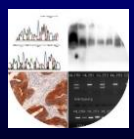
New Developments in the laboratory

Mohammad Ilyas



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Overview

- Digital Pathology
  - Utility / TuPaQ / HMM
- Next Generation Sequencing
  - Principles and utility
- Liquid biopsy
  - Utility and limitations

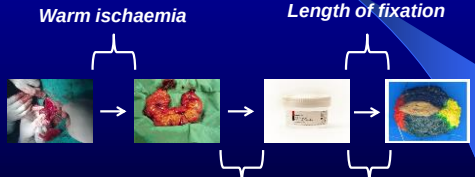
Digital Pathology

- Digital Pathology in service delivery
  - Mechanics of digitisation
  - Utility and limitations
- Digital Pathology in research
  - Tumour Parcellation and Quantification (TuPaQ)
  - Histogenic Molecular Mapping (HMM)

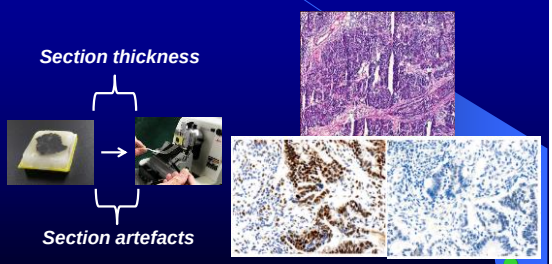
What is Digital Pathology?

- Digital Pathology involves taking a digital image of a tissue section
- The image can then be used for diagnosis as an alternative to the microscope
- The image data can be mined in several of ways for computer aided diagnosis
- Getting a digital image involves several steps, each of which is a source of varation

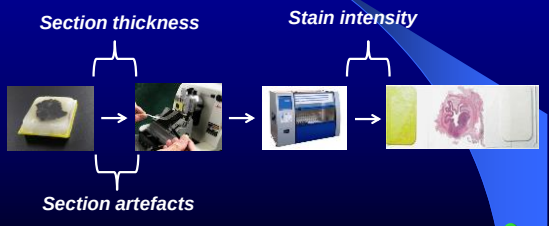
The digital pathology workflow



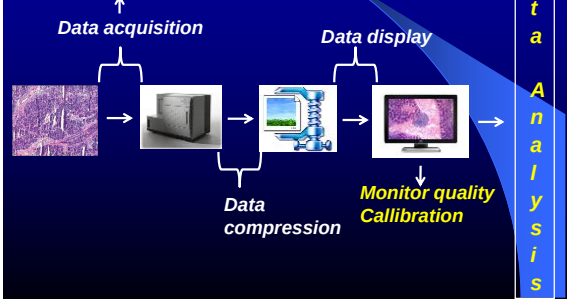
The digital pathology workflow



The digital pathology workflow



The digital pathology workflow



The digital pathology workflow

Data analysis

- Remote diagnosis
- Multiple user
- Frozen section
- Teaching
- Image analysis
- CBIR
- Imaging cytometry

Utility of Digital Pathology

- Images can be used for diagnostic work (FDA approved)
- Images can be used for instant second opinion
- Images can be used for accurate quantification
- Computer aided diagnosis may automate and facilitate tasks

Can digital images be used for diagnosis?

Validation of digital pathology imaging for primary histopathological diagnosis

David R J Snead,[1,2] Yee-Wah Tsang,[1,2] [illegible] Kimani,[3] Richard Crossman,[3] Nasir M Rajpoot,[2,4] Elaine Blessing,[1] [illegible] Gopalakrishnan,[1] Paul Matthews,[1] Navid Mo[illegible]han,[1] [illegible] Read-Jones,[1] Shatrughan Sah,[1] Emma Simmons,[1] [illegible],[1] Sari Suortamo,[1] Yen Yeo,[1] [illegible] El Daly[1] & Ian A Cree[1,2]

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Snead D R J, Tsang Y-W, Meskiri[illegible], Crossman R, Rajpoot N M, Blessing E, Chen K, Gopalakrishnan K, Matthews [illegible] N, Read-Jones S, Sah S, Simmons E, Sinha B, Suortamo S, Yeo Y, Daly [illegible] (2015) Histopathology. DOI: 10.1111/his.12879

- Over 3000 cases - DM is non-inferior to OM

Can digital images be used for difficult diagnosis?

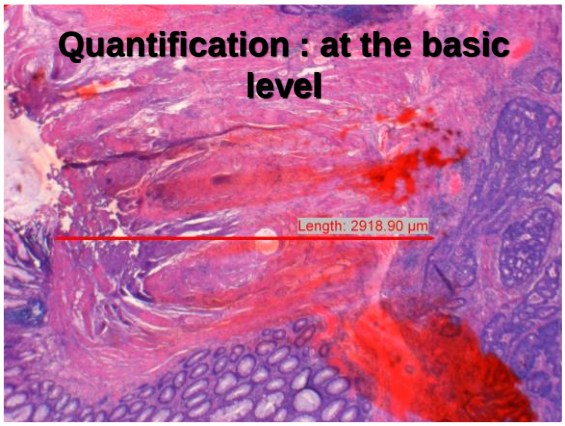
Digital microscopy is non-inferior to optical microscopy in the diagnosis of dysplasia in Barrett's oesophagus:

A validation study

C. Marchessoux
Healthcare division
Kortrijk, Belgium

P.V. Kaye, A. Mukherjee, S. Paine, A. Haider, M. Ilyas
University of Nottingham & QMC
Nottingham, UK

Quantification : at the basic level



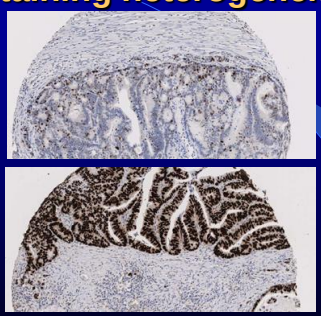
Limitations of Digital Pathology

- Image acquisition can be problematic and needs QC
- Images are large and data management (compression / transmission / storage) are problematic
- Viewing environment needs to be optimised
- There is no cost case!

Image Analysis

- Digital images can be in interrogated in many ways to yield information which may facilitate or enhance diagnosis
- Deep learning methods are being used to mine data
- Recurrent themes are:
  - Tumour segmentation for biomarkers
  - Image registration for multiple biomarkers

Staining heterogeneity



Does TuPaQ have the answer?

Tissue Segmentation

$$\frac{\partial \phi}{\partial t} = spf\left(I\left(x\right)\right) \cdot \alpha \|\nabla \phi\|, \tag{1}$$

$$spf\left(I\left(x\right)\right) := \frac{I\left(x\right) - \frac{e^{+}(C)+e^{-}(C)}{2}}{\max_{x \in \Omega}\left(\left|I\left(x\right) - \frac{e^{+}(C)+e^{-}(C)}{2}\right|\right)}, \tag{2}$$

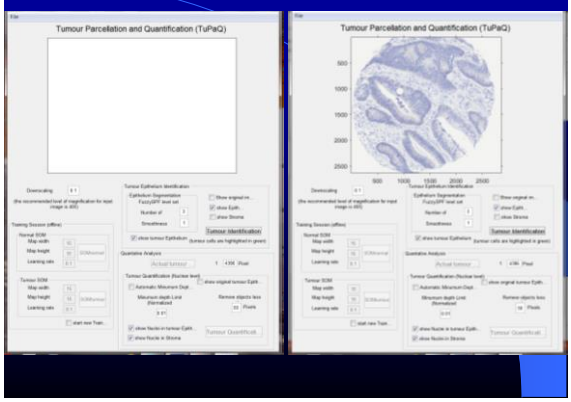
$$WAI_1 = \frac{1}{n}\sum_x WAI(x). \tag{17}$$

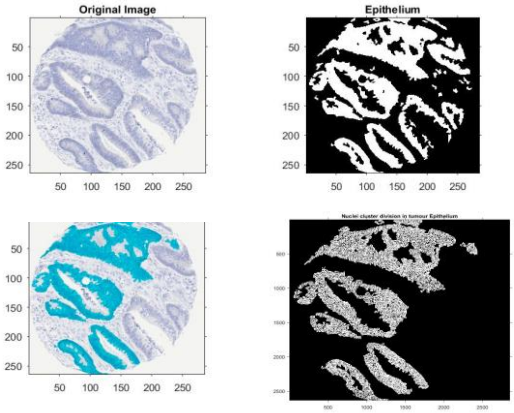
$$WAI_2 = \begin{cases} WAI(x)_{\frac{n+1}{2}} \text{for n is odd} \\ \frac{1}{2}(WAI(x)_{\frac{n}{2}} + WAI(x)_{\frac{n}{2}+1}) \text{for n is even} \end{cases} \tag{18}$$

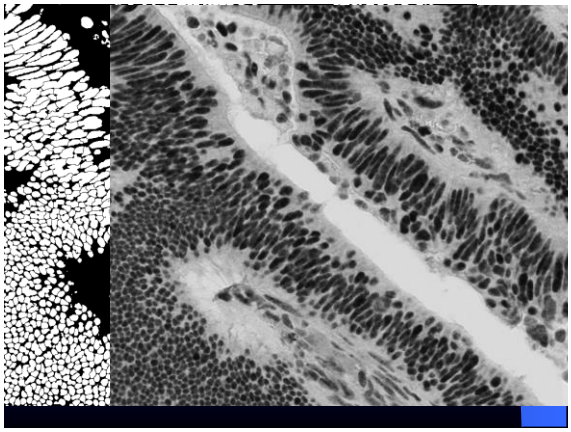
$$WAI_3 = \frac{1}{n}\sum_x (WAI(x) - WAI_1). \tag{19}$$

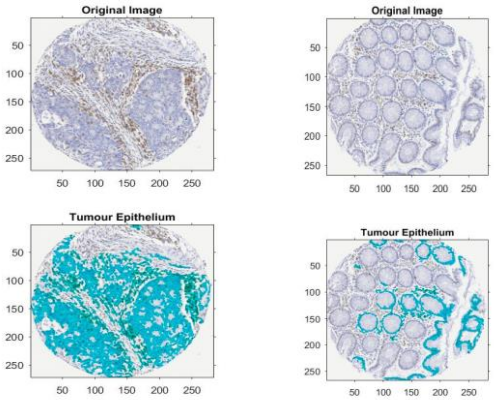
$$WAI_4 = \frac{\frac{1}{n}\sum_x (WAI(x) - WAI_1)^3}{\left(\sqrt{\frac{1}{n}\sum_x (WAI(x) - WAI_1)^2}\right)^3}, \tag{20}$$

$$WAI_5 = \frac{\sum_x (WAI(x) - WAI_1)^4}{\frac{1}{n}\left(\sum_x (WAI(x) - WAI_1)^2\right)^2}, \tag{21}$$





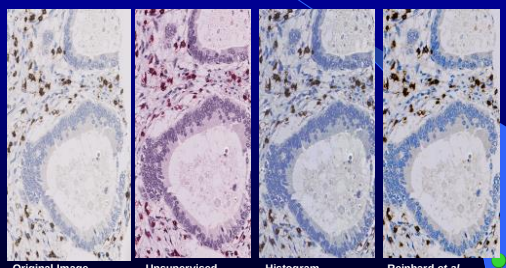




Tissue Segmentation

- The best algorithm works in a supervised way
- It is not quite perfect so we are working improve tissue analysis by stain normalization
- A number of methods have been tried
- Next is biomarker quantification

Image pre-processing



Histogenic Molecular Mapping

- Histogenic molecular mapping (HMM) is a means of mapping multiple markers form IHC onto a single composite “map” of a tissue section
- It assumes that immediately adjacent sections are similar and objects can be mapped onto each other
- A panel of biomarkers can be used to then create a “map” of activated pathways

Histogenic Molecular Mapping

- HMM requires
  - Handling of large images
  - Tissue registration
  - Tissue segmentation
  - Biomarker evaluation and quantification

Image Registration

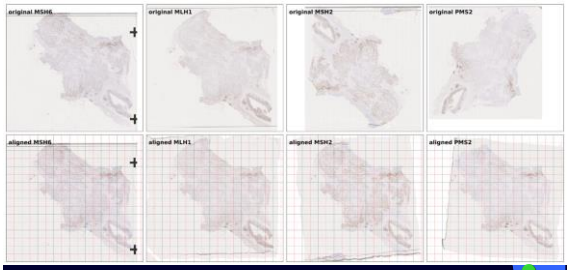
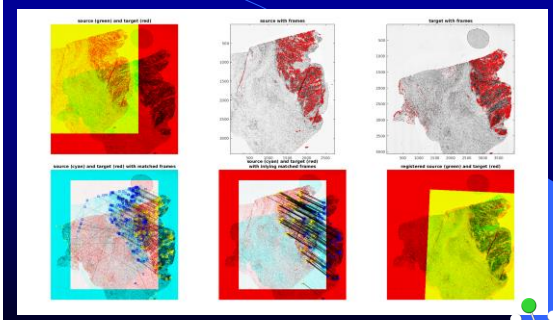
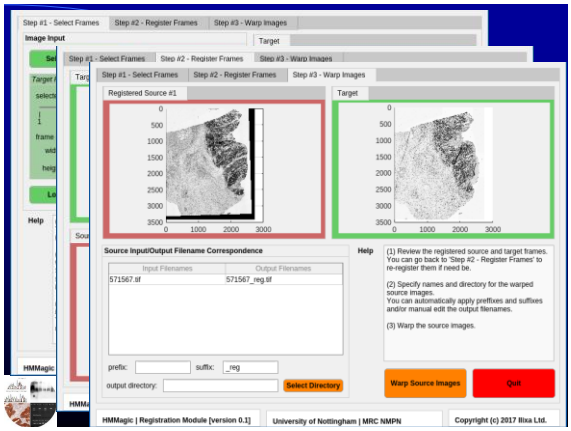
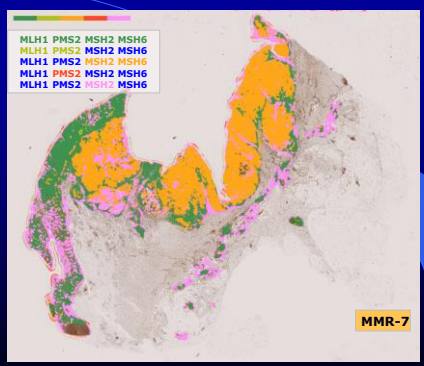


Image registration is a major problem due to variation in positioning and arterfacts

Image Registration







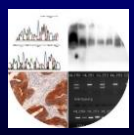
TuPaQ and HMM

- Both should be available soon(!) as a stand-alone packages
- They should deal with images in all formats
- Pathways can then be mapped out
- Biomarker assessment should be accurate

Learning point: Digital Pathology

- Digital Pathology involves image acquisition, data compression and data display.
- Automated image analysis on digital images can improve certain tasks such as biomarker quantification.

New Developments in the laboratory: Next Generation Sequencing



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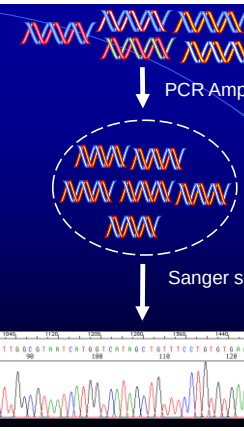
Next Generation Sequencing

- What is NGS?
- The sequencing process
- The NGS workflow (LSD)
- Utility and limitations

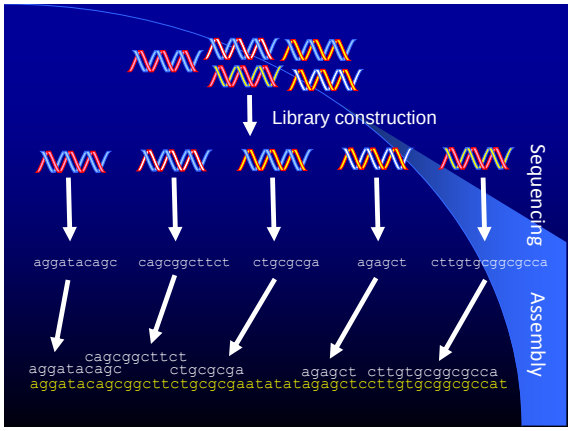
Principles of Sequencing

- Usual type of sequencing is called “Sanger sequencing”
- A pool of molecules is sequenced and the “net” sequence is captured
- Several sequencing chemistries are available with different sensitivities
- Low frequency alleles can however be lost in the background noise



What is NGS?

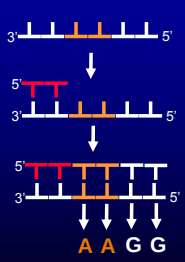
- NGS is also known as massively parallel sequencing
- It allows millions of sequencing reactions to be performed simultaneously
- Individual molecules are captured, sequenced and then assembled
- Template may be PCR products, probe captured DNA, genomic DNA, RNA, miRNA



Principles of Sequencing

- Sequencing is based on DNA replication
- Single DNA strands are used as template and bases are added to synthesis new strands
- Bases can be identified as they are added using radioactivity / fluorescence / pH etc.
- The sequence can be inferred from these signals

Sequencing by synthesis



(1) Starting with a single strand of DNA

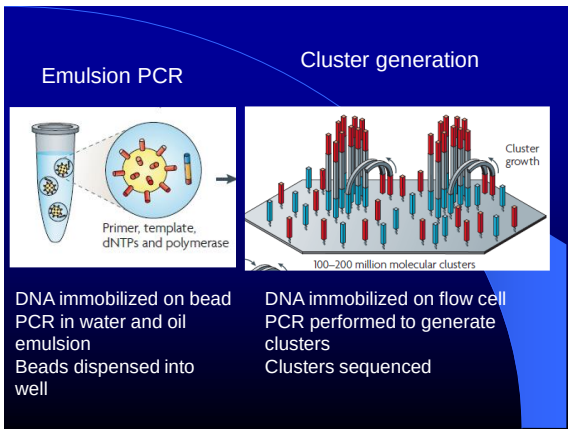
(2) A primer is added

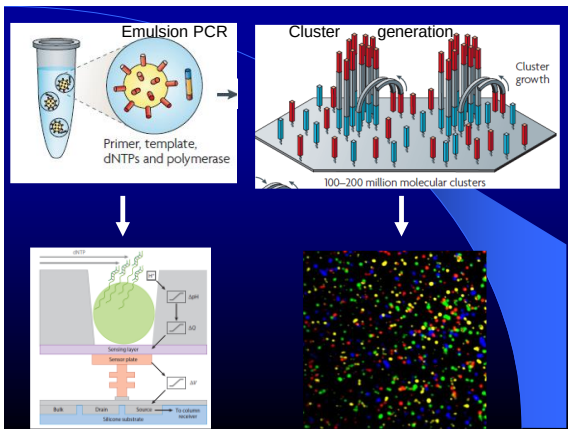
(3) Bases are added

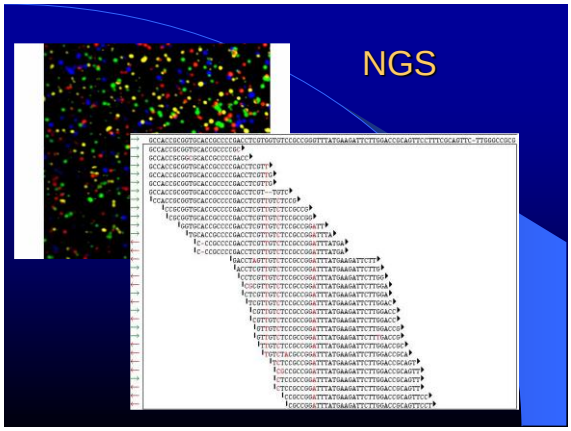
(4) A signal is recorded for each base

Sequencing reaction

- Libraries are converted to single stranded DNA
- Each molecule is immobilized and undergoes clonal amplification
- Sequencing is performed by synthesizing a new strand
- Nucleotide incorporation is detected by changes in fluorescence / pH / pyrophosphate







Next Generation Sequencing

- NGS can be broken down into three simple steps:
- Library preparation
- Sequencing reaction
- Data assembly

NGS workflow

Template

↓

Library preparation

↓

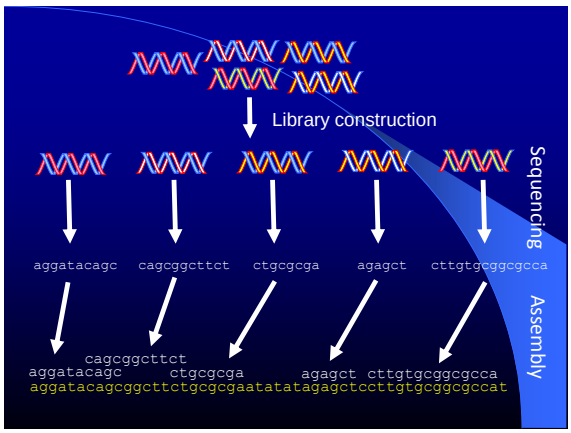
Sequencing

↓

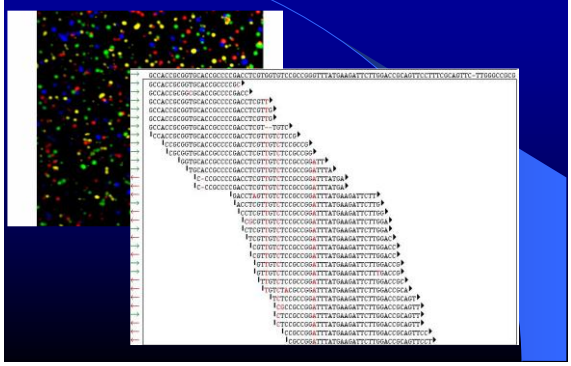
Data analysis

LSD

- The library is your template which has been barcoded
- Libraries can be enriched for specific sequences
- Sequences can be aligned against a *reference sequence*
- Sequenced can be aligned without a reference sequence using overlaps – called *de-novo assembly*



De-Novo sequence assembly



Summary 1

- NGS technology allows massively parallel sequencing – each molecule is sequenced individually
- It depends on Watson-Crick base pairing
- Libraries are samples which have been fragmented and to which adapters have been added to allow sequencing
- Sequence assembly can be done using a reference sequence or *de-novo*

NGS assays

- All nucleic acids can be sequenced using NGS
- For DNA, assays include:
  - Whole Genome Sequencing (WGS): everything (exons, introns, regulatory elements, structural elements)
  - Whole Exome Sequencing (WES): this is just the coding regions of the genome (comprising approximately 2% of genome)

NGS assays

- Targeted sequencing: only certain selected regions of the genome

- For WES and targeted sequencing, the library needs to be enriched by hybridisation capture or PCR
- Each assay has a limited number of reactions – the more target sequence, the lower the sequencing depth

NGS assays

- For RNA, the assay is know as RNA-Seq
- It can be used for quantifying mRNA (i.e. expression profiling), miRNA, lncRNA and footprinting rRNA
- Specialist assays include:
  - Methyl-Seq: used to identify methylated regions in the DNA
  - ChIP-Seq: used to identify DNA-protein interactions (e.g. transcription factors)

Utility and limitations of NGS

- NGS can:
  - Identify point mutations and indels
  - Identify copy number changes
  - Identify structural changes
  - Precisely quantify gene expression

Utility and limitations of NGS

- In addition:
  - WGS is good at identifying structural variants but not good at identifying single nucleotide variations / indels. Vice versa for targeted sequencing
  - There are constraints from the small size of sequence such as missing large indels
  - There are platform specific issues e.g. homopolymers

Utility and limitations of NGS

- Is there anything that NGS cannot do?

- Not much!

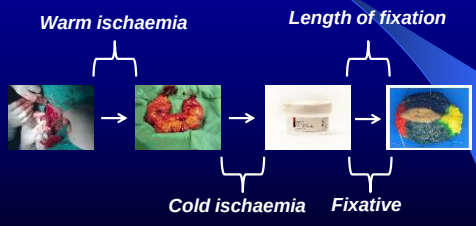
- However:

- There are template issues (previously discussed)

- There are interpretation issues (discussed later)

- There are turnaround time issues

The digital pathology workflow



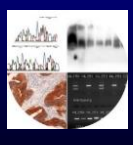
Summary 2

- NGS technology can interrogate all nucleic acid templates
- It can inform on a variety of different types of mutation and will probably replace a number of other tests
- Different assays have different strengths
- There are technical and interpretative issues

Learning points: NGS

- Next Generation Sequencing (NGS) has three steps i.e. (i) library preparation, (ii) sequencing, (iii) data assembly.
- NGS can be performed at varying scales (whole genome sequencing / whole exome sequencing / targeted sequencing) to reveal different types of information.

New Developments in the laboratory: Liquid biopsy



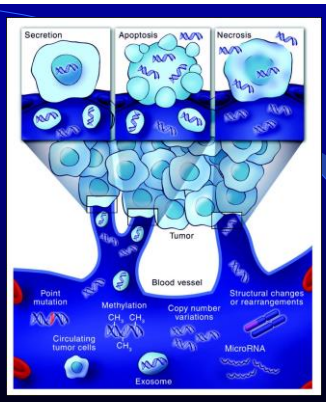
http://www.nmpn.info/

Liquid biopsy

- What is Liquid Biopsy?
- Technical considerations
- Utility and limitations

Circulating biomarkers

- Can interrogate cellular components shed into the bloodstream through tissue damage
- Includes cfDNA, RNA, miRNA, exosomes
- Can also interrogate circulating tumour cells – both for the purposes of genotyping and for culture
- It is also called the “liquid biopsy”



Luis A. Diaz Jr, and Alberto Bardelli JCO 2014;32:579-586

NGH – circulating biomarkers

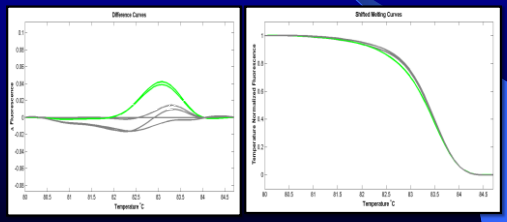
- Tumour screening
- Tumour presence after surgery
- Tumour recurrence
- Tumour response

- Tumour profiling for predictive testing, heterogeneity, prognosis etc.

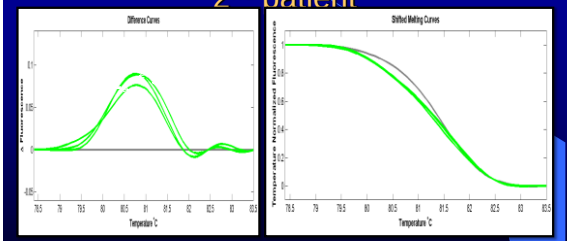
cfDNA as a monitor of surgery

- We reasoned that surgical clearance of tumour could be monitored through testing tumour cfDNA
- Plasma has been collected from patients prior to surgery and every day after surgery until discharge
- DNA was extracted using standard kits
- We screened for mutations using HRM analysis

4th patient: BRAF (Exon 15)



PIK3CA E9
2nd patient



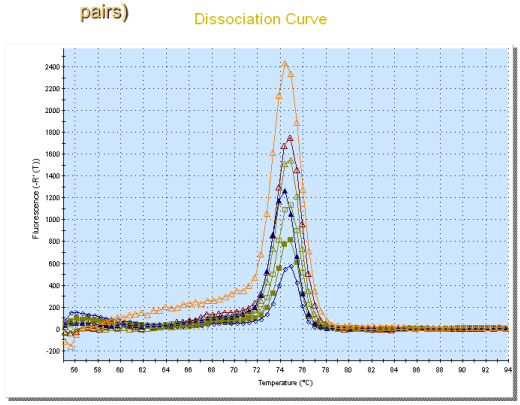
cfDNA as a monitor of surgery

- Initial data very promising with evidence of clearance and non-clearance of tumour
- Data were replicated by two different students
- However, there was variation between mutations and samples e.g. one mutation would appear to be cleared but another wouldn't ; mutations would reappear after clearance
- Tests had to be re-optimised!

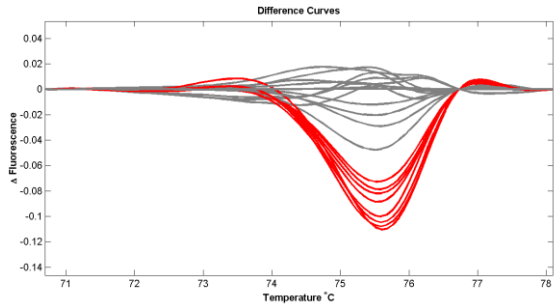
Sample number	58bp (K2)	105bp (K4)	158bp (PTEN 3)	194bp (TP53 5)
5 Day 1	29.52	32.57	36.90	35.94
6 Day 1	29.27	33.79	35.93	No Ct
7 Day 1	31.48	34.70	38.05	No Ct
8 Day 1	30.45	35.95	38.90	No Ct
9 Day 1	31.08	No Ct	34.25	No Ct
10 Day 1	30.04	33.87	36.14	No Ct
11 Day 1	30.54	33.43	37.36	No Ct
12 Day 1	29.36	33.21	35.32	36.88
13 Day 1	29.74	34.28	36.79	No Ct
14 Day 1	32.17	34.87	35.60	37.40
15 Day 1	36.19	No Ct	39.58	No Ct
16 Day 1	32.54	35.31	38.76	No Ct
Norm	30.46	32.26	34.91	37.72
NTC	37.25	No Ct	39.48	No Ct

Green = correct peak, Orange = Slightly odd peak, Red = No peak

PTEN exon 5a (52 base pairs)



Right shift occur's in the sample's with late take off. Sample 11 Day 1 and sample 12 Day 1.



cfDNA as a monitor of surgery

- Further testing of samples shows that size of PCR product is important
- We now design primers for a maximum of 100bp
- Further testing also shows that poor quality samples are developing artefacts
- These are samples with late take-off in the amplification plot although it is uncertain whether this is due to low DNA quantity or other factors

Utility of Liquid Biopsy in cancer

- Tumour screening
- Tumour presence after surgery
- Tumour recurrence
- Tumour response

- Tumour profiling for predictive testing, heterogeneity, prognosis etc.

Learning points: Liquid Biopsy

- Liquid biopsy is the analysis of nucleic acids, exosomes or tumour cells circulating in the blood.
- Testing circulating nucleic acids in constrained by fragmentation of template and low quantity of template.

Overview

- Digital Pathology
  - Utility / TuPaQ / HMM
- Next Generation Sequencing
  - Principles and utility
- Liquid biopsy
  - Utility and limitations