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Factors that drive the increasing use of FFPE tissue in basic and translational cancer research

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Abstract

The decision to use 10% neutral buffered formalin fixed, paraffin embedded (FFPE) archival pathology material may be dictated by the cancer research question or analytical technique, or may be governed by national ethical, legal and social implications (ELSI), biobank, and sample availability and access policy. Biobanked samples of common tumors are likely to be available, but not all samples will be annotated with treatment and outcomes data and this may limit their application. Tumors that are rare or very small exist mostly in FFPE pathology archives. Pathology departments worldwide contain millions of FFPE archival samples, but there are challenges to availability. Pathology departments lack resources for retrieving materials for research or for having pathologists select precise areas in paraffin blocks, a critical quality control step. When samples must be sourced from several pathology departments, different fixation and tissue processing approaches create variability in quality. Researchers must decide what sample quality and quality tolerance fit their specific purpose and whether sample enrichment is required. Recent publications report variable success with techniques modified to examine all common species of molecular targets in FFPE samples. Rigorous quality management may be particularly important in sample preparation for next generation sequencing and for optimizing the quality of extracted proteins for proteomics studies. Unpredictable failures, including unpublished ones, likely are related to pre-analytical factors, unstable molecular targets, biological and clinical sampling factors associated with specific tissue types or suboptimal quality management of pathology archives. Reproducible results depend on adherence to pre-analytical phase standards for molecular in vitro diagnostic analyses for DNA, RNA and in particular, extracted proteins. With continuing adaptations of techniques for application to FFPE, the potential to acquire much larger numbers of FFPE samples and the greater convenience of using FFPE in assays for precision medicine, the choice of material in the future will become increasingly biased toward FFPE samples from pathology archives. Recognition that FFPE samples may harbor greater variation in quality than frozen samples for several reasons, including variations in fixation and tissue processing, requires that FFPE results be validated provided a cohort of frozen tissue samples is available.

Key words: biobank, cancer, consent, DNA, FFPE, frozen tissue, molecular target, paraffin processing, pathologists, pathology archive, pre-analysis, protein extraction, reproducibility, RNA, standards

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When fresh human tissue is obtained by biopsy or surgery for pathological examination and diagnosis, it typically is fixed in 10% neutral buffered formalin or alternative fixative, processed through graded alcohols and embedded in paraffin blocks, i.e., formalin fixed, paraffin embedded (FFPE) blocks. In most instances a single or small number of 5 µm sections is cut from the face of the block and stained for microscopic analysis. Only a limited number of defined clinical situations, e.g., intraoperative consultations, renal and muscle biopsies, require tissue samples to be preserved by other methods e.g., frozen, for diagnostic evaluation. Diagnostic material including slides and remaining FFPE blocks is retained in the pathology department and this collection often is known as the pathology archive. The suitability of this archive to support the combination of morphological and immunohistochemical analysis, which remain the cornerstones of clinical pathology diagnosis, have consolidated the role of the FFPE preservation format for clinical use.

The collection, processing and storage of human biospecimens such as blood, body fluids and tissue with associated patient data for either clinical or research purposes constitutes what is commonly known as biobanking (Liu and Pollard 2015), although a wider definition encompasses sample collections of all biological types and sources (Hewitt and Watson 2013, Kinkorová 2016). Human tissue sample biobanking has medical, ethical, research, legal, political, commercial and economic ramifications (Mee et al. 2013, Bjugn et al. 2015) in addition to its role in facilitating diagnosis and precision medicine, i.e., treating each patient according to his or her personal and genetic characteristics (Liu and Pollard 2015) as well as translational health research. By contrast to the standard clinical process, when patients provide informed consent for research biobanking, fresh tissue that is surplus to diagnosis is most often preserved by freezing, e.g., placed in an ultralow temperature or a vapor phase of liquid nitrogen freezer. This approach is taken to preserve cells and subcellular components including nucleic acids and proteins optimally for use in research projects. Although preservation as frozen tissue and frozen blocks has long been regarded as the gold standard for preservation of tissues for research, FFPE samples from pathology archives now are widely used for research.

We review and examine here the factors that may govern the research decision to use clinical FFPE tissue, the main product associated with the clinical pathology department archive, for basic and translational cancer research.

General factors

When researchers pursue a defined project and question, they require appropriate samples and linked data. This material may be available in the existing "retrospective collections" held in pathology archives or in biobanks. Alternatively the project may require the creation of a "bespoke or prospective collection" if the required samples have not been biobanked previously or if an unavailable specific collection and data protocol are needed. Researchers interested in rare diseases or in obtaining samples of tumors not usually resected surgically, such as small cell lung carcinoma, face significant challenges with obtaining samples collected specifically for research by either retrospective or prospective protocols, and a limited amount of clinical diagnostic FFPE tissue is all that is likely to be available. Even common tumor types can become scarce, because of changes in clinical requirements for diagnosis that have occurred over time, e.g., lung adenocarcinoma.

Prospective and retrospective collections

Bespoke prospective collections have been regarded as higher quality and more efficient than retrospective biobanked collections, because of the initially defined purpose that initiated the collection. The need for a prospective collection, however, usually delays the progress of the research and such collections undoubtedly account for many unused old samples that are held in freezers around the world; only part of the sample collection may have been used initially, or grant funding may have been terminated with no mechanism in place for others to access or identify the samples. By contrast, biobanks that collect and store samples to maintain a retrospective collection usually can provide sufficient cases to facilitate multiple studies using different criteria and without delay. Unfortunately, many biobanks fail to develop targets for their collections (Meredith et al. 2015) and, by definition, these collections contain many samples that do not meet specific study criteria, are never selected and therefore become unused samples also. As a result, biobanks of frozen samples exist on all continents and the problem is compounded by the fact that many are no longer identifiable or findable and may be of dubious quality. Despite the large number of high quality samples in reputable biobanks, a much greater potential source of material for research is the pathology archive.

FFPE and ELSI

Whereas the universal standard is to obtain patient consent for collection and use of biobanked samples (usually frozen samples) for research, FFPE tissue is derived primarily from pathology archives and the standards for its subsequent use for research vary among regions. In Ireland and Canada, consent requires approaching the patient for consent or obtaining a waiver of consent from an ethics review committee as well as approval by a pathologist or hospital committee. An example of the type of research where a waiver is the appropriate and only feasible mechanism to allow the research to progress is the type of study where long term patient outcome data is required to define the starting patient cohort, then the corresponding samples from initial diagnosis and subsequent relapse events can be obtained only from clinical FFPE materials (West et al. 2011). Another example is where unusual or rare samples are needed and sufficient numbers of cases are likely to be obtainable only from several pathology archives and diagnoses spanning many years. Unfortunately, ethical, legal and social frameworks regarding access to clinical FFPE tissue samples are not consistent among countries (Kaye et al. 2016). Greater awareness by an educated and informed public of biobanking and research that depends on biobanks is needed to support the improvement of mechanisms that facilitate the use of human tissues in research (Gaskell and Gottweis 2011).

Opt out consent

Opt out consent (https://www.federa.org/sites/default/files/digital_version_first_part_code_of_conduct_in_uk_2011_12092012.pdf) has existed in Belgium and The Netherlands for many years, and is allowed in a new European Commission directive on protection of personal data (https://ec.europa.eu/info/strategy/justice-and-fundamental-rights/data-protection_en) and in a recent declaration by The World Medical Association (https://www.wma.net/policies-post/wma-declaration-of-taipei-on-ethical-considerations-regarding-health-databases-and-biobanks/). The opt out system is pragmatic and reduces biobanks' operational costs (Riegman and van Veen 2011). Individual patients rather than an IRB or medical ethics committee make the decision regarding use of residual FFPE tissue, but opt out requires specific ethical, legal, institutional and informed societal support. Detailed discussion of the opt out system is beyond the scope of this review but is clearly warranted in view of the increased use of FFPE in cancer research.

Sustainability and costs

Although research biobanks and pathology archives play complementary roles in supporting research, the sustainability of these types of collections is quite different. Maintaining a biobank that stores frozen samples is expensive and funding frequently is derived from several sources including the hospital, university, grants and industry (Barnes et al. 2014, Henderson et al. 2013). A biobank's budget is vulnerable, which requires periodic re-evaluation of its goals, inventory and sample collection policies to achieve sustainability (Vaught et al. 2011, Parry-Jones 2014, Albert et al. 2014). A major biobank cost that is greater than that for equipment, consumables or data management systems is personnel costs, including personnel required for obtaining informed consent.

Pathology departments have a more dependable healthcare budget and are obliged to process and store tissue, and to issue reports on all collected specimens without exception. Patient specimens are received in large batches and are examined systematically and sequentially by a pathologist, then processed. The FFPE blocks created are durable and less costly to store than frozen tissue. Pathology and inventory data also are maintained as part of the clinical patient chart, though often not in a format conducive to efficient search and retrieval using research criteria as in a biobank.

Special biobank requirements and integration with pathology

Biobanked frozen samples are more hazardous than FFPE samples, and have special shipping requirements (Grizzle et al. 2010). Freezers require alarm monitors and CO_2 back-up systems or liquid nitrogen replenishment, and make inefficient use of expensive laboratory space. Integration of the research biobank with the pathology department can lead to mutual benefits by sharing facilities, costs, power and space (Grizzle et al. 1998, Lawler et al. 2016). Pathology archival samples

for approved projects may be stored in a biobank and retrieved efficiently for research, provided there is adequate sample tracking and an urgent response to clinical care requirements. Importantly, pathology and biobank staff must work closely together and understand their respective rules of engagement.

FFPE blocks

Major challenges to the use of archival FFPE blocks for cancer research are the ELSI, referred to above, and that FFPE blocks are generated from procedural specimens submitted to pathology without quality control (see "sample processing and standards" below). Certain pathologists may be unwilling to share for research tissue that they (wrongly) perceive to be theirs. Surgical specimens or biopsies, mislabeled in the operating theaters or clinics (Makary et al. 2007) or in pathology, and mislabeled blocks or slides clearly have the potential to jeopardize clinical care or invalidate research findings, but such errors are typically resolved safely by detailed investigation and careful clinical correlation (Tozbikian et al. 2017); they are greatly reduced by well-structured laboratory protocols, barcoding and sample tracking.

Ownership

In most jurisdictions, pathologists are considered custodians, but not owners, of patients' diagnostic FFPE tissue. US courts, however, have ruled that tissues are under the control of the healthcare facility. There have been attempts to define two mutually exclusive categories of excised human tissues, "diagnostic" and "research," for attributing ownership of the former to institutions and defining rules that govern their use in research (Cheung et al. 2013). This proposal has been debated in Canada and rejected by the large majority of clinicians and researchers - see Cheung et al (2013) letters in response http://www.cmaj.ca/content/185/2/135/tab-e-letters

Access to FFPE and cost recovery

Researchers may apply for use of FFPE samples in the archives for research projects approved by an ethics review committee, which may or may not waive the requirement for patient consent. Although there is a perception that pathologists present a barrier to researcher access to tissues, the challenge is that pathology departments are not structured or resourced to identify and undertake efficiently the work to retrieve samples for research. Even if the research criteria for selection of samples are simple or specific sample identifiers are already known, prior to the technical staff locating blocks and slides, which may be stored off-site and under a service contract, and cutting new sections for the researchers, a pathologist must review each patient's report and pathology slides and blocks to determine suitability of individual blocks. This is necessary for several reasons. First, on behalf of the patient, depleting material that might be essential for subsequent clinical review for patient care must be prevented. Second, blocks are intended to support delineation of multiple diagnostic features. For tumor specimens these features include the status of resection margins and presence of pathology accompanying the target lesion; even blocks that represent the target lesion vary significantly in their composition such as the percentage of viable tumor cells. Also the optimal block identified in the original pathology report might be greatly depleted owing to additional sectioning for immunohistochemical stains carried out at the time of diagnosis or previously committed to another research project. Thus, the pathologist can be required both for quality control of samples released for research and for maintaining adequate diagnostic tissue in the pathology department to have new slides stained from some blocks. For the research biobank, these decisions and most of this work has already been performed at the time of harvesting and allocation of samples to the biobank and any charges or costs have been integrated into the operational model. Furthermore the research biobank also usually assumes more expenses up front, including consent and procedural expenses. By contrast, the FFPE archival block is collected and stored under the clinical procedure consent and the cost of original FFPE processing and storage is absorbed by the hospital. Cost recovery charges, therefore, are clearly justifiable (Vaught et al. 2011, Parry-Jones 2014, Albert et al. 2014), although understandably the above subtleties are not always appreciated fully by all researchers.

Sample processing and quality control

Biospecimen science is the systematic study of the factors that affect sample quality and downstream analysis of frozen and fixed samples

(Moore et al. 2012, Engel et al. 2014). Where known and applicable, details should be reported to facilitate reproducibility of the study (Moore et al. 2012). Although often ignored by researchers accessing pathology archives, pre-analytical variables, reviewed by Bass et al. (2014), affect both FFPE archival and frozen biobank samples and include patient factors such as co-morbidities and stress, warm ischemia during surgery, perioperative events, operative changes, cold ischemia and the microenvironment from which the sample was removed. Certain pre-analytical variables can be recorded, e.g., ambient temperature, transport time and time from arrival to freezing/fixation; however, many pre-analytical factors are unknown, partly understood or patient-specific. Some variables are specific to frozen biobank samples, including freeze-thaw cycles and degradation of labile components, such as RNA, that can occur during storage. Other variables are specific to FFPE archival specimens, such as the use of inadequately buffered formalin or fixatives other than formalin, specimen size, inadequate formalin perfusion of the specimen, duration of fixation, delayed pathological examination and a delay from dissection to processing (van Maldegem et al. 2008, Zhou et al. 2015). Tissue processing variables include the use of xylene substitutes, differences in the time or chemistries of specific tissue processor steps (Grizzle WE, personal communication) and the establishment of a hydrophobic environment on transfer of the tissue to xylene (Otali et al. 2009). Additional variables include the duration of FFPE block storage (Combs et al. 2016) and exposure of FFPE blocks to hazards such as rodents and mold.

Immunohistochemistry

Fifty years ago, fresh frozen tissue was considered essential for preserving antigenicity for immunohistochemical staining (Nakane and Pierce 1966). Within the ensuing 10 years, however, modifications of the immunoperoxidase method (Taylor and Burns 1974, Huang et al. 1976, Kurzon and Sternberger 1978), with or without the use of antigen retrieval techniques (Denk et al. 1977, Cuevas et al. 1994, Shi et al. 1991, 2011) render FFPE tissue eminently suitable for detecting a wide variety of intracellular and surface antigens by immunohistochemistry for diagnostic pathology practice, basic and translational research and validation of biomarkers. Tissue stabilization reagents such as PAXGENE and BHP are reported to enhance antigenicity (Kap et al. 2011, Mueller et al. 2011). The quality of FFPE samples for immunostaining can deteriorate gradually, however, with long-term storage for certain antigens other than actin and keratin (Combs et al. 2016, Grillo et al. 2015). Indeed, unstained FFPE sections can begin to lose antigenicity within several weeks (Jacobs et al. 1996), possibly due to exposure of molecules in thin sections to the atmosphere.

Quality assurance and quality control

FFPE archival samples can be important for quality assurance of frozen biobanked samples and vice versa. Composition can be determined fairly well by frozen section analysis (Mee et al. 2013), but reference to an adjacent FFPE block often is better. Comparison of assay performance and research results from studies using frozen tissue with those using an adjacent FFPE block can help confirm the performance of antibodies (Mee et al. 2011, Greytak et al. 2015) by extracting protein for western blots. Quality control is not limited to evaluating the quantity and quality of extracted DNA and RNA and a pathologist's examination, but may require macrodissection or laser capture microdissection (Emmert-Buck et al. 1996, Baldelli et al. 2015, Varley et al. 2014) to enrich the sample for tumor cells. There is a spectrum from unsatisfactory to acceptable and insufficient attention to quality control greatly contributes to the lack of reproducibility of scientific research findings (Begley and Ioannidis 2015, Grizzle et al. 2015).

Research question, molecular target and technique to be used

The suitability of FFPE tissue for research depends on the research question that is asked, the molecular target and the technique that is considered for use. Samples that are "imperfect" for certain research projects may be appropriate for others. A detailed strategy to quantify and stratify sample quality appropriate for specific research techniques has been proposed by the ISBER biospecimen science working group (Betsou et al. 2016). The technique to be used requires knowledgeable, adaptable and expert technical staff in addition to appropriate laboratory instrumentation. Commonly, FFPE tissue requires especially rigorous pre-analytical phase preparation prior to using a technique originally designed for frozen tissue (see below). Antigen

retrieval by heat pretreatment of FFPE sections is part of diagnostic pathology practice for immunohistochemical staining and assays etc. (Shi et al. 2011). Not all results from translational research laboratories can be "readily reproduced," however, because tissue preparation methods are too variable or because of the involvement of other factors such as poor quality assurance or sample selection bias (Yeo et al. 2014, Neumeister et al. 2014, Atherton et al. 2016).

Biological studies associated with multi-site clinical projects and trials that seek to establish the role of new biomarkers and associated targeted therapies usually rely on retrieval of existing FFPE archival tissue samples, derived from multiple pathology departments (Sparano et al. 2015). Formalin fixation and tissue processing (Otali et al. 2009, Atherton et al. 2016), however, are not uniform in all specimen types, particularly in older resection specimens or from institution to institution. Therefore, critical decisions for advances in patient treatment might be based on examination of FFPE sections that may not be comparable. Therefore, it would be highly desirable, for both consistency and clinical care, to establish objective and reliable tissue quality controls for FFPE sections (Yeo et al. 2014, Neumeister et al. 2014).

Analysis of chromosomes, DNA, RNA, MicroRNA, and proteins in FFPE tissue

FFPE samples may be all that is available to a researcher or may be the optimal sample for pursuing a specific research question. What are its limitations in selected molecular investigations?

Chromosomes

Many molecular techniques for assessing aspects of DNA have been modified successfully for application to FFPE tissue (Dietel 2016). FFPE tissue can be used to detect chromosomal aberrations using microarray comparative genomic hybridization (Toffoli et al. 2014, Pinto et al. 2016). FFPE tissue also can be used to probe specific genetic sequences and structural or numerical chromosomal aberrations by fluorescence in situ hybridization (FISH) (Lim and Lim 2017). FISH assays have significant clinical diagnostic utility for soft tissue tumors (Horn et al. 2014) and are employed routinely on FFPE sections of breast cancer tissue to determine whether a patient has multiple copies of the HER2 gene, which predicts that trastuzumab (Herceptin) therapy would be beneficial (Griggs et al. 2017, Morey et al. 2016).

DNA

Although there is better quality DNA in fresh or frozen tissue, it has long been known that DNA can be extracted for gel-based analysis from FFPE tissue (Watson et al. 1993) and improved extraction methods continue to be reported (Potluri et al. 2015). Nevertheless, DNA extracted from FFPE cancer tissue harbors potential sequence artifacts (Do and Dobravic 2015) and is associated with higher false-negative and false-positive rates of mutation than frozen tissue (Gallegos Ruiz et al. 2007). This is because formalin fixation forms cross-links, causes DNA degradation and introduces chemical contaminants that may affect experimental reagents. Errors in mutation detection associated with low numbers of functional DNA copies and DNA de-amination artifacts account for most false positives, although newer methodologies may be able to reduce these errors significantly (Bourgon et al. 2014).

Next generation sequencing (NGS)

Quality management and awareness of limitations of FFPE are vitally important (Bourgon et al. 2014, Grizzle et al. 2015, de Abreu et al. 2016). Rigorous quality management and FFPE tissue pre-preparation, including optimized library preparation, are required for clinical application of NGS (de Abreu et al. 2016, Bolognesi et al. 2016) applied to FFPE DNA to detect reproducibly copy number variations and single nucleotide variations (Shao et al. 2016). These investigators reported 100% concordance of mutation profiles between discovery and validation cohorts when comparing an optimized NGS approach to conventional assays, including PCR, FISH and IHC, applied to clinical lung adenocarcinoma samples. Bolognesi et al. (2016) employed a microchip-based digital sorter to obtain pure cell populations for NGS from FFPE tissue. Astolfi et al. (2015) reported that if DNA in FFPE was of high quality, results of whole exomic sequencing were equivalent to those obtained from frozen tissue samples. Others have reported equivalent frozen and FFPE tissue data based on a small series of cases (Munchel et al. 2015, Bonfiglio et al. 2016). Whole exomic sequencing data from FFPE gDNA extracted from samples of gastrointestinal stromal tumors and melanomas, however, were not consistently comparable to frozen tissue results; this was attributed to the fact

that certain clinical samples yield lower quality DNA and might be the only samples available for important clinical decision making (Astolfi et al. 2015, De Paoli-Iseppi et al. 2016). Bioinformatics analysis, essential for interpretation of NGS data, must be adapted to FFPE-based data. Standardization (see below) is essential for translational and clinical applications.

RNA

RNA can be extracted from FFPE tissue, but it is degraded into smaller fragments than in frozen tissue (Micke et al. 2006, Sun et al. 2016). Kashofer et al. (2013) examined the impact of pre-analytical factors and quality control measures required to improve the reliability of RNA extraction from FFPE. Small amounts of input RNA can be detected and quantified by reverse transcription quantitative real-time polymerase chain reaction (RT-qPCR) (Dama et al. 2016), which is more sensitive than FISH or immunohistochemistry (Lung et al. 2016). Paluch et al. (2017) reported that a targeted RNA sequencing panel profiled mRNA expression levels reliably in FFPE samples of ovarian carcinoma and was concordant with results from matching mRNA expression from freshly frozen samples and determined by RT-PCR. Cieslik et al. (2015) reported improved results from RNA sequencing of degraded RNA using an exome-capture transcriptome protocol. An mRNA-based in situ mutation detection technique based on padlock probes showed differences in mutant allele frequencies within two morphologically homogeneous colorectal cancers (El-Heliebi et al. 2017). Morten et al. (2016) reported variable success in detecting a p53 mRNA isoform (delta40p53) using two different assays applied to matched frozen and FFPE tissue samples. The apparently capricious detection of mRNA in FFPE tissue might be due to differences between individual mRNA molecules, the variable impact of known pre-analytical variables or suboptimal quality management.

MicroRNA (mi-RNA)

Mi-RNAs are easier to retrieve from FFPE than mRNA owing to their small size and relative stability (Howe 2017). Based on analysis of pathology archival samples, biomarkers based on mi-RNA features determined from FFPE tissues are therefore potentially very useful for developing precision medical strategies (Caramés et al. 2016, Wong et al. 2016); however, mi-RNA in FFPE material may not be immune to pre-analytical variables. The global mean yield of mi-RNA is lower with increased fixation time and in older paraffin blocks (Boisen et al. 2015). Furthermore, How et al. (2015) reported that a prognostic 9-mi-RNA signature set for cervical carcinoma identified in frozen samples could not be validated in an independent cohort of FFPE samples and that concordance between frozen and FFPE samples was lacking. A 2-miRNA classifier (miR-21 + miR-155), validated in small cohorts of EUS-FNA and FFPE needle biopsies, exhibited 81.5% sensitivity and 85.7% specificity in distinguishing pancreatic cancer from benign pancreatic lesions (Frampton et al. 2016). Kakimoto et al. (2016) reported that mi-RNA stability in FFPE tissue correlated with guanine and cytosine (GC) content: GC-rich mi-RNAs were less degraded than those with < 40% GC content. Therefore, intratumor and interpatient heterogeneity of mi-RNA expression in FFPE tissue (Jepsen et al. 2016) should not be unexpected.

Proteins

FFPE tissue-based proteomics is an alternative approach to discovery and evaluation of biomarkers for precision medicine. Proteins are much less amenable to extraction from FFPE, however, than from frozen tissue (Thompson et al. 2013, Shi et al. 2013, Steiner et al. 2014). More importantly, by contrast to the often more forgiving assays of DNA and RNA, the quality of extracted protein is an important factor. Individual phosphorylated proteins may show increased, decreased, or stable immunohistochemical expression with increasing delays before formalin fixation (Vassilakopoulou et al. 2015, Atherton et al. 2016, Grizzle et al. 2016). Reverse phase protein arrays (RPPA) immobilize and profile the entire protein repertoire of the sample (Paweletz et al. 2001). RPPA was used for the Cancer Genome Atlas project (Weinstein et al. 2013) and has enhanced utility when integrated with other analytical platforms (Lu et al. 2016). Mass spectrometry-based quantitative proteomics traditionally has been performed on fresh or frozen tissue but its application to FFPE is expensive and challenging (Steiner et al. 2014, Lu et al. 2016).

Perhaps the most critical issue for proteomics studies is the quantitative recovery of proteins from the FFPE sample prior to downstream analysis (Shi et al. 2013). Ethanol and xylene-free deparaffinization using hot distilled water only provides

an increased yield of proteins extracted from archival paraffin blocks (Mansour et al. 2014). Heat-induced antigen retrieval (Shi et al. 2006), based on antigen retrieval for immunohistochemistry (Shi et al. 1991), and elevated hydrostatic pressure (Fowler et al. 2008) also have been reported to increase the yield of proteins extracted from FFPE tissue. Recent progress with optimization of proteomics platforms has enabled the threshold for analysis to be lowered to single 10 µm sections (Hughes et al. 2016).

Kojima et al. (2012) reported a robust method for protein extraction using heat-induced antigen retrieval and trypsin digestion, with validation in small cohorts of FFPE human pancreatic cancer and matched FFPE and frozen mouse pancreatic tissue samples. Complete solubilization of FFPE tissue sections has been proposed as part of the protein extraction procedure, but there is no consensus concerning the optimal protocol for protein extraction from FFPE tissues (Shi et al. 2013). Moreover, there are many types of mass spectrometry instrumentation that may produce divergent results from aliquots of the same FFPE sample. A result is not necessarily representative of the specific cell type under investigation in frozen or FFPE tissue and failure to identify a protein does not indicate its absence, because there are no practicable normalization approaches (analogous to housekeeping genes) for analyzing proteins. Despite these significant challenges, analysis of proteins by mass spectrometry can be a useful discovery tool if results are confirmed by other techniques.

Standards

The European Committee for Standardization has produced pre-analytical phase standards for protein, DNA and RNA extraction from FFPE

https://standards.cen.eu/dyn/www/f?p=204:110:0::::FSP_PROJECT:41046&cs=1FD7275F5AE4E23C9D30AF3C7F4824F6E,

https://standards.cen.eu/dyn/www/f?p=204:110:0::::FSP_PROJECT:41044&cs=118071B8B9C4BA81C9C3680A98DEC61D8,

https://standards.cen.eu/dyn/www/f?p=204:110:0::::FSP_LANG_ID,FSP_PROJECT:25,41042&cs=1988037AFFA089882B7D6CD54AB81B5F2.

These documents are intended to standardize the pre-analytical process of sample collection and will soon be published as ISO international standards. Where standardization is not possible, documentation helps identify the many potential variables that may compromise a sample. The documentation of sample metadata is the key tool for identifying sources of variation that can lead to incorrect measurements. Identifying a source of variation may direct the exclusion of a sample from a particular cohort or form the basis for reassessment of the adherence to pre-analytical protocols before collecting additional samples. General adoption of the pre-analytical standards will create more possibilities for the use of FFPE samples for medical research with greater exchangeability of equivalent samples that will enable multicenter studies to become more successful. The development of each set of new therapies and diagnostic tests is followed by continuous improvements in tools, assays and methods, and success depends on reproducibility after validation for the intended uses. Validation is obligatory before a new method can be accepted for health care (Burke and Grizzle in press). The latter also is an argument for choosing or adapting approaches to be able to analyze FFPE materials early in the progress of a cancer research study.

Conclusion

The decision to use FFPE pathology archival material for cancer research may be dictated by the research question and analytical technique. Alternatively, the decision may be a compromise or no choice at all depending on the country, national ELSI, sample and biobank availability, and access policy. Biobanked frozen samples of common cancers are likely to be available, but determinants such as associated treatments and availability of outcomes data may limit their application. Microscopic lesions and rare tumors will, for all practical purposes, exist mostly in FFPE pathology archival samples and may need to be obtained from several pathology departments to accumulate adequate numbers; however, care should be exercised to avoid biases that may be caused by site differences including different fixation and tissue processing approaches (Atherton et al. 2016, Burke and Grizzle in press). Investigators must decide the sample quality that is fit to answer a specific research question. Recent publications report variable successes with techniques modified to examine all common molecular targets in FFPE samples. Unpredictable failures likely are related to pre-analytical factors, unstable molecular targets or

biological and clinical sampling factors associated with specific tissue types. More reproducible results should follow adherence to pre-analytical phase standards for molecular diagnostic analyses in vitro for DNA, RNA, and in particular, extracted proteins. Experienced personnel and appropriate equipment are essential. With continuing adaptations of techniques for application to FFPE, the greater potential to acquire larger numbers of FFPE samples, and the greater convenience of using FFPE in assays for precision medicine, the choice of material in the future will be increasingly biased toward FFPE samples from pathology archives (Hughes et al. 2010). The realization that FFPE may be of poorer quality, because more variations are introduced by fixation and tissue processing to paraffin than in freezing tissue, however, would require that FFPE results be verified using a cohort of frozen tissue samples for complete understanding of the disease process.

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