Supporting material

Multiplexed single-cell analysis of fine needle aspirates allows accurate diagnosis of salivary gland tumors

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**Supporting Figure 1.** Sample processing of FNA samples. A. FNA samples were obtained *ex vivo* using 22G needle on surgically removed tumor tissue. B. Cells in each FNA sample were cytocentrifuged by Cytospin and then attached on a glass slide. C. Cells were imaged on the glass slide. An example image of H&E stain of an FNA sample (salivary duct carcinoma) is presented on the right.
Supporting Figure 2. Overview of the chemistry enabling FAST imaging. The FAST linker synthesis starts with a lysine scaffold contains a PEG₄ linker for efficient antibody conjugation: i. TSTU, DIPEA; ii. H₂N-PEG₄-CO₂H; iii. DCM/TFA (20%); iv. rTCO-PNP, DIPEA; v. piperidine (7.5%). The linker was functionalized with AF488, AF555 or AF647. To generate the quencher, BHQ3-amine was coupled with HTz-PEG₅-NHS to yield BHQ3-Tz in one step. The fluorescent signal of FAST-labeled antibodies can be efficiently quenched (>90%) with 10 μM of BHQ3-Tz in < 1 min³.
Supporting Figure 3. Validation of antibody specificity and biomarker expression across samples. To compare the FAST-FFPE staining to immunohistochemistry, we processed a number of FFPE sections of representative samples for both methods. Shown here is one example of mucoepidermoid carcinoma cases, expressing high levels of CK7, MUC1, p63. The staining patterns matched between FAST-FFPE and IHC. Reproducible staining patterns of the FAST antibodies were observed across multiple sections.
Supporting Figure 4. Biomarker detection in FFPE tissue sections of different SGT subtypes. To assess and compare the expression pattern and level of each biomarker measured by FAST antibody staining in FNA and tissue sections, FFPE tissue sections of representative samples for each subtype were imaged (PA, MuEC, LEC, and MyEC are shown). High expression of key molecular markers in each SGT subtypes was detected matching with the results from FAST-FNA (Fig.2).
Supporting Figure 5. Normalization and global threshold setting strategy. Normalization was necessary to account for the multiplicative batch effect in the fluorescent intensities measured in individual cells imaged for a given marker (EPCAM in this example). Samples were first normalized to the 10th percentile of signal to align the left edge of negative peak in each sample. Aligned data was combined for each marker to set the global threshold for the positive signal. Threshold was placed at the full width at half maximum (FWHM) away from the peak location of negative peak of the aggregate data.
Supporting Figure 6. ROC curves of PA diagnosis based on HMGA2, GFAP, or PLAG1. **A.** The positive fraction of HMGA2, GFAP, or PLAG1 expressing cells in each specimen of both PA and non-PA cases was used for ROC plots. The threshold was determined at the fraction value that maximizes the sum of sensitivity and specificity as shown in each table. Accuracy was calculated as the ratio of correctly identified specimens in all PA and non-PA samples included in the analysis. **B.** The positive fraction of each marker in PA and non-PA specimens are shown for comparison. Student’s t-test was used for statistical analysis.
Supporting Figure 7. Correlation between analysis results of single cell homogenate of tissues and its FNA. To validate FAST-FNA measurements, we performed a direct comparison between the results from single cell homogenate of surgically removed SGT tissues and its FNA samples obtained post-operatively. Bar graphs present the fraction of positively stained cells for each marker. In general there were good correlations between the two sample types, and the biomarker expression profiles were similar. Overall, the average Pearson correlation coefficient was $\rho=0.89$ with standard deviation of 0.02.
Supporting Figure 8. Correlation of nuclear NTRK measurement by FAST-FFPE and IHC. The fluorescent intensity of NTRK stain in FFPE tissue sections imaged by FAST-labeled antibody (FAST-FFPE) was compared to the signal intensity measured by IHC in consecutive sections. Each datapoint represents the signal measured in individual cells in representative tissue sections of FFPE and IHC. Signal intensity was measured after background subtraction and scaling to 1 as maximum value. The Pearson correlation coefficient was $r=0.70$. 
Supporting Figure 9. Summary of SGT specimen processing for various analyses presented in the study.
### Supporting Table 1. Antibodies utilized for FAST imaging to characterize various SGT subtypes. Additional information is included in Supporting Table 2.

| Marker | Target | Vendor | Cat #     | Clone | Isotype     |
|--------|--------|--------|-----------|-------|-------------|
| p63    | MuEC, MyEC | Abcam  | ab735     | 4A4   | Mouse IgG2a |
| NTRK   | SC     | Abcam  | ab76291   | EP1058Y | Rabbit IgG  |
| CD45   | Immune cells | BD Pharmingen | 555480 | Hi30 | Mouse IgG1 |
| EpCAM  | Tumor cells | Biolegend | 324202   | 9C4   | Mouse IgG2b |
| EGFR   | Tumor cells | Selleck Chemicals | A2000 | -     | Human IgG1  |
| Her2   | SDC    | BioXcell | BE0277   | 7.16.4 | Mouse IgG2a |
| Muc1   | MuEC   | Fitzgerald | 10-M93A  | M01102909 | Mouse IgG  |
| CK5/6  | MyEC, MuEC | Millipore | MAB1620 | D5/16B4 | Mouse IgG1 |
| GATA3  | SC     | Biolegend | 35-4900  | 1G8   | Mouse IgG1 |
| CD117  | AdCC   | Biolegend | 312302 | 104D2 | Mouse IgG1 |
| LEF1   | BCA    | Biorad  | AF7647    | Polyclonal | Goat IgG |
| GATA15 | SC, SDC | BD Biosciences | 558686 | L50-823 | Mouse IgG1 |
| CD18   | Tumor cells | Biolegend | 915106  | D6    | Mouse IgG2a |
| AR     | SDC    | CST     | 5153     | D6F11 | Rabbit IgG |
| GFAP   | PA     | Biolegend | 644702  | 2E1.E9 | Mouse IgG2b |
| CK7    | MuEC, SDC, AdC | Biolegend | 601601  | W16155A | Rat IgG2a |
| TrkB   | SC     | R&D     | MAB3971  | 72509 | Mouse IgG1 |
| HMGA2  | PA     | CST     | 8179S    | D1A7  | Rabbit IgG |
| Myb    | AdCC   | R&D     | AF6209   | Polyclonal | Sheep IgG |
| NR4A3  | ACC    | Millipore sigma | HPA043360 | Polyclonal | Rabbit IgG |
| s100A4 | SC, MyEC | Biolegend | 810101  | S100A4 | Rabbit IgG |
| TrkC   | SC     | R&D     | AF373    | Polyclonal | Goat IgG |
| PLAG1  | PA     | Novus Biologicals | H00005324-M02 | 3B7 | Mouse IgG2a |
| Sox10  | ACC, MyEC | R&D     | AF2864   | Polyclonal | Goat IgG |
| Muc5ac | MuEC   | ThermoFisher | MA5-12175 | 45M1 | Mouse IgG1 |
| aSMA   | Fibroblasts | ThermoFisher | 14-9760-80 | 1A4 | Mouse IgG2a |
| panCK  | MuEC, ACC | Biolegend | 914204  | AE-1/AE-3 | Mouse IgG1 |
| Rabbit IgG | 2' for NTRK | Biolegend | 410404  | 6B9G9 | Mouse IgG1 |
| Isotype control N/A | BioXcell | BE0083 | MOPC-21 | Mouse IgG1 |
| Isotype control N/A | BioXcell | BE0085 | C1.18.4 | Mouse IgG2a |
| Isotype control N/A | BioXcell | BE0086 | MPC-11 | Mouse IgG2b |
| Isotype control N/A | Biolegend | 910801 | Poly29108 | Rabbit IgG |
| Isotype control N/A | R&D | AB-108-C | Polyclonal | Goat IgG |
| Isotype control N/A | R&D | 5-001-A | Polyclonal | Sheep IgG |
| Isotype control N/A | BioXcell | BE0089 | 2A3 | Rat IgG2a |

**Tumor subtype abbreviations**: MyEC, myoepithelium carcinoma; MuEC, mucoepidermoid carcinoma; SC, secretory carcinoma; SDC, salivary duct carcinoma; ACC, acinic cell carcinoma; AdCC, adenoid cystic carcinoma; PA, pleomorphic adenoma; BCA, basal cell adenoma.
Supporting Table 2. Biomarkers commonly used in SGT subtype analysis and the cell lines used for antibody testing of each biomarker.

| Marker | Target | Pattern | Reference | Cell line |
|--------|--------|---------|-----------|-----------|
| NR4A3  | ACC    | N       | Am J Surg Patho, 2019; 43(9):1264-72 | A431 |
| SOX10  | ACC, MyEC | N   | Int J Mol Sci, 2021;22:6776 | U-2 OS |
| CD117  | AdCC   | M/C    | Int J Mol Sci, 2021;22:6776 | HEK293 |
| MYB    | AdCC   | N      | Int J Mol Sci, 2021;22:6776 | THP-1 |
| LEF1   | BCA    | N      | Diagn Cytopathol, 2017;45(12):1078-83 | HEK293 |
| TP63   | MuEC   | N      | Int J Mol Sci, 2021;22:6772 | RT4 |
| MUC1   | MuEC   | C      | Int J Mol Sci, 2021;22:6772 | U-2 OS |
| MUC4   | MuEC   | C      | Int J Mol Sci, 2021;22:6772 | MCF-7 |
| MUC5AC | MuEC   | C      | Int J Mol Sci, 2021;22:6772 | A549 |
| GFAP   | PA     | C      | Int. J. Mol. Sci. 2021, 22(13), 6771 | U-2 OS |
| HMGAL1 | PA     | N      | Genes Chromosom Cancer 2009;48(1):69-82 | A431, U-2 OS |
| PLAG1  | PA     | N      | Int J Mol Sci, 2021;22:6776 | U-2 OS |
| panNTRK| SC     | N/C    | Histopathology, 2019;75(1):54-62 | CAPAN-2 |
| GCDFP15| SC, SDC| C      | Am J Surg Patho, 2010; 34(5)599-608 | MDA-MB231 |
| TRKB   | SC     | C      | Ann Diagn Pathol, 2021; 50(9):151673 | U-2 OS |
| S100   | SC, MyEC | C    | Am J Surg Patho, 2010; 34(5)599-608 | A549 |
| TRKC   | SC     | N      | Oncogene 2013; 32:3698–3710 (2013) | A549, U-2 OS |
| GATA3  | SC, SDC| N      | Head and Neck Pathol. 2013;7(4):311-5 | A431, U-2 OS |
| AR     | SDC    | N/C    | Int J Cancer, 2018;143(4):758-766 | U-2 OS |
| EpCAM  | Tumor cells | M    | Arch Oral Biol. 2017;79:87-94; Pathology 2018; 50(7) | A431 |
| EGFR   | Tumor cells | M    | Oral Oncol, 2012; 48(10):991-996 | A431 |
| HER2   | SDC    | C/M    | Diagn Histopathol, 2020; 26(4): 159-164 | BT474 |
| CK5/6  | MyEC, MuEC | C    | Int J Mol Sci, 2021;22:6771 | A431 |
| AFP    | Tumor cells | C    | J Nihon Univ Sch Dent, 1992; 34:240-248 | MCF-7 |
| CK18   | Tumor cells | C    | J Oral Biol Craniofac Res. 2014; 4(2): 127–134 | A431, U-2 OS |
| CK7    | MuEC, SDC, AdCC | C    | Int J Mol Sci, 2021;22:6771 | RT4 |
| panCK  | MuEC, ACC | C    | Int J Mol Sci, 2021;22:6771 | MCF-7 |
| SMA    | Fibroblasts | C     | Front Biosci. 2010;15:226-236 | A431 |
| CD45   | Immune cells | M    | Annu Rev Immunol, 1994; 12, 85–116 | PBMC |

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### Supporting Table 3. Cost estimate.

| Items                      | Unit price ($) | Cost per sample ($) |
|----------------------------|----------------|---------------------|
| Antibody conjugation       | 11.37 per antibody (10 ul reaction) | 0.57 |
| Glass slide                | 0.62 each slide | 0.62 |
| Octospot                   | 2.14 each slide | 2.14 |
| Antibodies                 |                |                     |
| anti-p63                   | 2.10 / μl      | 0.53 |
| anti-NTRK                  | 8.57 / μg      | 2.14 |
| anti-CD45                  | 0.84 / μg      | 0.21 |
| anti-EpCAM                 | 1.75 / μg      | 0.44 |
| anti-EGFR                  | 0.14 / μg      | 0.04 |
| anti-Her2                  | 0.15 / μg      | 0.04 |
| anti-Muc1                  | 0.25 / μg      | 0.06 |
| anti-CK5/6                 | 0.29 / μg      | 0.07 |
| anti-Muc4                  | 3.98 / μg      | 0.99 |
| anti-AFP                   | 2.95 / μg      | 0.74 |
| anti-CD117                 | 1.50 / μg      | 0.38 |
| anti-LEF1                  | 4.36 / μg      | 1.09 |
| anti-GATA3                 | 3.48 / μg      | 0.87 |
| anti-GCDFP15               | 1.95 / μg      | 0.49 |
| anti-CK18                  | 1.85 / μg      | 0.46 |
| anti-AR                    | 5.51 / μg      | 1.38 |
| anti-GFAP                  | 2.50 / μg      | 0.63 |
| anti-CK7                   | 2.15 / μg      | 0.54 |
| anti-TrkB                  | 5.16 / μg      | 1.29 |
| anti-HMG2                  | 4.89 / μg      | 1.22 |
| anti-Myb                   | 4.76 / μg      | 1.19 |
| anti-NR4A3                 | 7.16 / μl      | 1.79 |
| anti-s100A4                | 1.95 / μg      | 0.49 |
| anti-TrkC                  | 5.16 / μg      | 1.29 |
| anti-PLAG1                 | 8.58 / μg      | 2.15 |
| anti-Sox10                 | 5.40 / μg      | 1.35 |
| anti-Muc5ac                | 4.04 / μg      | 1.01 |
| anti-aSMA                  | 4.20 / μg      | 1.05 |
| anti-panCK                 | 1.95 / μg      | 0.49 |
| anti-Rabbit IgG            | 2.75 / μg      | 0.69 |
| Mouse IgG1 isotype         | 0.15 / μg      | 0.04 |
| Mouse IgG2a isotype        | 0.15 / μg      | 0.04 |
| Mouse IgG2b isotype        | 0.15 / μg      | 0.04 |
| Rabbit IgG isotype         | 1.82 / μg      | 0.46 |
| Goat IgG isotype           | 0.11 / μg      | 0.03 |
|                  |         |      |
|------------------|---------|------|
| Sheep IgG isotype| 0.11 / μg | 0.03 |
| Rat IgG2a isotype| 0.15 / μg | 0.04 |
| **Total**        |         | **29.12** |