Tissue handling for molecular pathology

This article reflects the outputs of a workshop held on 19 July 2017 and attended by cellular pathologists, biomedical scientists and others involved in establishing genomic testing for cancer as part of the 100,000 Genomes Project. It addressed a topic of increasing importance: how best to handle surgical specimens from the point of removal, such that optimal results will be obtained from molecular analysis as well as conventional microscopy and immunohistochemistry.

Professor Louise Jones

Dr Clare Craig

Impact of tissue handling

Pathologists are facing a number of challenges that come with the rapid progress in molecular diagnostics for cancer, including whole genome sequencing (WGS). One key issue is the need to optimise and standardise tissue handling in order to achieve high quality sequencing while retaining adequate morphology for diagnosis. In the Chief Medical Officer for England's annual report, Dame Sally Davies has emphasised that cancer genomics should become part of routine care. This presents challenges but also potential opportunities.[1]

During the Pilot and Initiation Implementation Phases of the 100,000 Genomes Project, extensive experimental work was undertaken to optimise and standardise the process of formalin fixation and to adapt the DNA extraction process to minimise DNA damage. However, while there was some improvement seen, WGS on formalin-fixed paraffin-embedded (FFPE) tissue continued to be substantially suboptimal, leading to the conclusion that, for optimal WGS, fresh tissue is required.

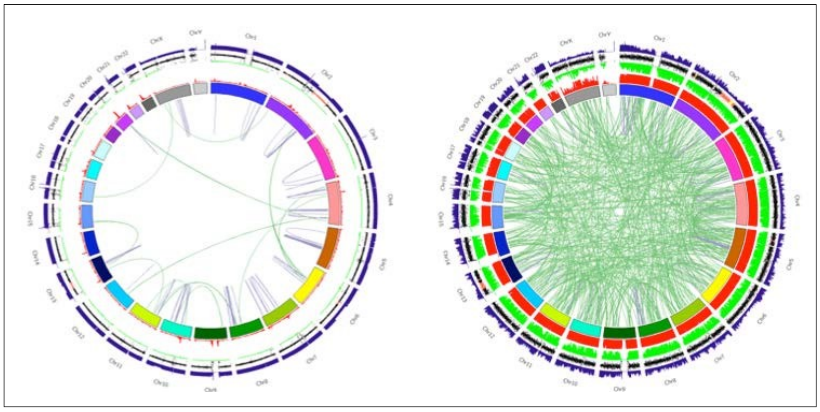

Figure 1: Artefactual mutations caused by formalin fixation. This plot illustrates the distribution of somatic variants in a fresh frozen (FF) and FFPE sample from the same prostatic cancer across the genome. It shows the greatly increased number of mutations introduced due to formalin-induced DNA damage. Chromosomes are arranged sequentially around the circumference as indicated. Structural variants (SVs) are indicated by arcs inside the plot; translocations are indicated in green, inversions are indicated in purple. Track 1 (innermost track): chromosomes; Track 2 (in red): number of somatic SNVs; Track 3 (in green): number of somatic indels; Track 4: ratio of normalised depth of coverage for tumour vs normal. CNV losses are indicated in red, CNV gains are indicated in green, copy-neutral LOH regions are indicated in yellow; Track 5 (outermost track, in blue): absolute depth of coverage in tumour sample.

What does formalin do to DNA?

Formaldehyde is highly reactive with DNA bases and proteins, leading to forming protein–DNA, interstrand DNA, and protein–protein crosslinking and DNA–formaldehyde adducts.[2] The result is a combination of direct fragmentation, abasic sites and deamination of cytosine to uracil or thymine causing false-positive nucleotide variants (see figure 1). The consequence is generation of artefactual mutations (false positives) making the reliable detection of structural variants and mutational patterns such as signatures extremely difficult.

What can be done to avoid formalin artefacts?

Approaches to overcome these problems with formalin and to routinely harvest fresh tissue will impact on a range of aspects of histopathology practice. An important issue is that, with centralisation of pathology services, theatres and laboratories are often not co-located, so any tissue that leaves theatres fresh will inevitably be exposed to a prolonged cold ischaemia time before reaching the laboratory, especially at weekends. This poses challenges in ensuring maintenance of morphological and nucleic acid integrity. A potential advantage is that samples arriving fresh in the laboratory can be fixed using an appropriate volume of formalin and for a controlled period of time. However, changing the process of tissue handling has the potential to impact on morphology and immunohistochemical results, which needs to be considered. Any possibility of using alternative fixatives will have similar consequences.

Should formalin be removed from theatres?

The impetus to consider a formalin-free theatre for improved molecular pathology has been overtaken by other drivers. Theatre nurses are increasingly motivated to reduce formalin exposure for health and safety reasons. Hospitals that have removed formalin from theatres and substituted tissue handling methods using refrigeration have discovered other benefits including: reduced weight of samples for handling by staff; reduced bulk for trans-

portation and storage; cost savings on purchasing formalin, as well as on cleaning and disposal; and ease of sampling fresh tissue for genomics. Pathology departments should consider working closely with operating theatres when proposing any such change, as there could be even greater savings from the theatre budget than from the laboratory.

As fresh tissue sampling moves from a research tool to a part of standard diagnostic care, it is necessary that it fits into workable routine laboratory pathways. An obvious way to do this is to take fresh tissue for molecular analysis when samples are opened prior to formalin fixation. The drawback is that this means samples being kept fresh until pathologists, laboratory staff and a laboratory bench are ready to receive them.

How good is our current formalin fixation 'gold standard'?

Pathologists have valid concerns over the effect of prolonged cold ischaemic time on fresh specimens. It would be naive to imply that current practice does not also suffer from similar problems. While recognising that some hospitals have co-localised surgical theatres and pathology departments with good portering, for many this is not the reality. Where patients have a resection performed at an off-site theatre or before a weekend, there still may be delays in receipt of the sample, though this is currently considered acceptable since the sample is in formalin.

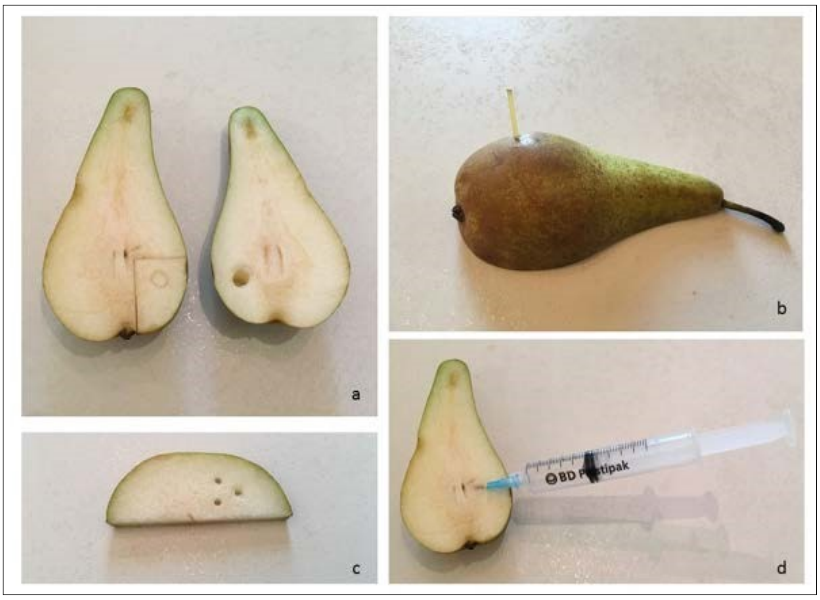

Figure 2: Small tumour sampling techniques: a) Mirror blocking. The fresh sample can be kept frozen until confident that it is surplus to histopathological diagnostic requirements. The area opposite the fresh sample is marked for tumour content assessment. b) Core biopsy, which can be taken from an opened or unopened sample. c) Fresh blocking and punch biopsies, which can be collated to ensure enough DNA. Tumour content can be assessed on surrounding material. d) Fine needle aspiration, which results in a better DNA yield per cell and is helpful in desmoplastic tumours where tumour content would otherwise be too low.

However, formalin penetrates tissue slowly with a decreasing rate over time: penetration slows from 3.6 mm at one hour, to 18 mm at 24 hours and after 100 hours penetration would still only be to a depth of 36 mm.[3] Thus, particularly for larger samples, tissue at the centre of the resection will effectively be unfixed at room temperature, with consequent impact on morphological (and nucleic acid) quality. In addition, theatre staff and others may feel that once a sample is in a bucket of formalin, it is safe to keep it there for an extended period and the sense of responsibility for delivering to the laboratory in as timely a fashion as possible is reduced.

Any concerns over the effects of delayed fixation need to be seen in context. There are bound to be differences between samples opened promptly and fixed optimally, and samples that are refrigerated first. However, a fairer comparison would be between samples that are refrigerated until a pathologist is ready to sample them fresh and those that are left unopened in formalin until they can be dealt with in the laboratory. Validation should only need to prove that refrigeration is as good as current best practice not as good as optimal fixation.

Controlled fixation

There is increasing evidence that restricting the time that tissues are exposed to formalin improves the false-negative and false-positive rate for immunohistochemistry and in situ hybridisation. Given the treatment impact of these tests, instigating controlled fixation is an important consideration when trying to improve quality in histopathology laboratories. The optimal time for tissue fixation is somewhat dependent on types of tissue, with guidelines for colorectal samples being longer than for many other sample types. For these types, an upper limit for time in formalin has been suggested as 24 hours,[4] whereas others suggest samples should not be fixed for longer than 12 hours.[5] Whichever length of time is considered optimal for a given sample type, the ability to control this, and ensure that the entire specimen is exposed to formalin, must certainly be seen as an advantage and a move towards greater standardisation in tissue handling.

Alternative fixatives

There are some tumour types for which collecting fresh tissue is particularly challenging. These include, for example, prostate cancer, where it is often impossible to discern the cancer macroscopically, and melanomas, where keeping the entire architecture intact is critical for diagnosis. Where samples are fixed in formalin-free fixatives such as Paxgene[6] a diagnosis can be made based on paraffin embedded tissue and then material for DNA extraction can be selected in a precise way from the paraffin blocks. Such alternative fixatives are not without their own challenges. It is critical that these cases are processed on a separate processor as any contact

with formalin will damage the DNA and negate the effect. Also, while reports suggest morphology and immunohistochemistry are comparable with formalin, further work is needed to validate the impact of alternative fixatives on grading and immunohistochemistry.

Sampling techniques for fresh tissue from surgical resections

There are few laboratories with spare cut up bench capacity, which means sampling fresh resection samples as they arrive from theatre can be logistically challenging. If sufficient fridge space is available, then keeping fresh samples refrigerated and batching them for opening can work well.

A key concern for all pathologists is to ensure that any changes to tissue handling processes do not compromise the diagnostic process and provision of all required prognostic parameters. For large tumours it usually is possible to sample the tumour fresh without impacting on diagnostic sampling (depending on proximity to key margins), but the potential to impact on diagnostics can be more of a challenge when tumours are small. Options for sampling small tumours include: taking a mirror full face for a paraffin block and for a frozen sample; using a biopsy gun to remove a core of tissue; aspirating the tumour and washing the needle into phosphate-buffered saline; or taking fresh tissue blocks and then removing small punch biopsies of relevant material (see figure 2). Some of these techniques suit particular tumour types better than others. For example, taking a core biopsy from a fresh breast tumour is straightforward, but this is more challenging for renal tumours which are often much softer when fresh.

Conclusion

Introducing new laboratory processes must be executed with care and due consideration for the impact on the wide variety of diagnostic tests performed on tissues. However, context is critical and consideration should also be given to the variation in practice that currently exists. This is particularly true for how samples are handled prior to fixation where there is vast inter-sample variation in cold ischaemia time and fixation time.

Collecting fresh tumour samples presents its own difficulties and pathologists round the country have risen to the challenge: from setting up systems to alert the laboratory that a fresh sample is imminent, to perfecting techniques for sampling small tumours without interfering with the histological diagnosis, and with several centres now trialling approaches such as vacuum-packing.

A working group has been formed to facilitate Genomics England and NHS England to work with the Royal College of Pathologists to explore the logistical and governance issues of removal of formalin from theatre, the evidence required and how this transition will be managed.

For videos of both individual talks from the workshop and descriptions of sampling procedures, please see the following link: https://www.genomicsengland.co.uk/information-for-gmc-staff/cancer-programme/transforming-nhs-services/

Professor Louise Jones
Professor of Breast Pathology, Barts Cancer Institute
Clinical Lead for Molecular Pathology, Genomics England

Dr Clare Craig
Clinical Lead for Cancer Recruitment
Genomics England

References

1. Davis DS. *Annual report of the Chief Medical Officer 2016: Generation Genome.* 2017. https://www.gov.uk/government/uploads/system/uploads/attachment_data/file/631043/CMO_annual_report_generation_genome.pdf
2. Do H, Dobrovic A. Sequence artifacts in DNA from formalin-fixed tissues: causes and strategies for minimization. *Clin Chem* 2015;61:64–71.
3. Medawar PB. The rate of penetration of fixatives. *J R Microsc Soc* 1941;61:46–57.
4. Pollard K, Lunny D, Holgate CS, Jackson P, Bird CC. Fixation, processing, and immunochemical reagent effects on preservation of T-lymphocyte surface membrane antigens in paraffin-embedded tissue. *J Histochem Cytochem* 1987;35:1329–1338.
5. Williams JH, Mepham BL, Wright DH. Tissue preparation for immunocytochemistry. *J Clin Pathol* 1997;50:422–428.
6. Mathieson W et al. A critical evaluation of the PAXgene tissue fixation system: morphology, immunohistochemistry, molecular biology, and proteomics. *Am J Clin Pathol* 2016;146:25–40.