Protoblock - A biological standard for formalin fixed samples

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BACKGROUND

Formalin-fixed, paraffin-embedded (FFPE) is the gold-standard in pathology tissue storage, representing the largest collections of patient material. Their reliable use for DNA analyses could open a trove of potential samples for microbiome research and are currently being recognised as viable source material for these studies. However, there several key features that limit bacterial-related data generation from this material:

i) DNA damage; ***ii)*** Low bacterial biomass (exacerbating contamination and host DNA effects); ***iii)*** Lack of suitable sample prep methods (leading to bias).

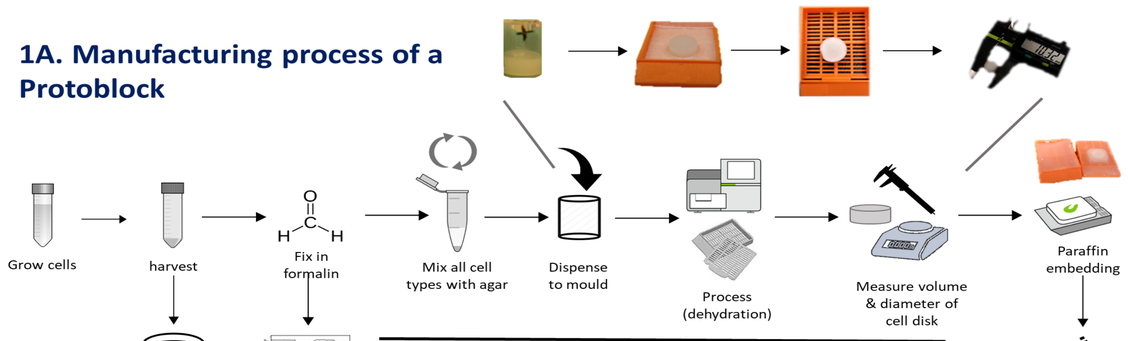
The development and systematic use of reliable standards is a key priority for microbiome research. **More than perhaps any other sample type, FFPE tissue urgently requires the development of standards to ensure the validity and reproducibility of results.** A model that serves as a standard for microbiome analysis of FFPE samples requires:

1) A defined bacterial and host cell load, *2)* Exposure to the same treatment as FFPE specimens, *3)* A format that enables the same treatment as the source material.

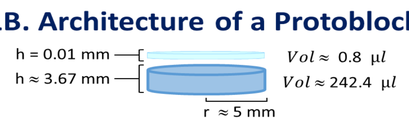
Here we present the **Protoblock**, to serve as a biological standard for FFPE samples. The Protoblock is a cell matrix, which can be populated with cell types and numbers, such as to resemble those of the FFPE tissue specimens. It can be integrated in the workflow at either the FFPE processing stage for prospective studies, or at the sample prep stage for retrospective studies, allowing the assessment of either workflows, highlighting caveats that need consideration in sequencing results.

MAKING THE PROTOBLOCK

1A. Manufacturing process of a Protoblock



1B. Architecture of a Protoblock

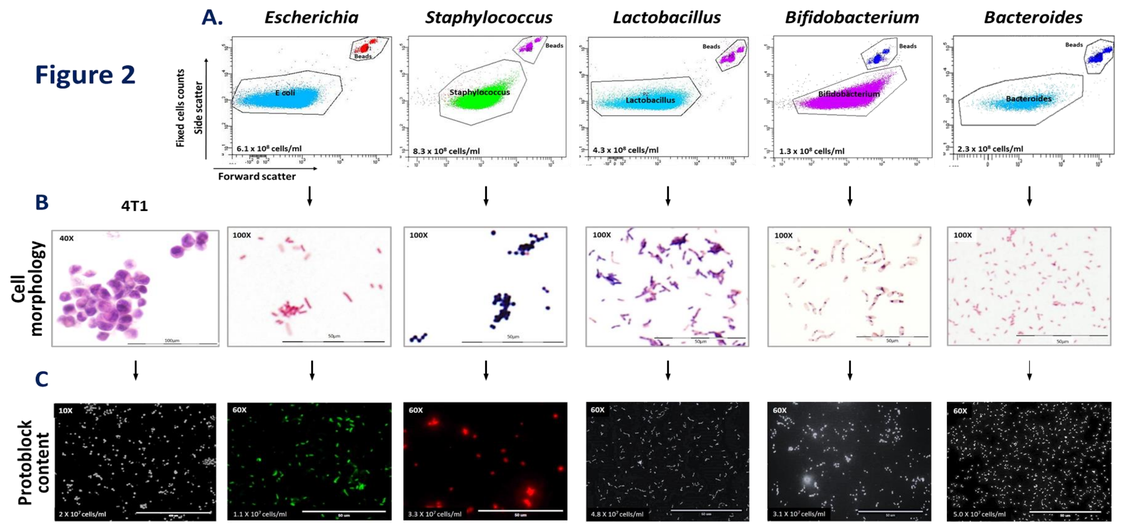


(i) Mammalian and bacterial cells are grown, formalin fixed and counted (cytometry) and the volume of cell suspensions are normalised to content; **(ii)** The cell suspensions are embedded in an agar matrix and solidified into a regular shape. **(iii)** The solidified cell matrix is processed with routine FFPE processing protocols and verified by microscopy **(Fig 1A)**. **(iv)** A slide's cell population is calculated by multiplying the cell content per microliter of block by the volume of a slide **(Fig 1B).**

VALIDATING THE PROTOBLOCK

Comparable ratios of a mix of 5 bacterial strains and 4T1 cells (in the same order of magnitude). Estimated cell content was confirmed by immunofluorescence microscopy in blocks containing individual cell types (Fig 2c) and mixed cell content. Cell wall/membrane integrity was assessed by Gram or H&E staining (Fig 2b).

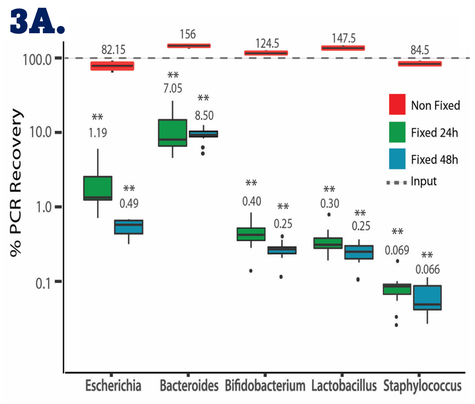
Figure 2



WHAT THE PROTOBLOCK CAN TELL ABOUT FFPE MATERIAL?

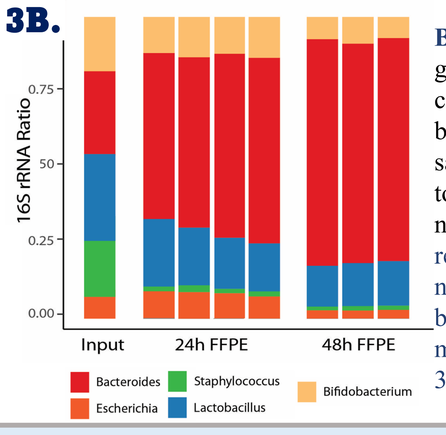
Bias in sample composition

3A.



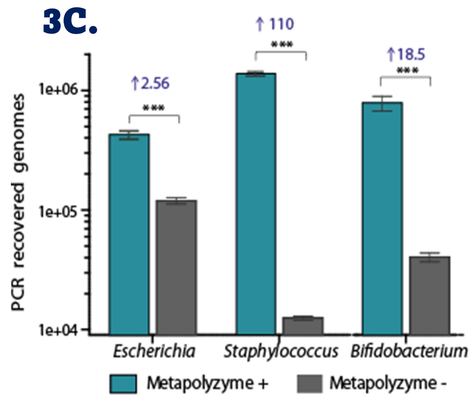
PCR readability. DNA from Protoblocks (with 5 strains) was purified with QIAGEN FFPE DNA kit. DNA recovery was measured via qPCR of strain specific 460bp DNA fragments. A >10-fold reduction of amplifiable DNA for FFPE samples was evident, with longer fixations leading to reduced recovery. The baseline recovery of bacterial FFPE DNA (~460 bp fragments) is ≤ 2-log the input (Fig 3A).

3B.



Bias. 16S rRNA gene sequencing confirmed a clear bias of the sample prep towards Gram-negative bacteria, revealing the need for a bacterial lysis mechanism (Fig 3B).

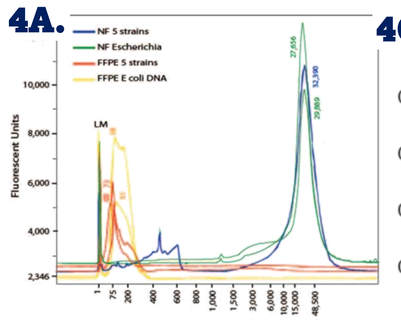
3C.



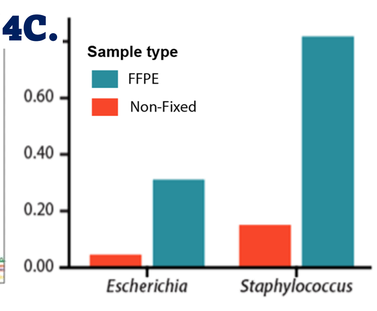
Assessing lysis bias. Protoblocks were treated with/ without a bacteriolytic enzyme cocktail (Metapolyzyme) and analysed via qPCR. For all strains tested, a marked increase in qPCR recovery was evident in samples treated with Metapolyzyme. A bacterial lysis mechanisms is required to guarantee the uniformity of cross-taxa lysis (Fig 3C).

Degree of DNA damage

4A.

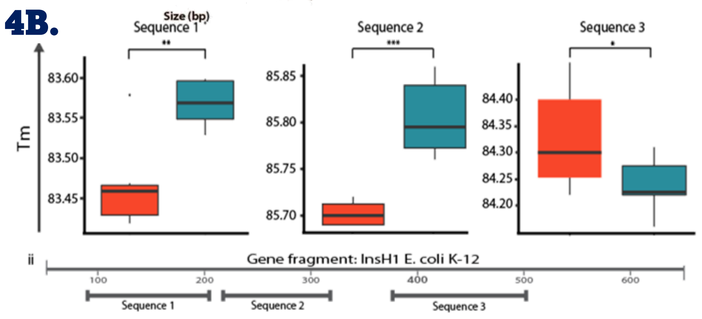


4C.



DNA fragmentation. DNA purified from Protoblocks (FFPE) and Non-Fixed (NF) samples with matched bacterial contents were analysed for integrity (Bioanalyser). NF bacterial DNA was highly integral ($\bar{x}$ = 31,100 bp, *Genomic Quality Number* (GQN) > 6.6). FFPE DNA was highly fragmented ($\bar{x}$ = 110-143 bp, GQN = 0.1) (Fig 4A) **Samples with GQN ≤ 0.3 are not suitable for sequencing analyses*

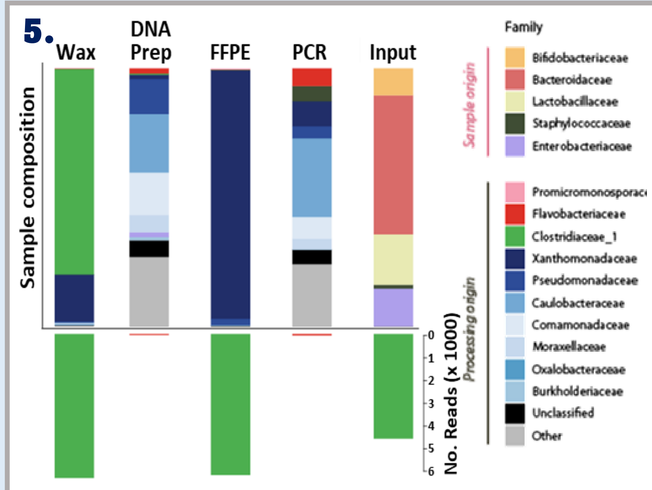
4B.



DNA sequence quality. High-resolution melt of 3 contiguous DNA fragments (≅100 bp) from the *E. coli InsH1* gene was performed in Protoblock DNA. Tm shifts indicative of DNA sequence alterations were observed in all fragments (Fig 4B). Alterations were confirmed by WGS, where a higher number of sequence artefacts (chimeras and SNPs) were found in FFPE samples, when compared with their NF reference (Fig 4C).

Sources of Contamination

5.



Characterising contaminants of FFPE Specimens.
The Protoblock detects contaminants of clinical FFPE samples. A set of controls were tested: *(i)*Wax: a corner from the sample blocks; *(ii)*FFPE: Sterile agar exposed to the FFPE processing workflow; *(iii)*DNA prep control: empty DNA-prep reactions; (iv) PCR: 16S PCR reactions loaded with microbial DNA free water.

The number of reads of each control make evident the detected contaminants (Fig 5). The taxonomic classifications of reads in controls differ completely from those of the Protoblocks. The bacterial biomass in Protoblocks is higher than that of clinical FFPE samples. Thus, the level of contamination poses a significant risk to the accuracy of any sequence analysis of clinical FFPE samples.

CONCLUSION

FFPE tissue is still far from ideal for microbiome studies. However, given the limited availability of rare 'fresh' samples, unlocking the potential of FFPE samples for the microbiome analysis of patient tumours, can improve our understanding of the role of intratumoural bacteria in cancer – *such as the associations between their functions and clinical features of tumour subtypes and responses to immunotherapy*. For this to be a reality, a robust quality control system, including standards, need to be developed. While FFPE microbiome research is still in dire need of optimisation, the Protoblock is well placed for use in optimisation of methods moving the field forward.

FUNDING

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