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Automated single cell expression profiling in intact tissue by highly multiplexed in situ hybridization

A team of Stanford researchers has developed a highly sensitive, single molecule in situ hybridization system called Proximity Ligation- in situ hybridization (PLISH), which merges the specificity of proximity ligation, the sensitivity of probe tiling, and the high signal intensity of rolling circle amplification. PLISH enables efficient, high level multiplexing by iterative detect-image-erase cycles of four transcripts per round following a single hybridization step, with automated calculation of single cell expression profiles. Downstream data analysis can include clustering algorithms and t-SNE display, but unlike with tissue dissociative approaches for single cell profiling, spatial and morphological data is retained for each cell. Thus, PLISH enables spatial re-mapping of individual cells or cell classes back onto the tissue, allowing for inference of signaling centers, for instance, by localizing cells expressing ligands and cognate receptors. PLISH is highly versatile, performing well on archival FFPE (formalin fixed, paraffin embedded) or cryo-embedded tissue, and is compatible with conventional immunostaining for correlation of RNA and protein expression. PLISH is technically simple and inexpensive, employing conventional fluorescence microscopy and open source software, and can therefore be performed by any laboratory without requiring investment in new equipment.

This technology can be used in combination with immunohistochemistry on primary tissue samples, including formalin-fixed and cryopreserved patient samples. Because PLISH is inexpensive and technically simple, it can be used by even non-expert biomedical researchers for in situ profiling to provide spatial context of newly-defined cell populations and reveal mechanistic aspects of human disease pathophysiology either alone or in conjunction with single-cell sequencing. In addition, it could potentially be adapted for diagnostics applications such as liquid biopsy or rare cell identification.

Stage of Research
The inventors have demonstrated the utility of PLISH for rapid, automated and unbiased cell-type classification by measuring five distinct RNA species in cryopreserved and formalin-fixed, paraffin-embedded (FFPE) lung tissue from mouse and human. These studies validated sensitivity, specificity, speed, signal strength and compatibility with immunohistochemistry. The inventors continue their research to increase the efficiency of high-level multiplexing by optimization of tissue fixation, probe design strategy, and different methods for more rapid detect-image-erase cycles.

Applications

- **Low level combinatorial in situ hybridization (ISH) +/- immunostaining** for:
    - *research* - co-stain for up to five transcripts and/or proteins in histological sections to identify and localize cell types of interest, correlate RNA (mRNA, non-coding RNA, etc.) and protein expression by cells, determine subcellular localization of RNAs of interest, integrate gene expression with morphologic and histologic features in tissue
    - **In situ single cell expression profiling** for:
        - *research* - validate single cell RNA-sequencing (sc-RNAseq) results, localize and map novel cell classes inferred from sc-RNAseq, empirically identify and map molecularly distinct cell sub-types, interrogate and map activity of entire signaling pathways in tissue by co-staining ligand and receptor families
        - *diagnostics* using primary patient samples with potential end user applications such as:
            - quality control and optimization of in vitro directed differentiation protocols of induced pluripotent stem cells
            - analysis of archived tissue - FFPE samples and legacy tissue bank samples of common or rare diseases
            - identify and/or validate disease biomarkers based on specific tissue context

Advantages

- **Multiplexed in situ analysis:**
  - unlimited capacity for multiplexing ISH with single hybridization step followed by detect-image-erase of 4 transcripts per cycle
  - "on-deman" capacity to analyze any gene of interest, without requiring pre-existing antibodies or other reagents
  - provides spatial context and localization of individual cells of interest in intact tissue
- **Inexpensive and technically simple:**
  - commodity oligonucleotides and enzymes (reagents cost ~$1/gene vs. >$1000 for conventional in situ kits)
  - no specialized equipment - signal detection with conventional fluorescent microscope
  - optimized probes reduce both background signal and the number of probes required for imaging
- **Fast** - single-step barcoding with rapid label-image-erase cycles and data collection
- **Specific and sensitive detection:**
  - specificity from coincidence detection
  - targets multiple sites in RNA sequence to detect low-abundance transcripts
  - high signal-to-noise from enzymatic amplification renders tissue autofluorescence inconsequen
  - single molecule resolution with low false positive rate (less than 0.05 RNA counts per cell)
  - enables deep profiling with increasingly complex mixtures of hybridization probes
- **Compatible** with:
  - immunohistochemistry - can be performed concurrently
  - archival FFPE tissue samples

Publications

- Monica Nagendran, Daniel P. Riordan, Pehr B. Harbury, Tushar J. Desai, Automated cell-type classification in intact tissues by single-cell molecular profiling, eLife, Jan 10, 2018 (eLife 2018;7:e30510).

Patents

- Published Application: 20200224243

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