Application Note MMI-CC-003 – Biomarker Research

Molecular profiling of breast cancer intra-tumor heterogeneity for the development of novel biomarkers

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Abstract

Tumor heterogeneity often challenges cancer diagnosis. In addition, a lack of fresh frozen tissue specimens and nucleic acid degradation in archival tissue can negatively impact cancer diagnostics.

However, as tumor heterogeneity is a known mechanism for the development of drug resistance, it is essential to characterize heterogeneous tumor tissue. Moreover, the quantitative measurement of biomarkers in archival material is useful in oncology research with access to libraries of clinically annotated material, in which retrospective studies can validate potential biomarkers and their clinical outcome correlation.

In this study, our research team optimized quantification of RNA in archival material. The gene expression assay described in this manuscript is a novel, quick, and multiplex method that can accurately classify breast cancer into the different molecular subtypes. Heterogeneous tumors were subjected to laser microdissection using the MMI CellCut system to separate morphologically different tissue areas for subsequent expression analysis.

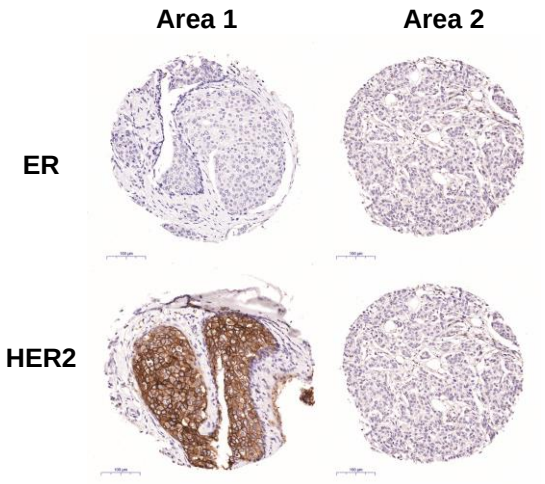

Figure 1: Immunohistochemistry profile of a heterogeneous tumor with Area 1 staining positive for HER2 (with gene amplification) and negative for ER, while Area 2 is staining negative (non-amplified) and weak positive ER.

Introduction

Optimization of RNA based assays using archival formalin-fixed paraffin-embedded (FFPE) material is challenging due to variability in surgical tissue processing and degradation of RNA caused by formalin used for tissue integrity preservation[1,2]. To overcome the limitation of performing accurate gene expression studies from archival material, our group used the bDNA multiplex

magnetic bead assay. Instead of enzymatic amplification of a target template, the bDNA technology uses hybridization of specific probes and amplification of a reporter signal[3]. The short recognition sequences of the capture and detection probes are designed to hybridize to short fragments of target RNA[4]. In addition, the use of tissue homogenates as the direct starting material in this assay, overcomes the inevitable loss of RNA that results from assays requiring prior RNA extraction and purification. Signal amplification, the use of short recognition sequences, and the exclusion of a purification step, contribute to the reduction in technical variation of the assay. The technology provides the possibility to multiplex the assay (up to 80 RNA targets) and measure the expression of a panel of targets from low material input such as from laser microdissected material.

Molecular classification of breast cancer is a process that interrogates molecular markers to categorize patient tumors into three molecular classes, i.e., luminal, human epidermal growth factor receptor 2 (HER2)-enriched, and basal subtype (Figure 1). The HER2-enriched subtype is well defined, with high expression of HER2 receptor, often due to the ERBB2 gene amplification, combined with low or absent estrogen receptor (ER) and progesterone receptor (PgR). The luminal subtype is generally positive for ER and the basal subtype are in general negative for the three receptors (HER2, ER, PgR), and significantly overlaps with the triple negative breast cancer (TNBC) diagnostic subtype[5,6]. Other markers are used to determine epithelial and mesenchymal characteristics. Fibronectin (FN1) is a main component of the breast tissue mesenchymal compartment. Increased FN1 expression is accompanied by high Ki67 staining, and shows a signature for a more invasive tumor[7,8] and is associated with metastasis[9].

Tumor area selection for breast cancer transcriptional subtyping has recurrently been performed by macrodissection[10,11]. To overcome tissue heterogeneity and increase sensitivity, we have reliably combined classical tissue staining with laser microdissection and multiplex molecular profiling methods. As a proof of principle, two distinct breast cancer clones have been defined by their epithelial mesenchymal signature and metastatic potential. The workflow of the described protocol can be easily translated to the current clinical setup and used to selectively isolate and characterize tissue subtypes using targeted mRNA profiling.

Material and Methods

1. Tissue Preparation

1. Using appropriate H&E procedures[12], stained reference tissue sections were prepared and the slides were digitally scanned for reference during microdissection.
2. Using a microtome, 20 µm tissue sections were cut onto an RNAse-free water bath at 40 °C. The sections were collected on membrane slides for laser microdissection using clean, RNAse-decontaminated equipment and materials.
3. The membrane slides were allowed to dry at 37 °C in a drying incubator overnight. Using the appropriate H&E procedure[13], the slides were stained with molecular biology grade solutions while increasing the staining time of hematoxylin to 6 min. Additionally, the membrane slides were stained with the appropriate immunohistochemical protocols[14].

2. Laser Microdissection

1. The slide limits were set in the software and the stage movement as well as the laser settings were calibrated.
2. The stained membrane slides were scanned using the 4x objective.
3. The target areas were selected and encircled for microdissection on the membrane slide. A minimum area of 42 mm^2 was laser microdissected. To determine the volume of homogenizing buffer to be used, the

automated record of the dissected area was activated.
4. The cap-lift mechanism was used to retrieve the dissected section onto the diffuser caps of labeled tubes attached to the appropriate appendage.

3. Tissue Lysis

1. The required homogenizing mixture volume for sample lysis using homogenizing solution and proteinase K at a 60:1 ratio was prepared.
2. 2.4 µL of homogenizing solution were pipetted into each tube for each 1 mm^2 of tissue area, vortexed for 10 s at the maximum speed, and centrifuged at room temperature for 5 s at 2,500 x g.
3. The tubes were incubated in a heating block at 65 °C for 12-18 h with shaking at 600 rpm.
4. The lysates were centrifuged at 21,000 x g for 5 min at room temperature.
5. Using a pipette, the clear supernatant was carefully aspirated and dispensed into a labeled fresh tube.
6. The clear supernatant can be stored in a -80 °C freezer.

4. Hybridization-based Assay and Data Analysis

RNA profiles were assayed using branched DNA capture and signal amplification (Quantigene 2.0) combined with xMAP bead technology as previously described (Baldacchino et al., 2018, JoVE).

Results

The described method has been applied for the simultaneous measurement of 40 transcripts in H&E stained, microdissected (Figure 2) highly degraded FFPE material.

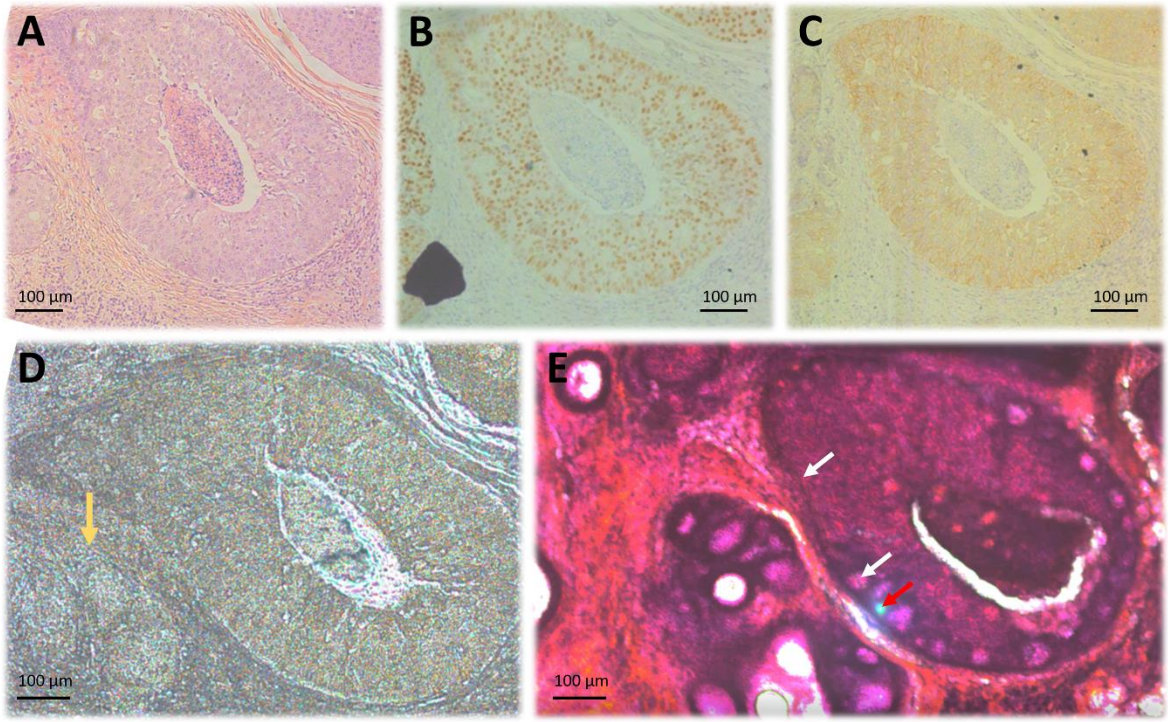

Figure 2: ***Laser microdissection of FFPE tissues.*** *(**A**) H&E stained slide; (**B**) Immunohistochemical staining for ER expression; (**C**) HER2 immunohistochemical staining; (**D**) Unstained 20 µm section on laser microdissection membrane slides. The yellow arrow indicates an area of invasive tumor that is not clearly demarcated due to lack of staining. (**E**) A 20 µm section stained with H&E for better delineation of areas of interest. White arrows indicate laser dissection trail while the Red arrow shows the laser focus during dissection. All illustrations were captured at 10x magnification using the Nikon Eclipse Ti microscope.*

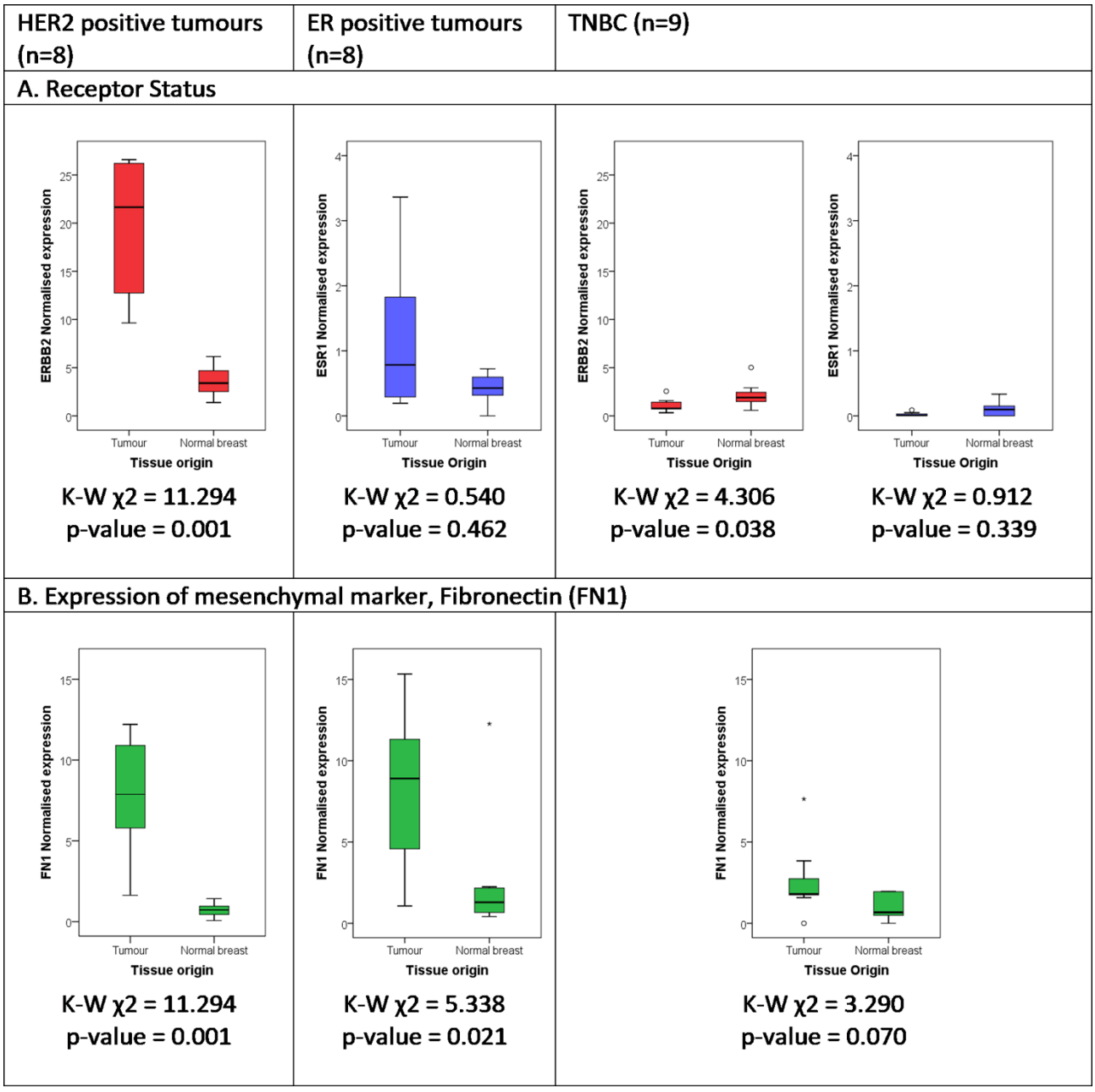

Figure 3: ***Expression of (A) receptor status and (B) mesenchymal marker FN1, in breast tumor compared to matched normal.*** *The classical diagnostic breast cancer subtypes are defined as per diagnostic result using immunohistochemistry and fluorescence in situ hybridization (FISH) for HER2 equivocal immunohistochemical staining. The normalized expression of (**A**) HER2 and ER, (**B**) FN1 measured by the hybridization-based assay is illustrated in HER2 positive, ER positive, and TNBC cases compared to patient matched normal breast tissue. The Kruskal Wallis Test Statistic (K-W χ2) shows that the expression of HER2 is significantly ($p < 0.05$) higher in the tumor tissue as opposed to the matched normal tissue in the HER2 positive cohort and significantly lower in the TNBC cohort. ER expression was not found to be significantly higher in the tumor tissue as opposed to the matched normal in the ER positive and TNBC cohorts. FN1 is significantly higher in the tumor tissue in the HER2 and ER positive subtypes and shows a trend towards being significantly elevated in tumor tissue also in the TNBC cohort.*

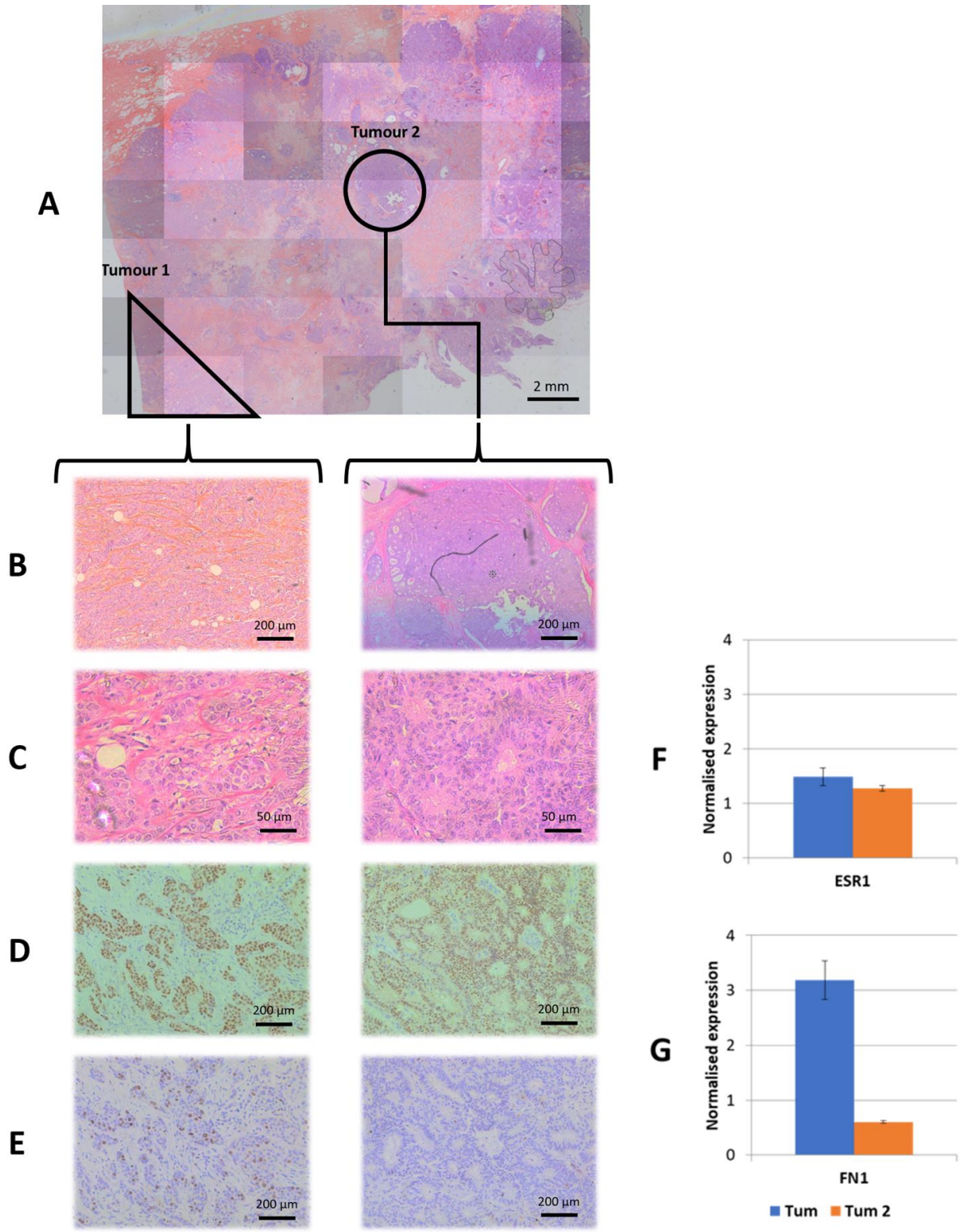

Figure 4: ***Case study: tumor heterogeneity.*** *Morphologically distinct tumors were microdissected and treated as distinct samples. (**A**) The master scan of the H&E section. (**B**, **C**) A 10x and 40x magnification, respectively for each tumor morphology identified. (**D**) Immunohistochemical staining for ER at 10x magnification. (**E**) Immunohistochemical staining for Ki67 at 10x magnification showing a higher mitotic activity in tumor 1. (**F**) Normalized expression levels for the ESR1 gene in each tumor showing relatively high and equal expression between tumors as expected from the immunohistochemical result. (**G**) Normalized expression levels of FN1, a mesenchymal marker, where increased FN1 expression is accompanied by high Ki67 staining showing a signature for a more invasive tumor. The inverse is observed in tumor 2, which appears to be a slower proliferating tumor with a lower malignant potential represented by reduced FN1 expression.*

Using this method, we show the accurate characterization of receptor status (Figure 3), classification of tumors into luminal and basal molecular subtypes[15], and differential expression of the mesenchymal marker, FN1, when comparing tumor and matched control tissue (Figure 4), in the various receptor positive and negative subtypes.

Discussion

A bead-based multiplex bDNA assay was optimized to quantify gene expression on degraded RNA derived from FFPE breast cancer tissue and normal breast ducts. Optimizing the assay involved developing an algorithm to classify breast cancer tumors in luminal and basal subtypes utilizing 8 well-known biomarkers and 5 potential normalizing genes. Data normalization was done using permutations of the normalizing genes. The selection of the normalizing genes was based on the best prediction of receptor status using the Luminal/Basal classifier genes.

The method can be adapted for use in other diagnostic and research areas following adequate selection of the normalizing gene set. One important application of this method in the research sector is the measurement of biomarkers in archival material that is well annotated with clinical outcomes. This could validate potential predictive markers in retrospective studies, quickly and accurately, and avoid long-term prospective studies that are awaiting disease-free survival and overall survival data.

This method also has a wide range of possible applications in the diagnosis of tumors and is adapted to the current diagnostic workflow. The main advantages of this method in the diagnostic field include: (1) implementation of high-throughput assays, (2) excluding subjectivity and equivocal results originating from image-based measurements, (3) accurate detection of multiple targets simultaneously, which enhance accuracy and minimize the use of precious patient samples, and (4) no requirement for highly specialized facilities and human resources. The optimized sampling process, together with the low amount of material required for the bead-based multiplex assay, allows further investigation of tumor heterogeneity. By using laser microdissection to accurately separate multiple foci of malignant tissue from the same patient section, it is possible to compare multiple gene expression between them as well as with matched normal tissue (Figure 4). Low material input is vital for diagnostic application on tumor biopsies that provide limited tumor tissue. The capacity of the assay to measure gene expression from degraded RNA samples allows easy transportation of samples for analysis within an institution or to outside servicing laboratories. In addition, whole section analysis was also possible using H&E stained material.

To summarize, the use of bDNA technology in combination with magnetic bead technology and the selection of the proper panel of target genes, will provide the added advantage of measuring gene expression directly in tissue lysates derived from small amounts of patient material, including microdissected material, exosomes, and circulating tumor cells. In addition to detection of tumor heterogeneity, the proper use of panels has the potential to detect tumor-derived exosomes for early diagnostics and early detection of relapses. Since there is no need for a nucleic acid amplification step, the signal amplification using the bDNA technology, combined with the bead-based multiplex, measures multiple gene expression in clinically-annotated archival material and provide a resource for biomarker validation.

References

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For further information on this study and related work, please refer to Baldacchino et al., 2018, JoVE and Scerri et al, 2019, Methods