mutation.[21] Formalin solution containing "DNAse neutralizing" EDTA is better in preserving tissues.[22] Although it is possible to extract proteins from formalin-fixed tissue, the rate of penetration of formalin is variable and hence the deeper layers of tissue show significant fluctuation in the phosphoprotein levels in large specimens. The conditions recommended for the use of formaldehyde as a tissue nucleic acid fixative are (a) minimum prefixation lag (<2 h), (b) use of cold 10% buffered formalin with addition of EDTA, (c) use of adequate volume of formalin fixative (nearly 20 times the volume of tissue as formalin rapidly becomes hydrated to form methylene glycol), and (d) duration of fixation of 3–6 h.[23] The best practical way is to sample multiple, small representative tissue bits and fix in appropriate volume.

RNA isolated from formalin-fixed, paraffin-embedded blocks when subjected to heating for 2 h at 70°C than routine 10 min at 60°C is found to yield better cDNA.[24] DNA preservation is good and suitable for genetic studies in the brain tissue preserved at indicate -80oC clearly up to 35 years. Acceptable DNA recovery is possible from tissues fixed in formalin for a short period in buffered formalin. DNA mutations cannot be analyzed in tissues fixed in formalin for more than 6 months. In tissues fixed in formalin for years, it is almost impossible to recognize point mutations, deletions, and triplet expansions.[25]

Formalin, though cost-effective, easy to prepare, and preserves morphology, causes unpredictable and reversible loss of immunoreactivity altering the antigen-binding sites. Immunohistochemistry has become practical and a mandatory technique to detect abnormal protein expression of altered genes in cancer. The main application includes detection of differentiation antigens for classifying the undifferentiated tumors in routine histology, immunophenotyping of lymphomas, characterizing neuroendocrine, and soft tissue tumors as well as various growth factors and receptors. Immunohistochemistry is also used for the diagnosis of various infectious agents by using specific antibodies to viral, bacterial, and fungal antigens to monitor bedside management. One of the important factors controlling the success of immunochemistry is proper tissue fixation, processing, unmasking of antigenic epitopes. Only *in situ* proteins can be identified qualitatively by immunohistochemistry and its intracellular localization, but not the quantitative estimation in a formalin-fixed tissues. International Agency for Cancer Research (IARC) has labeled formalin as class I carcinogen.[26]

FORMALIN-FREE FIXATION FOR DIAGNOSIS AND RESEARCH

These limitations in formalin fixation have led to search for "formalin-free fixatives" to use for archiving tissues in routine practice and cancer biobanks for high-throughput microarray studies. Carnoy's fixative (six parts ethanol, three parts chloroform, and one part glacial acetic acid) is optimal for preservation of nucleic acids in tissues and RNA can be extracted.[27] But because of chloroform, lipids are extracted, though histology is preserved well. Methacarn (Methanol instead of ethanol in Carnoy's fixative) fixation is a novel tool for analysis of the gene expression profile in paraffin-embedded tissue specimens.[28] Modified methacarn (a mixture of methanol and acetic acid) is gentle on tissue membranes, and immunochemistry can be performed with a shorter incubation period and higher dilution of antibodies.[29] Modified zinc-based fixative is a nonformalin, reliable, cost-effective, nontoxic fixative with excellent integrity of RNA and DNA for molecular

analysis, and antigen retrieval by heat is not needed for immunohistochemistry.[30] DNA sequences up to 2.4 kb and RNA fragments up to 362 bp in length could be successfully amplified. However, the zinc fixatives can cause shrinkage of tissues distorting the histology.[31] Ethanol-based fixatives do not create covalent bonds, eliminates water of hydration, inactivates proteases, and maintains the conformation and function of the proteins. "FineFIX" fixative containing a mixture of ethanol, glycerol, polyvinyl alcohol, and monomeric carbohydrates in distilled water prevents tissue shrinkage, vacuolation, and nuclear pyknosis. This fixative is suitable for long-term fixation, proteomic analysis, and allows laser capture microdissection of cells for molecular and proteomic studies, comparable to fresh tissues. FineFIX-treated tissues are found to allow better DNA (formalin up to 350 bp FineFIX over 2400 bp) and RNA (formalin 100–200 bases: FinoFix up to 600 bases) analysis. It fixes tissues rapidly, and histological artifacts are minimal. The proteins extracted from FineFIX-treated samples are comparable in quality with those obtained from fresh frozen tissue, thus facilitating conventional protein analysis.[32] Multipurpose ethanol-based fixatives containing phosphatase and kinase inhibitors preserve phosphoproteins and maintain histomorphology for frozen sectioning and paraffin embedding. RNA integrity is maintained for about 72 h at room temperature and allows immunohistochemistry and proteomic analysis.[33] HOPE fixation (Hepes-glutamic acid buffer mediated organic solvent protein effect): This protection solution comprising of a mixture of amino acids at pH 5.8–6.4 penetrates tissue by diffusion preserving the morphology and proteins and antigenic structure for differential analysis by immunohistochemistry and enzyme histochemistry. Good quality of DNA and RNA can be extracted even after a period of many years from HOPE-fixed tissue specimens. Absence of cross-linking and greater yield of extractable nucleic acids makes it a suitable fixative for tissue banking.[34] Acetone–methylbenzoate xylene (AMeX) fixative preserves the morphology, and high molecular weight DNA and good quality RNA can be extracted.[35] Universal molecular fixative (UMFIX) is a methanol/polyethylene glycol-based fixative and is useful for small biopsy tissues such as stereotactic biopsies/endoscopic biopsies to amplify small amplicons by RT-PCR. Tissue can be fixed at room temperature, and immunoreactivity of UNIFIX-fixed tissue is comparable to formalin-fixed tissue. The quality and quantity of mRNA and DNA extracted from frozen and UNIFIX-fixed tissue are similar, and high molecular RNA can be extracted by laser capture microdissection from UNIFIX-fixed paraffin embedded tissues.[36] RCL2 is a formalin-free fixative containing ethanol, acetic acid, and complex carbohydrate, and RCL2 fixed tissue can be kept at room temperature while at -20°C when high molecular quality is required. Morphology, tinctorial quality, and immunoreactivity are better than formalin-fixed tissue. Quality of RNA and protein profile from RCL2-fixed paraffin embedded tissue is comparable to frozen sections.[37,38]

An Italian company has commercialized a vacuum-sealed device as an ideal way to transport large fresh specimens in a formalin-free atmosphere from the operation theater to pathology department for diagnosis and tissue banking. Because of vacuum, there is no chance of drying and the technique is excellent for histochemical and molecular biological studies.[39] The histomorphology is found to be well preserved up to 5–9 days and provides excellent material for tissue banking. Histochemistry, immunohistochemistry, and FISH techniques can be carried out, and the quality of nucleic acid is better if refrigerated under vacuum. This can also be used for transportation of specimens for banking and transplantation.

The branch of biotechnology and industry is ready to meet the challenges in tissue preservation, storage, and processing, a critical issue in cancer tissue banking. With the sequencing of human genome in 2003 and the development of microchip and microarray has given an insight into the molecular interior of the cells, which a pathologist with microscope cannot detect. The scientific utility of the molecular biological and proteomic data obtained from archived tissue especially is dependent on specimen quality. In the era of revolution in biotechnology, molecular genetics and proteomics, questions like, "How does the DNA sequence differ among individuals in the society?" and "What makes an individual prone for a certain form of disease or brain tumor?" can be addressed. Many novel genes and their translated protein have been identified, some named as biomarkers, by screening the archival tissues.

Attempts at RNA isolation from sections on which standard immunostaining has been carried out has been found not possible, the detrimental factors being the blocking step, the commercially available antibody diluents, and the amplification kits.[17] PBS buffer diluted antibodies greatly improve the recovery of RNA from the cells.

Multiple antigen localization by immunohistochemistry is an important and yet to be well established technology to screen multiple antigens in the brain tumor and brain tissue. Usually multiple primary antibodies raised in different animals or of diverse IgG isotypes are utilized

to facilitate specific labeling. However, the suitable primary antibodies are usually not available for chosen antigen localization. Kim *et al.*[40] and Pirici *et al.*[41] have developed a technique of using removable chromogen stain and efficient "antibody stripping technique" on formalin-fixed paraffin-embedded sections to facilitate sequential immunostaining with different primary antibodies on the same section regardless of the animal species in which antibody is raised. They used 10% SDS, Tris–HCl, β-mercaptoethanol as the antibody stripping solution and found it to be better than $KMnO_4$ or Glycine/SDS. The technology is found to be relatively robust. The methodology is found useful for a global view of protein colocalization and differential expression in the heterogeneous cellular population in tumor and the normal brain in the same section. This adds to the tissue microarray technology for handling multiple antigens in multiple tissue samples, when standardized for the laboratory. This avoids the need for confocal microscopy for routine use, though resolution does not match that of confocal microscopy for multiple antigen localization.

PROTEOMIC ANALYSIS

Formalin-fixed and paraffin-embedded tissue archives represent the largest clinically annotated biospecimen resource in many of the medical centers involved in oncology research using newer molecular biology techniques developed. Advances in mass spectroscopy-based proteomic analysis have facilitated global protein expression profiling and biomarker discovery. For the validation, formalin-fixed paraffin-embedded tissues are used extensively. The heat induced antigen retrieval strategies for immunohistochemistry has been found to offer partial cross-link reversal, enabling the use of such tissues for proteomic validation studies. Nirmalan[42] have described a new heat-induced antigen retrieval strategy using SDS-containing Lammli buffer for efficient intact protein recovery from formalin-fixed paraffin-embedded tissues for Western blot analysis. They achieved efficient extraction of full length, nondegraded immunoreactive protein, comparable to frozen tissue. However, they observed variability in the extraction and expression efficiency of membrane-associated proteins.[42]

In a routine histopathology laboratory, in hospital departments of pathology, all tissues either big or small are fixed in formalin and paraffin embedded. After a few sections are cut for histopathological examination, the tissues are archived for varying length of periods, extending to decades. These archival tissues become precious resource material especially for the studies of molecular epidemiology, brain tumor classification, study of brain tumor genetic heterogeneity, and rare syndromic diseases and case studies with very long follow-up periods, with known therapy outcomes and evaluating response to new treatment regimens.

Formalin though compromises the DNA and RNA integrity, it is found possible to analyze them by PCR/RT-PCR, keeping the limitations in mind. In contrast, protein analysis is impractical due to the covalent bonds between proteins in the tissue resulting in poor quantitative analysis of protein expression. On the other hand, ethanol/methanol-based fixatives, though coagulative in nature, do not create covalent bonds between proteins, eliminate the specific molecules of water of hydration, and thus preserve the molecular conformation and function. Hence, the alcohol-based fixatives facilitate conventional proteomic analysis. Two alcohol-based fixatives RCL-2 and FineFIX (Milestone SrL, Bergano, Italy) are found to be very useful for proteomic and molecular genetic studies, comparable to fresh frozen and archived tissues at -80°C.[37]

Cancer is only one facet of human diseases. Infections, neurodegenerative diseases, diabetes, and cerebrovascular–cardiovascular diseases add to the list of human diseases. Biobanks are emerging to collect the tissues in these disorders following standard and stringent conditions to facilitate research on human tissue, with a fond hope of finding new leads for cure.

The emergence of transmissible and serious infection diseases like Acquired Immuno Deficiency Syndrome (HIV/AIDS), Hepatitis C, Creutzfeldt–Jacob disease, antibiotic-resistant tuberculosis, *Staphylococcus* and *Enterococcus*, and JCV/BHK viral infection have necessitated increased focus on safety of health care workers especially in fresh tissue biobanks. Following the universal precautions during collection, freezing, and retrieval for distribution is mandatory. The plasma sample collected prior to surgery has to be tested for HIV-1, Hepatitis B, and Hep C. *Mycobacterium tuberculosis* and other tropical diseases are tested for as a safety measure. This information has to be made available to the researchers as a mandatory protocol, to prevent accidental infections to the technical personnel and the investigators. The fresh tissue dissection should be carried out in a biohazard hood dedicated for dissection, retrieval, and distribution of the tissue, and it should be designed with negative pressure to control airflow in the space dedicated and avoid environmental contamination. All the instruments, work areas, and the exterior of all the specimen containers should be decontaminated using 10% solution of sodium hypochlorite (common household bleach). A well-tested and approved tissue

disposal protocol has to be followed. These procedures in reality are not new to a pathology department receiving the fresh specimens for diagnosis though not practiced consistently for various reasons. Biobanks, because of inherent nature of storing fresh tumor tissues and distributing them, tissue-handling procedures need to be stringent and of international standards to avoid unexpected events.

Most of the international biobanks do not consider biospecimens to be tradable commodities and thus operate as "not-for-profit" organizations. They depend on government and/or charitable organization for funds, and minimal charges are levied to the researchers for transport and other incidental expenses. To facilitate broader specimen collection, international biobank networks are established, sharing the material based on agreed upon scientific, ethical, and legal principles. Biobanking is an activity requiring dedicated staff to obtain patient consent and collection of samples. These can be established in a hospital pathology department, as the need of diagnosis dictates that sample flow through these departments. It is ideal that the pathologist must examine resected specimens first, take samples for diagnosis, before the sample is stored for research. In view of operational ease in small biobanks, the staff members at the collection site are the custodians of the samples they collect. In a network situation, a portion of the collection is made available to researchers at other sites, the members providing financial support for the network. This model reduces the perceived ownership of the specimen by the surgeons and pathologists and thus promotes science and philosophy of sharing between the scientific fraternity.

CAN INDIA INITIATE A BIOBANK AND THE PATHOLOGIST MEET THE CHALLENGE TO PROMOTE RESEARCH IN NEURO-ONCOLOGY AND NEUROPATHOLOGY?

Because of short sightedness of the medical specialists and indifferent attitude of pathologists in the hospitals in India, the idea of biobanking to promote research and participate in probing the human disease has not received its deserved attention. Similarly, the reluctance on the part of pathologists and the trainees to conduct postmortem studies and preserve the tissues fresh has contributed to it. India because of varied ethnic population can be a rich source of research material, leading to seminal contributions in geographic pathology. The patterns of cancer in India are found to be different, highlighting the genetic and environmental factors modulating the expression and progression of neoplasia. A Cancer Biobank dedicated to a few specific varieties of neoplasia is established at ACTREC-TMH, Mumbai, and is used for cancer research. Human Brain Tissue Repository for Neurobiological studies has been functioning at Department of Neuropathology, NIMHANS, Bangalore, for the past 15 years. The training in pathology has changed from the Victorian era of simple histopathology on formalin-fixed, paraffin-embedded study to molecular biological and genetic pathology, thanks to the keen interest evinced by the basic scientists. Molecular genetic studies have become mandatory in cancer hospitals in India. Basic scientists are involved in proteomic and genomic analysis of human cancer, to identify peripheral biomarkers for early diagnosis and predicting recurrence and prognosis. In all these studies, the pathologist continues to be the central figure, starting from collection of specimen for diagnosis, storing, archiving the material, and developing a database thus actively contributing to cancer biobanking. In many of the laboratories, diagnostic pathology histopathology specimens including tumor specimens are discarded periodically as a "biological waste." Storing these annotated formalin-fixed specimens as paraffin block archives represents transforming a biological waste to "precious biological resource" for research. A few dedicated regional centers of archived tissue bank networks can be developed in the country, sharing the credit for it as a consortium. It is mandatory to ensure proper, willing consent from the patient prior to surgery, to utilize the remaining tissue after the diagnosis for research and teaching with facility to distribute it to investigators maintaining confidentiality. The managers of Biobanks of archival tissue need to be aware of the limitations and problems in the usually practiced techniques, be abreast with recent technology and be involved in a constant dialogue with the basic scientists. With good effort, the pathology departments can become small biorepositories initially archiving properly annotated formalin-fixed tissue and paraffin blocks and later fresh tissues. To make a beginning, the regional pathologists can consider forming a network and developing a database of regional cancers, diagnosed by modern technology. A proactive approach should be undertaken to form regional referral centers to assist each other in diagnosis, and sharing well-characterized material for research. "We, the pathologist need to evolve a new level of awareness that the tissues in the bucket is now one of the most important parts of the patient, because analysis of this tissue will determine all treatment decisions downstream," said Carolin Compton, Chief of NCI Biorepository, USA. Biobanking has prodded the biotechnologist with challenges to come up with innovations and pass it to the industry to deliver solutions to the pathologist and the managers of biobanks.

Let the pathologist become the hub of the team identifying the relevant questions, enrolling the basic scientist, neurosurgeon, biotechnologist, and industry to evolve practical and cost-effective answers for storage, easy retrieval, sharing, innovative analysis thus enhancing integrated job opportunities to the next generation clinical investigators and onco-pathologists. A pathologist, as a diagnostician, is part of the team of surgeons, imageologists, basic scientists, and biotechnologists for the bedside patient care and new discoveries. A biobank in a medical center turns a biological waste of cancer tissue after diagnosis into a precious resource for research and a knowledge bank for the future.

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REFERENCES

1. Polge C. Fertilizing capacity of bull spermatozoa after freezing at 79 degrees C. Nature 1952;169:626-57.
2. Rowley SD, Anderson GL. Effect of DMSO exposure without cryopreservation on hematopoietic progenitor cells. Bone Marrow Transplant 1993;11:389-93.
3. Júnior AM, Arrais CA, Saboya R, Velasques RD, Junqueira PL, Dulley FL. Neurotoxicity associated with dimethylsulfoxide-preserved hematopoietic progenitor cell infusion. Bone Marrow Transplant 2008;41:95-96.
4. Abdelkefi A, Lakhal A, Moojat N, Hamed LB, Fekih J, Ladeb S, *et al.* Othman TB. Severe neurotoxicity associated with dimethyl sulphoxide following PBSCT. Bone Marrow Transplant 2009; 44:323-4.
5. Hanna J, Hubel A. Preservation of stem cells. Organogenesis 2009;5:134-7.
6. Cross SS, Start RD, Smith JH. Does delay in fixation affect the number of mitotic figures in processed tissue? J Clin Pathol 1990;43:597-9.
7. Lievre A, Chapusot C, Bouvier AM, Zinzindozoue F, Piard F, Roignot P, *et al.* Clinical value of mitochondrial mutations in colorectal cancer. J Clin Oncol 2005;23:3517-28.
8. Bra-goszewski P, Kupryjanczyk J, Bartnik E, Rachinger A, Ostrowski J. Limited clinical relevance of mitochondrial DNA mutation and gene expression analysis in ovarian cancer. BMC Cancer 2008;8:292.
9. Schraml P, Kononen J, Bubendorf L, Moch H, Bissig H, Nocito A, *et al*. Tissue microarrays for gene amplification surveys in many different tumor types. Clin Cancer Res 1999;5:1966-75.
10. Jewell SD, Srinivasan M, McCart LM, Williams N, Grizzle IH, MD, LiVolsi V, *et al.* Analysis of the molecular quality of human tissues. An experience from the Cooperative Human Tissue Network. Am J Clin Pathol 2002;118:733-41.
11. Zhong H, Simons JW. Direct comparison of GAPDH, beta-actin, cyclophilin, and 28S rRNA as internal standards for quantifying RNA levels under hypoxia. Biochem Biophys Res Commun 1999;259: 523-6.
12. Tso JY, Sun XH, Kao TH, Reece KS, Wu R. Isolation and characterization of rat and human glyceraldehyde-3-phosphate dehydrogenase cDNAs: Genomic complexity and molecular evolution of the gene. Nucleic Acids Res 1985;13:2485-502.
13. Margan SH, Handelsman DJ, Mann S, Russell P, Rogers J, Khadra MH. *et al.* Quality of nucleic acids extracted from fresh prostatic tissue obtained from TURP procedures. J Urol 2000;163:613-5.
14. Berger A, Bruschek M, Grethen C, Sperl W, Kofler B. Poor storage and handling of tissue mimics mitochondrial DNA depletion. Diagn Mol Pathol 2001;10:55-9.
15. Caggana M, Benjamin MB, Little JB, Liber HL, Kelsey KT. Single-strand conformation polymorphisms can be used to detect T cell receptor gene rearrangements: An application to the *in vivo* hprt mutation assay. Mutagenesis 1991;6:375-9.
16. Harish G, Venkateshappa C, Mahadevan A, Pruthi N, Srinivas Bharath MM, Shankar SK. Effect of premortem and postmortem factors on the distribution and preservation of antioxidant activities in the cytosol and synaptosomes of human brains. Biopreserv Biobank 2012;10:253-65.
17. van Maldegem F, de Wit M, Morsink F, Musler A, Weegenaar J, van Noesel CJ. Effects of processing delay, formalin fixation, and immunohistochemistry on RNA Recovery From Formalin-fixed Paraffin-embedded Tissue Sections. Diagn Mol Pathol 2008;17:51-8.
18. Blow N. Technology Feature Tissue preparation: Tissue issues. Nature 2007;448:959-62.
19. Baker AF, Dragovich T, Ihle NT, Williams R, Fenoglio-Preiser C, Powis G. Stability of phosphoprotein as a biological marker of tumor signaling. Clin Cancer Res 2005;11:4338-40.
20. Tokuda Y, Nakamura T, Satonaka K, Maeda S, Doi K, Baba S, *et al*. Fundamental study on the mechanism of DNA degradation in tissues fixed in formaldehyde. J Clin Pathol 1990;43:748-51.
21. Williams C, Pontén F, Moberg C, Söderkvist P, Uhlén M, Pontén J, *et al*. A high frequency of sequence alterations is due to formalin fixation of archival specimens. Am J Pathol 1999;155:1467-71.
22. Yagi N, Satonaka K, Horio M, Shimogaki H, Tokuda Y, Maeda S. The role of DNase and EDTA on DNA degradation in formaldehyde fixed tissues. Biotech Histochem 1996;71:123-9.
23. Srinivasan M, Sedmak D, Jewell S. Effect of fixatives and tissue processing on the content and integrity of nucleic acids. Am J Pathol 2002;161:1961-71.
24. Masuda N, Ohnishi T, Kawamoto S, Monden M, Okubo K. Analysis of chemical modification of RNA from formalin-fixed samples and optimization of molecular biology applications for such samples. Nucleic Acids Res 1999;27:4436-43.
25. Ferrer I, Armstrong J, Capellari S, Parchi P, Arzberger T, Bell J, *et al*. Effects of formalin fixation, paraffin embedding, and time of storage on DNA preservation in brain tissue: A BrainNet Europe study. Brain Pathol 2007;17:297-303.
26. World Health Organization International Agency For Research On Cancer IARC Monographs on the Evaluation of Carcinogenic Risks to Humans. Vol. 88. Formaldehyde, 2-Butoxyethanol and 1-*tert*-Butoxypropan-2-ol, 2006. 27. Foss RD, Find all citations by this author (default).
27. Foss RD, Guha-Thakurta N, Conran RM, Gutman P. Effects of fixative and fixation time on the extraction and polymerase chain reaction amplification of RNA from paraffin-embedded tissue. Comparison of two housekeeping gene mRNA controls. Diagn Mol Pathol 1994;3:148-55.
28. Shibutani M, Uneyama C, Miyazaki K, Toyoda K, Hirose M. Methacarn fixation: A novel tool for analysis of gene expressions in paraffin-embedded tissue specimens. Lab Invest 2000;80:199-208.
29. Mitchell D, Ibrahim S, Gusterson BA. Improved immunohistochemical localization of tissue antigens using modified methacarn fixation. J Histochem Cytochem 1985;33:491-5.
30. Wester K, Asplund A, Bäckvall H, Micke P, Derveniece A, Hartmane I. *et al*. Zinc-based fixative improves preservation of genomic DNA and proteins in histoprocessing of human tissues. Lab Invest 2003;83:889-99.
31. Lykidis D, Van Noorden S, Armstrong A, Spencer-Dene B, Li J, Zhuang Z, *et al*. Novel zinc-based fixative for high quality DNA, RNA and protein analysis. Nucleic Acids Res 2007;35:e85.
32. Stanta G, Mucelli SP, Petrera F, Bonin S, Bussolati G. A novel fixative improves opportunities of nucleic acids and proteomic analysis in human archive's tissues. Diagn Mol Pathol 2006;15:115-23.
33. Virginia Espina V, Mueller C, Edmiston K, Sciro M, Emanuel F, Petricoin EF, *et al*. Tissue is alive: New technologies are needed to address the problems of protein biomarker pre-analytical variability. Proteomics Clin Appl 2009;3:874-82.
34. Wiedorn JO, Goldmann T, Kühl H, Mehraein Y, Scherthan H, Niketeghad F, *et al*. HOPE Fixation: A Novel Fixing Method and Paraffin-embedding Technique for Human Soft Tissues. Pathol Res Pract 2001;197:823-6.

35. Sato Y, Mukai K, Furuya S, Kameya T, Hirohashi S. The AMeX method: A multipurpose tissue-processing and paraffin-embedding method. Extraction of protein and application to immunoblotting. Am J Pathol 1992;140:775-9.
36. Vincek V, Nassiri M, Block N, Welsh CF, Nadji M, Morales AR. Methodology for preservation of high molecular-weight RNA in paraffin-embedded tissue: Application for laser-capture microdissection. Diagn Mol Pathol 2005;14:127-33.
37. Delfour C, Roger P, Berthe CB, Rochaix P, Kalfa N, Raynaud P, *et al*. RCL2, a New Fixative, Preserves Morphology and Nucleic Acid Integrity in Paraffin-Embedded Breast Carcinoma and Microdissected Breast Tumor Cells. J Mol Diagn 2006;8:157-69.
38. Preusser M, Plumer S, Dirnberger E, Hainfellner JA, Mannhalter C. Fixation of brain tumor biopsy specimens with RCL2 results in well-preserved histomorphology, immunohistochemistry and nucleic acids. Brain Pathol 2010;20:1010-20.
39. Bussolati G, Chiusa L, Cimino A, D'Armento G. Tissue transfer to pathology labs: Under vacuum is the safe alternative to formalin. Virchows Arch 2008;452:229-31.
40. Kim M, Soontornniyomkij V, Ji B, Zhou X. System-Wide Immunohistochemical Analysis of Protein Co-Localization. PLoS One 2012;7:1-9.
41. Pirici D, Mogoanta L, Kumar-Singh S, Pirici I, Margaritescu C, Simionescu C, *et al*. Antibody elution method for multiple immunohistochemistry on primary antibodies raised in the same species and of the same subtype. J Histochem Cytochem 2009;57:567-75.
42. Nirmalan NJ, Harnden P, Selby PJ, Banks RE. Development and validation of a novel protein extraction methodology for quantitation of protein expression in formalin-fixed paraffin-embedded tissues using western blotting. J Pathol 2009;217:497-506.

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