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Development and validation of mFISHseq: a diagnostic test using multiplexed RNA-FISH-guided laser capture microdissection RNA sequencing

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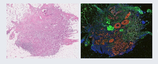
Background

- Breast cancer (BCa) is comprised of multiple histological and molecular subtypes, often displaying considerable intra-tumoral heterogeneity[1].
- Current diagnostic assays such as immunohistochemistry fail to adequately address the complex biology of BCa subtypes by only profiling a few biomarkers. While multigene assays provide more detailed characterization of tumor biology, they sacrifice critical spatial information[2-4]. Thus assays that capture the best of both worlds could be clinically useful.
- **Objective** – To address these limitations, we developed and validated mFISHseq, a novel, spatially informed tool that integrates multiplexed RNA fluorescent in situ hybridization (FISH) of the four main BCa biomarkers (*ESR1*/*PGR*/*ERBB2*/*MKI67*), which are used to guide laser capture microdissection (LCM) of regions of interest followed by RNA-sequencing.

Methods

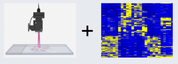
The mFISHseq (Multiplex8+) assay

H&E and Multiplexed RNA-FISH



ACD RNAScope Multiplex Fluorescent V2 Assay
- Estrogen (*ESR1*)
- Progesterone (*PGR*)
- Her2 (*ERBB2*)
- Ki67 (*MKI67*)

Laser capture microdissection (LCM) and RNA-SEQ



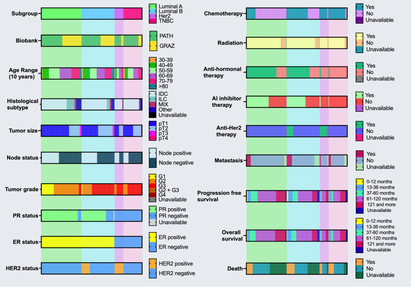
- H&E + Biomarker-guided capture of ROIs
- Takara SMARTer Stranded Total RNA-Seq Kit v3 – Pico Input Mammalian
- NovaSeq 6000 –100M reads/sample (2 x 100 PE)
- Signatures implemented via GeneFu[5]
  - PAM50, AIMS, PAM50 ROR-S

Analytical validity and molecular subtyping

- IHC vs mFISHseq - Area under the receiver operating characteristic curves (AUROC)
- Spearman correlations between FISH vs SEQ
- Subtype outcomes (Kaplan-Meier curves - overall survival) and concordance (Cohen's kappa)
- Effects of LCM vs no LCM on gene expression, subtyping, and risk groups

Retrospective cohort

- 1,082 FFPE breast tissues
- 2 biobanks (Biobank Graz, PATH Biobank), 1 hospital (Malaga), 2 companies
- Clinicopathological data (see right)
  - IHC and 18 years follow up
- Signed informed consent
- Approved by the Ethics Committee of the Bratislava Self-Governing Region (Ref. No. 05320/2020/HF) and the Ethics Commission of the Medical University of Graz on behalf of Biobank Graz (No. 34-354 ex 21/21, 1158-2022)



Results – Analytical Validity and Molecular Subtyping

1 mFISHseq shows excellent agreement with IHC biomarker status with 93% accuracy across training-test sets.

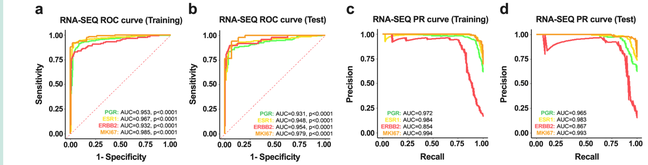


Figure 1. Analytical validity of mFISHseq compared to immunohistochemical data as assessed by area under the receiver operating characteristic (AUC-ROC) curves (**a**, **b**) and precision-recall (PR) curves (**c**, **d**) in 1,013 breast tumors stratified into 70:30 training (n=701) and test (n=312) datasets.

2 Continuous RNA-SEQ expression data shows similar expression patterns to IHC when stratified into semi-quantitative IHC scores.

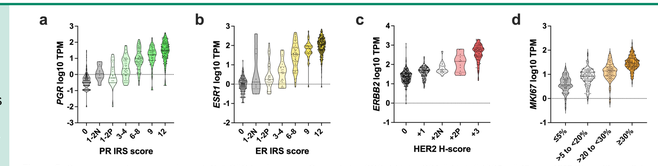


Figure 2. Graphs show the comparison of RNA-SEQ to biobank IHC results for *PGR*-PR (**a**), *ESR1*-ER (**b**), *ERBB2*-HER2 (**c**), and *MKI67*-Ki67 (**d**). Data are presented as a comparison of RNA-SEQ log10 normalized transcripts per million (TPM) values to either immunoreactivity (IRS) score for PR/ER, H-Score for HER2, or percent of positive cells for Ki67. Dotted lines in the violin plots show the median and quartiles.

3 RNA-SEQ and RNA-FISH show moderate to very strong correlations, highlighting the potential for cross-validation using two methods.

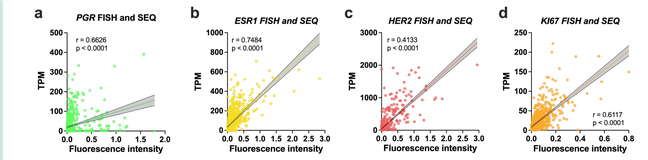


Figure 3. Graphs depict correlations between RNA-FISH (fluorescence intensity) and RNA-SEQ (transcripts per million, TPM) for *PGR* (**a**), *ESR1* (**b**), *ERBB2* (**c**), and *MKI67* (**d**). Correlations are Spearman's rho and the shaded region represents the 95% confidence bands of the best fit linear regression line.

4 mFISHseq's 293-gene molecular subtyping consensus clustering approach has superior prognostic performance and has substantial agreement with PAM50 and AIMS.

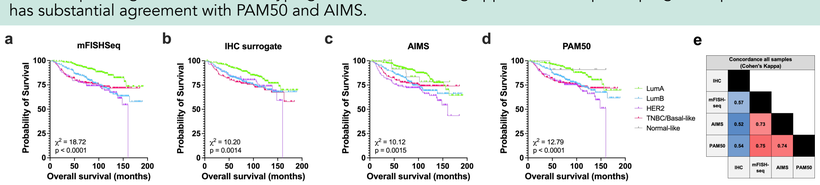


Figure 4. Kaplan-Meier (KM) survival curves comparing overall survival of molecular subtypes assigned by mFISHseq (**a**), IHC surrogate (**b**), AIMS (**c**), or PAM50 (**d**) subtyping schemes. The log-rank test was used to compare KM curves. (**e**) Cohen's kappa concordance between mFISHseq, IHC surrogate, AIMS, and PAM50 subtyping schemes for all samples.

Results – Laser Capture Microdissection

5 To ascertain the importance of laser capture microdissection (LCM), we compared LCM vs no LCM on 40 samples representative of each molecular subtype and with varying tumor content.

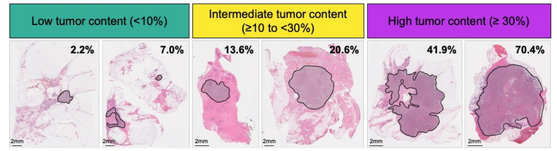


Figure 5. Photomicrographs depict examples of hematoxylin and eosin-stained resected tumor specimens (n=40) with low, intermediate, or high tumor content represented in shaded annotations. Tumor area was dissected and compared with adjacent sections that did not undergo LCM. Scale bars represents 2mm length.

6 LCM enriches for breast cancer biomarkers that are IHC-positive, irrespective of tumor content, and also increases tumor specific gene expression signals.

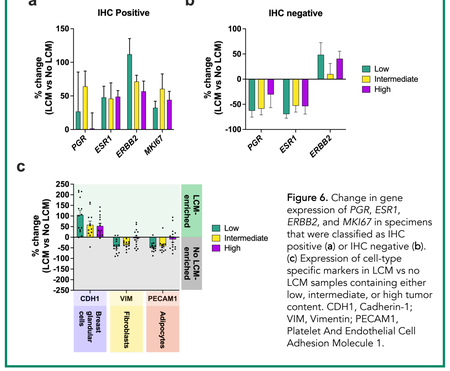


Figure 6. Change in gene expression of *PGR*, *ESR1*, *ERBB2*, and *MKI67* in specimens that were classified as IHC positive (**a**) or IHC negative (**b**). (**c**) Expression of cell-type specific markers in LCM vs no LCM samples containing either low, intermediate, or high tumor content. CDH1, Cadherin-1; VIM, Vimentin; PECAM1, Platelet And Endothelial Cell Adhesion Molecule 1.

7 41% of samples that did not undergo LCM are reclassified into a different subtype (e.g., Normal-like subtype) due to non-tumor tissue contamination.

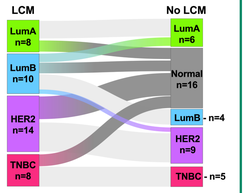


Figure 7. Sankey diagram illustrates the change in mFISHseq consensus subtypes in samples that underwent LCM or No LCM.

8 Prognostic risk assignment is susceptible to the presence of non-tumor tissue, with 41% of samples reclassified into a lower risk group and even 6 patients moving from high to low risk. This has major implications for (neo)adjuvant treatment.

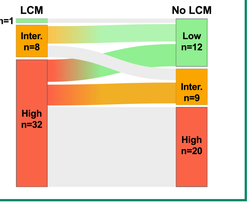


Figure. Sankey diagram illustrates the change in prognostic risk assignment using the PAM50 risk of recurrence by subtype (ROR-S) score in samples that underwent LCM or No LCM.

Summary and Conclusions

- mFISHseq (also known as Multiplex8+[6]) has excellent concordance with the gold-standard IHC. Combining RNA-FISH and -SEQ captures spatial and transcriptome information, while also cross-validating test results.
- In addition to highly accurate quantification of the four main BCa biomarkers, mFISHseq profiles the whole transcriptome, providing superior performance based on outcome in stratifying patients into molecular subtypes.
- LCM is an essential component of the mFISHseq workflow because it ensures removal of non-tumor tissue (e.g., stroma, immune, healthy), thus enhancing signal-to-noise for cancer-specific genes and ensuring accurate assignment of molecular subtypes and prognostic risk groups.

References: **1**. Yersal, O. & Barutca, S.. World J Clin Oncol 5, 412–424 (2014). **2**. Mackay, A. et al. JNCI: Journal of the National Cancer Institute 103, 662–673 (2011). **3**. Weigelt, B. et al. The Lancet Oncology 11, 339–349 (2010). **4**. Bartlett, J. M. S. et al. J Natl Cancer Inst 108, djw050 (2016). **5**. Gendoo, D. M. A. et al. Bioinformatics 32, 1097–1099 (2016). **6**. Paul, E.D. et al. medRxiv 2023.12.05.23299341; doi: https://doi.org/10.1101/2023.12.05.23299341 (2023).

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