



KEY FACTORS IN CLINICAL DECISION MAKING:

Assessment of reproducibility in multiplex immunohistochemistry

SUMMARY

Multiplex Immunohistochemistry (mIHC) is a powerful tool for the simultaneous detection of tissue-based biomarkers, revolutionizing traditional immunohistochemistry. mIHC facilitates assessments at the cellular level of different proteins, as well as their spatial arrangement on a single tissue section to determine their role in therapeutical outcome, thus enabling precision medicine in translational research and clinical practice. However, several technical requirements are important to performing high-quality staining and analysis and to obtaining high internal and external reproducibility of the results. For a mIHC assay to be considered validated, at a minimum, it must be demonstrated to be reproducible from an analytic perspective. Here we present the results of a validation test to verify the precision of the imaging mass cytometry (IMC) workflow, therefore the reproducibility of the data.

APPROACH

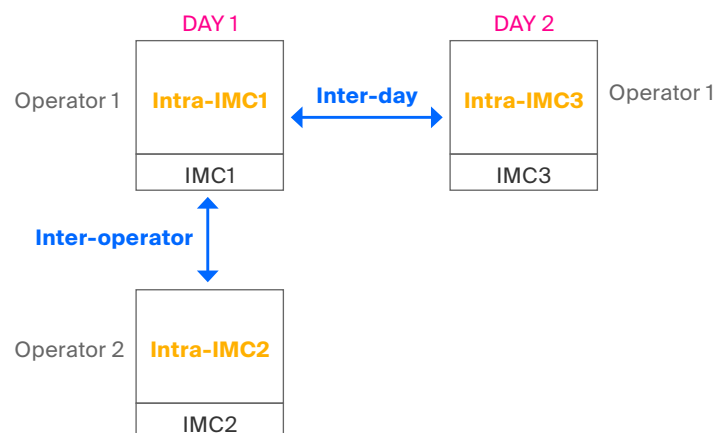
The mIHC workflow starts with antibody selection, optimization, and validation and ends with a digital image analysis. It is important to refine, standardize, optimize, and validate the end-to-end workflow in mIHC to obtain reproducible results to support large-scale multi-site trials and to enable their possible clinical application. To determine the reproducibility of markers in a mIHC panel, we must consider that a group of markers is being evaluated and that those markers have specific cell phenotypes across different sections, according to the abundance of specific cell phenotypes. Other external variables can interfere with the analysis; therefore, it is important to evaluate the precision of the assay by performing a comparison between different experimental conditions.

TABLE 1. Antibody panel

| TARGET GROUP | METAL | TARGET NAME |
|---------------|-------|-------------|
| Macrophage | 159Tb | CD68 |
| Vasculature | 141Pr | SMA |
| B lymphocytes | 161Dy | CD20 |
| T lymphocytes | 162Dy | CD8a |

In the presented approach, serial sections of tonsil tissue were stained with six (6) metal-tagged antibodies (Table 1) and operated by two different operators in two different days (Figure. 1).

Three (3) regions of interest (ROIs) were selected and ablated for each tissue section. Inter-IMC and intra-IMC reproducibility were assessed with the calculation of the coefficient of variation (CV%), standard error (SE) calculation and linear plotting in scatter plot.

FIGURE 1. Experimental plan



WORKFLOW

Twelve (12) consecutive tissue sections of FFPE Human Tonsil (non-pathological tissue) were used. Sections number 4, 8 and 12 stained with Hematoxylin and Eosin (H&E) were used to define regions that were maintained similar across the sections to be analyzed. According to the H&E staining, three (3) regions of interest (ROIs) framing geminal center, lymphoid follicles and squamous epithelium areas were selected for all the remaining sections used for IMC. The ROIs were grouped in 3, creating technical replicate groups in each slide to allow a consistent comparison between different acquisition (Fig. 2).

The generated data was analyzed with our proprietary digital algorithms. In brief, in order to compare the ROIs from different tissue sections, an overlay of the different images from the same group was performed. This allowed selection and extraction of a new common area from all the ROIs. Cell segmentation and classification to negate observer variability were then performed (Fig. 3) to generate the abundance of cell types within the region analyzed. The advantage of the digital analysis is to avoid the observer variability.

FIGURE 2. Graphical representation of the ROIs group

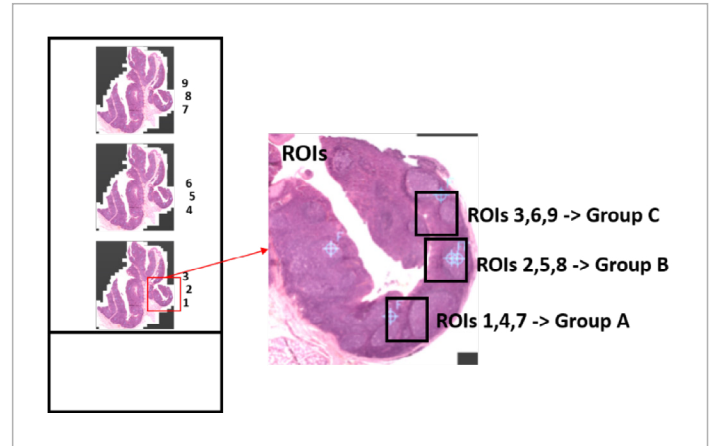
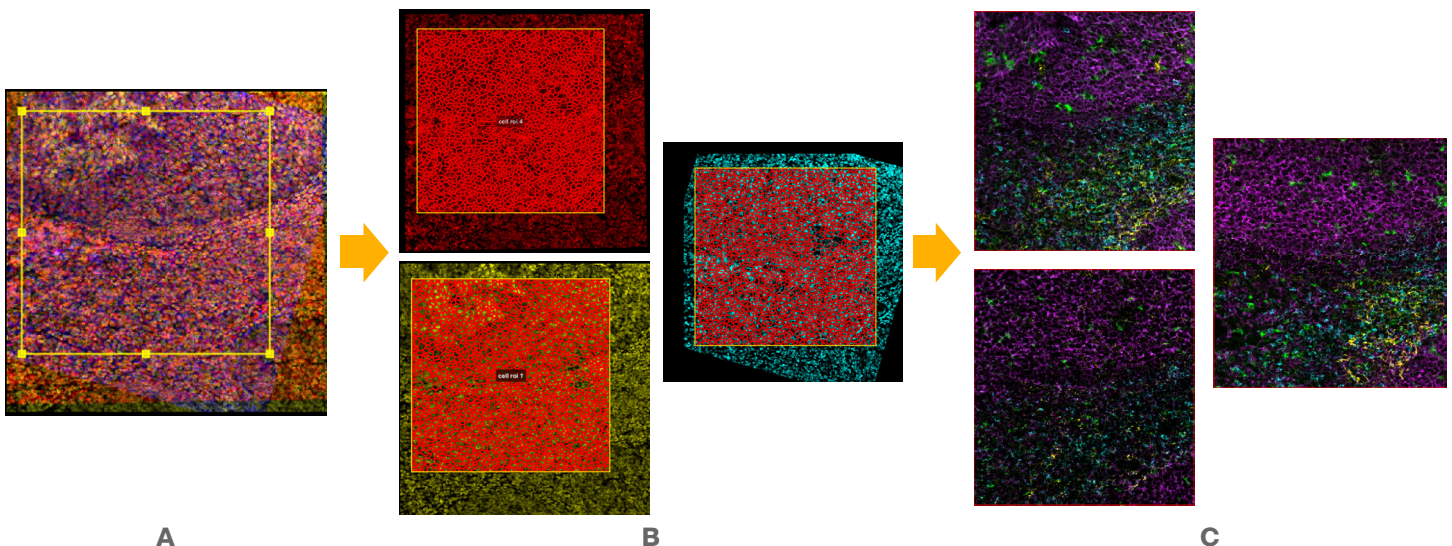


FIGURE 3 (A). Overlay of all group A ROIs to define a new common area, in yellow. (B) The cells in each new area common to all the images were segmented and (C) classified according to their staining. It is possible to appreciate the inter-IMC staining consistency. aSMA : Yellow, CD68 : Green, CD20 : Pink, CD8 : Cyan.





RESULTS

In Table 2, the results from the calculation of the intra and inter IMC reproducibility are presented. Intra-IMC reproducibility across all 3 markers (CD20, CD8a and CD68) was fairly good based on the calculated CVs. The SMA however showed higher variability. The comparison of the percent of positive cells for each marker inter-day and operator also showed a good concordance (Figure. 4), with the exception of the group C in IMC3. Accuracy from which each marker is representative of the data set was good based on the standard error.

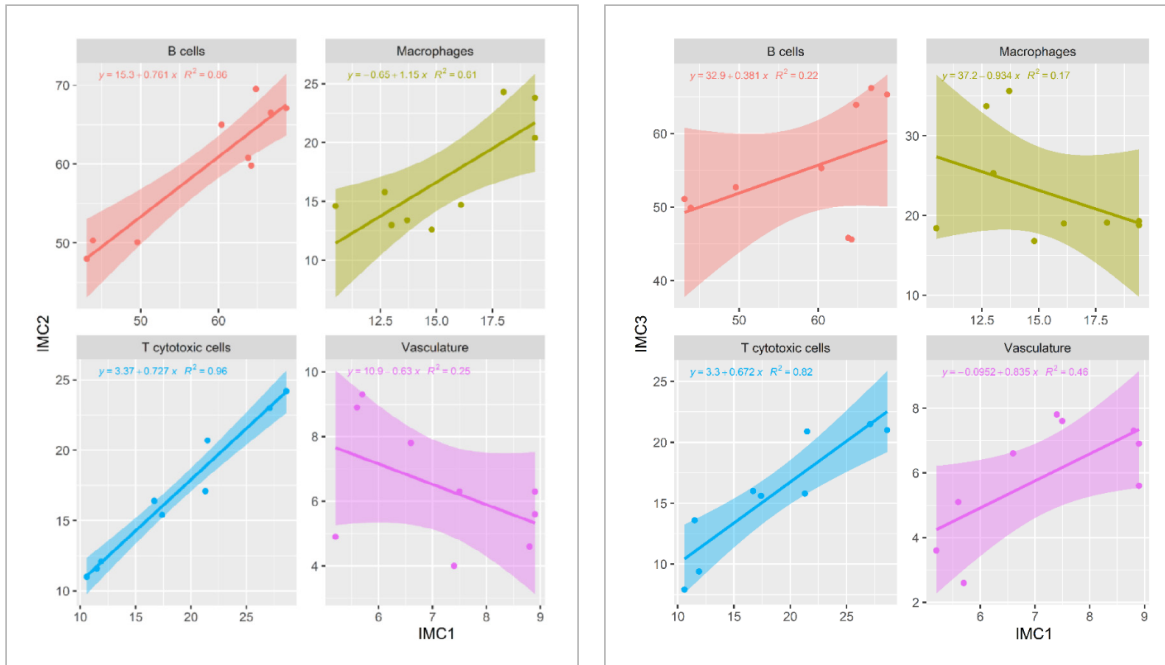
TABLE 2. Results of %CV (up) and SE (down) calculation The coefficient of variation (CV) is a relative measure of the dispersion of data around the mean and it is calculated by dividing the standard deviation by the mean. This value is expressed in percentage (CV%). The standard error (SE) is a value that reflects the standard deviation (SD) of the mean within a data set. This error was used to ensure the precision and reliability of the estimation.

| CV% | | B LYMPHOCYTES (CD20) | T CYTOTOXIC LYMPHOCYTES (CD8A) | VASCULATURE (SMA) | MACROPHAGES (CD68) | AVERAGE CV% | |
|-------------------------------|---------|----------------------|--------------------------------|-------------------|--------------------|-------------|--------|
| Intra-IMC1 | Group A | 7,8 | 14,5 | 9,9 | 5,0 | 9,3 | <10% |
| | Group B | 2,9 | 5,9 | 16,3 | 21,2 | 11,6 | 10-20% |
| | Group C | 3,3 | 13,4 | 4,8 | 3,9 | 6,4 | 20-30% |
| Intra-IMC2 | Group A | 2,6 | 7,9 | 24,0 | 9,3 | 10,9 | >30% |
| | Group B | 2,3 | 4,8 | 17,1 | 8,5 | 8,2 | |
| | Group C | 4,5 | 5,2 | 31,6 | 10,8 | 13,0 | |
| Intra-IMC3 | Group A | 2,7 | 1,5 | 3,3 | 1,3 | 2,2 | |
| | Group B | 1,8 | 28,7 | 10,7 | 6,3 | 11,9 | |
| | Group C | 11,3 | 1,3 | 33,4 | 17,4 | 15,8 | |
| Inter-operator (IMC1 vs IMC2) | Group A | 6,8 | 12,9 | 28,6 | 12,1 | 15,1 | |
| | Group B | 2,5 | 4,9 | 19,0 | 14,4 | 10,2 | |
| | Group C | 3,6 | 11,7 | 29,7 | 8,3 | 13,3 | |
| Inter-day (IMC1 vs IMC3) | Group A | 8,2 | 14,8 | 7,1 | 3,2 | 8,3 | |
| | Group B | 2,5 | 18,5 | 18,6 | 19,3 | 14,7 | |
| | Group C | 15,2 | 12,5 | 27,0 | 47,7 | 25,6 | |

| SE | | B LYMPHOCYTES (CD20) | T CYTOTOXIC LYMPHOCYTES (CD8A) | VASCULATURE (SMA) | MACROPHAGES (CD68) | AVERAGE SE |
|-------------------------------|---------|----------------------|--------------------------------|-------------------|--------------------|------------|
| Intra-IMC1 | Group A | 2,05 | 2,16 | 0,45 | 0,55 | 1,30 |
| | Group B | 1,13 | 0,38 | 0,77 | 1,69 | 0,99 |
| | Group C | 1,21 | 1,43 | 0,15 | 0,30 | 0,77 |
| Intra-IMC2 | Group A | 0,74 | 1,03 | 0,69 | 1,23 | 0,92 |
| | Group B | 0,92 | 0,32 | 0,65 | 0,68 | 0,64 |
| | Group C | 1,59 | 0,49 | 1,40 | 0,87 | 1,09 |
| Intra-IMC3 | Group A | 0,81 | 0,19 | 0,15 | 0,15 | 0,32 |
| | Group B | 0,67 | 1,71 | 0,39 | 0,66 | 0,86 |
| | Group C | 3,20 | 0,12 | 0,73 | 3,16 | 1,80 |
| Inter-operator (IMC1 vs IMC2) | Group A | 1,31 | 1,27 | 0,75 | 1,03 | 1,09 |
| | Group B | 0,68 | 0,23 | 0,57 | 0,82 | 0,58 |
| | Group C | 0,92 | 0,83 | 0,80 | 0,46 | 0,75 |
| Inter-day (IMC1 vs IMC3) | Group A | 1,61 | 1,41 | 0,22 | 0,25 | 0,88 |
| | Group B | 0,69 | 0,82 | 0,55 | 1,25 | 0,83 |
| | Group C | 3,46 | 0,88 | 0,51 | 4,35 | 2,30 |



FIGURE 4. Correlation plot for each marker, B cells (CD20), T cytotoxic cells (CD8a), Macrophages (CD68), Vasculature (aSMA) showing the (A) inter-operator and (B) inter-day concordance. A scatterplot is a type of data display that shows the relationship between two numerical variables. Each member of the dataset gets plotted as a point whose (x, y) coordinates relates to its values for the two variables.



CONCLUSION

In this work, we present reproducibility results aimed at understanding the impact of reproducibility in clinical translation research. The high intra-IMC concordance indicates a minimal variability of the staining. The good (<20%) average total inter-IMC, both with a variance of operator and day of experimentation, demonstrates the reproducibility of the technique. The marker evaluation is a key aspect of reproducibility: markers with abundant and specific cell expression, such as CD20 and CD8a in tonsils are easy to evaluate and give consistent expression evaluation across serial sections. For markers with variable geographic distribution across tissues and low expression, such as aSMA in the present tissue, reproducibility results are more challenging indicating the need to pursue a specific thoughtful customized qualification for each marker involved in clinical studies in order to get a reliable read out that will impact study outcomes.

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