Reproducibility in spatial biology: Reducing variables to improve the reliability of insight generation

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Lisa Arvidson[2], Reginaldo Prioli[2], Sam Jensen[2], James B. Hoying[3], Michael W. Golway[4], Michael J. Smith[1], Katie O. White[1], Richard A. Heil-Chapdelaine[1], Hideki Sasaki[1], Chi-Chou Huang[1], Tuan H. Phan[1], Melinda L. Angus-Hill[1]

[1]Leica Microsystems, Bellevue, WA; [2]Cell Signaling Technology, Danvers, MA; [3]Advanced Solutions Life Sciences, Manchester, NH; [4]Advanced solutions Life Sciences, Louisville, KY

Introduction & Aim

There is an ongoing crisis in reproducibility in scientific studies, and studies in spatial biology are no exception. Cell DIVE™ Multiplex imaging solution (Leica Microsystems) provides reliable workflow solutions to minimize variability from study to study. Cell DIVE allows probing and imaging of dozens of biomarkers on a whole tissue section with an iterative staining and dye inactivation workflow. At its core, Cell DIVE is designed to provide methods reproducibility, from tissue preparation, antigen retrieval, and sample imaging and slide storage. Cell DIVE is designed to work with directly conjugated primary antibodies, another source of variability. However, conjugated antibodies from Cell Signaling Technology (CST) are rigorously validated using stringent acceptance criteria to reduce variability. In addition, the use of recombinant antibodies, the consistent conjugate brightness and antibody degree-of-labeling reduce lot to lot variability, ensuring reliable conjugated antibodies for spatial biology studies. High resolution imaging results are obtained by consistent round to round imaging, consistent calibration and corrections, and reduction of human error using the BAB 200 liquid handling solution (Advanced Solutions Life Sciences). We present here, an iterative biomarker study using adjacent tissue sections probed with distinct lots of CST antibody panels, imaged in temporally separated batches using a Cell DIVE imager fitted with a BAB200 liquid handler. Following robust analysis (segmentation, phenotyping and statistical analysis), we report the reproducibility findings across parameters. Methods and results reproducibility in spatial biology is essential for reliable insight generation, giving confidence in the quality of future studies aimed at improving patient outcomes.

Results

Reproducibility is a critical component of cancer research, and several factors can affect the reproducibility of experimental results in spatial biology. Antibody validation is an essential step in cancer research to ensure that the antibodies used are specific and reliable. The validation process involves testing the antibody to confirm that it recognizes the target protein or molecule accurately and without cross-reactivity with other proteins. These antibodies must maintain robust staining of samples, between lots, and over time. Moreover, tissue samples must be collected, processed, and sectioned accurately to ensure the reproducibility and reliability of results. Furthermore, automated systems are increasingly being used in cancer research to enhance the reproducibility and efficiency of experiments. However, it is crucial to ensure that the automated systems used are properly validated and optimized to ensure their reliability and reproducibility. Here we test the reproducibility of the Cell DIVE solution fitted with a BAB 200 automated system and CST validated antibodies (see Methods and Materials and Fig. 1). Many variables ultimately contribute to the success of these experiments (Fig. 2).

To assess the reproducibility between batches, we use concordance measurements. Here, we compare the results obtained from each biomarker between batches, or from all biomarkers between batches, providing insight into the reliability and reproducibility of the experimental results. Pearson correlation coefficient and Lin's correlation coefficient, can be used to assess the concordance of results from a single biomarker or with all biomarkers, respectively. Pearson correlation for each biomarker, all samples are concordant (>0.5 with most achieving statistical significance). For Lin's coefficient, samples are concordant, though a few measures are outliers. The use of concordance measurements can help identify sources of variability and ensure that the results obtained from different experiments or assays are consistent and reliable. In conclusion, ensuring the reproducibility of the experimental results in spatial biology in cancer research requires attention to several critical factors, including antibody quality, standardized tissue sample sectioning, and automated processing of samples. The combination of CST validated biomarker antibodies and the BAB 200 Cell DIVE automated solution provide a high level of concordance across samples.

Figure 1.

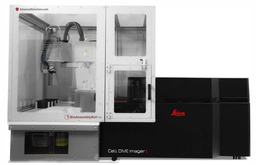
A Pancreatic Ductal Adenocarcinoma from Pantomics

	Group 1	Group 2	Group 3	Group 4	Group 5	Group 6
BATCH 1	SLIDE-1380	SLIDE-1383	SLIDE-1386	SLIDE-1389	SLIDE-1392	SLIDE-1395
BATCH 2	SLIDE-1381	SLIDE-1384	SLIDE-1387	SLIDE-1390	SLIDE-1393	SLIDE-1396

B

Round 1			
Channel	Biomarker AB	Dye	Clone
GREEN	VIM	AF488	D21H3
ORANGE	CD68	AF555	D4B9C
FAR RED	SURVIVIN	AF647	71G4B7
NIR	CD45	AF750	D9M8I
Round 2			
Channel	Biomarker AB	Dye	Clone
GREEN	SMA	AF488	D4K9N
ORANGE	CD3	AF555	D7A6E
FAR RED	CD56	AF647	E7X9M
NIR	CD8	AF750	D8A8Y
Round 3			
Channel	Biomarker AB	Dye	Clone
GREEN	NAK	AF488	D4Y7E
ORANGE	CD31	AF555	89C2
FAR RED	KI67	AF647	D2H10
NIR	CD11B	AF750	D6X1N

C



D

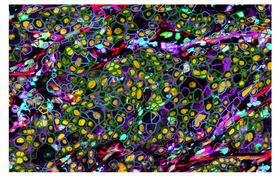


Figure 1: Experimental design of repeatability study (Figure 1). Pancreatic ductal adenocarcinoma slides were separated into two batches (Fig 1A). Slides were stained separately using three rounds of biomarker antibodies (Fig 1A). Note, each batch was stained using a different antibody lot. Fully automated staining and imaging was done using the Cell DIVE system fitted with the BAB 200 system (Fig. 1C). Cells were segmented and phenotyped for each biomarker antibody using AI based segmentation (Fig 1D). Cell counts for each biomarker were divided by total number of cells to give a percentage of biomarker positive cells out of total cells for each biomarker.

Methods & Materials

CST antibodies undergo a vigorous validation process to ensure antibody performance on FFPE tissue. All antibodies in this study were direct conjugates or off the shelf (OTS) commercial conjugates.Tissue was obtained from a commercial source (Pantomics; Table 1). Two batches of antibodies were diluted into two 96 well plate batches and used to stain separately two batches of slides via an automated liquid handling and plate handling application (BioAssembly Bot, BAB200). Slides were imaged on the Cell DIVE using four channels plus DAPI, with automatic AF removal, corrections and stitching. Fully stitched images were imported, fused, and segmented using proprietary software developed by Leica Microsystems. Following segmentation, biomarker positivity was measured as a percent of total cells. Statistical measures of concordance were applied to compare each batch of single cell phenotype measurements.

Conclusions

- Automated iterative staining and imaging of a 12 full tissue slides with 12 biomarker antibodies using the BAB 200 Cell DIVE multiplexed imaging solution.
- Multiplexed imaging has a number of variables from antibody quality and lot differences, to tissue preparation, and individual slide staining and imaging.
- CST antibodies combined with the BAB 200 Cell DIVE multiplexing solution provides reliability and uniformity at scale to multiplexed imaging studies.

Figure 2

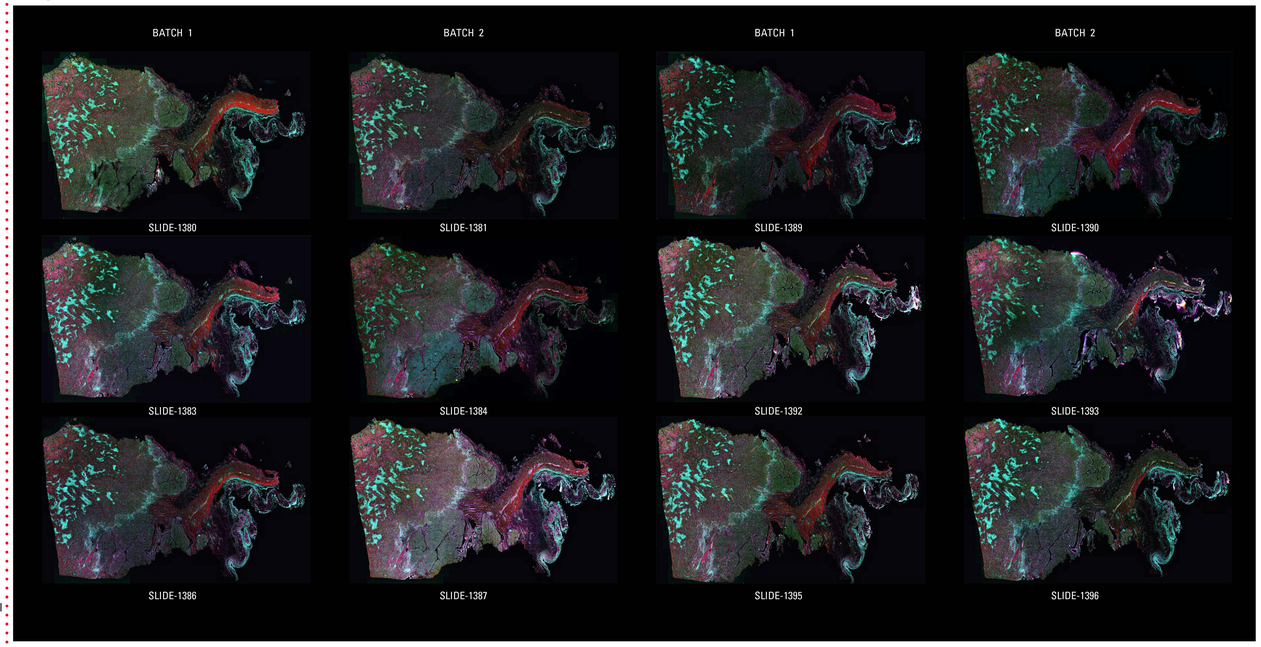


Figure 2: Visualization of all biomarkers across each slide. Notice overall similarities across slides.

Figure 3

A

Pearson Correlation Test	CD11B	CD31	CDE3	CD45	CD56	CD8A	CD68	KI67	NAK	SMA	SURV	VIM
Pearson Correlation Coefficient r	**0.855**	**0.710**	**0.702**	**0.777**	**0.848**	**0.620**	**0.900**	**0.962**	**0.866**	**0.883**	**0.990**	**0.927**
n	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00	6.00
t-distance	3.290933	2.0180598	1.971926	2.464826	3.19583237	1.580819	4.126475	7.018535	3.467397	3.767092	14.03435	4.940306
DF:	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00	4.00
p Value:	**0.03019**	**0.11375**	**0.11990**	**0.06933**	**0.03303**	**0.18907**	**0.01454**	**0.00217**	**0.02564**	**0.01965**	**0.00015**	**0.00782**

B

Lin's Coefficient	Slide1380	Slide1381		Slide1383	Slide1384		Slide1386	Slide1387		Slide1389	Slide1390		Slide1392	Slide1393		Slide1395	Slide1396	
CD11B	20.06%	17.99%	Ave	19.47%	22.51%	Ave	18.77%	20.36%	Ave	15.37%	19.70%	Ave	21.46%	19.11%	Ave	20.67%	21.26%	Ave
CD31	5.80%	4.70%	20.22%	5.26%	7.59%	18.82%	5.91%	6.29%	18.15%	4.09%	5.38%	12.04%	6.52%	15.75%	21.62%	5.78%	6.17%	20.65%
CD3E	6.24%	6.85%	16.78%	6.98%	14.90%	29.72%	5.77%	6.34%	26.74%	5.54%	9.30%	25.16%	7.11%	5.89%	28.96%	6.80%	5.24%	19.10%
CD45	42.08%	31.65%	Varp	36.88%	91.74%	Varp	32.77%	67.73%	Varp	25.01%	57.89%	Varp	40.02%	45.94%	Varp	38.37%	37.52%	Varp
CD56	1.09%	0.77%	4.98%	1.10%	6.81%	3.98%	0.79%	1.20%	2.89%	0.51%	1.32%	1.14%	1.60%	1.91%	5.27%	1.14%	1.21%	4.49%
CD68	10.18%	6.93%	2.90%	8.87%	63.77%	8.05%	7.48%	15.55%	8.82%	5.26%	10.45%	7.21%	10.23%	11.97%	9.79%	8.81%	8.83%	4.39%
CD8A	2.90%	3.23%	Correl	3.30%	4.32%	Correl	2.81%	3.15%	Correl	2.85%	2.97%	Correl	3.64%	3.36%	Correl	3.29%	2.96%	Correl
KI67	2.61%	5.35%	**0.987567**	3.28%	7.12%	**0.662531**	2.72%	4.30%	**0.956022**	2.27%	4.68%	**0.980852**	3.32%	5.39%	**0.802955**	3.74%	4.97%	**0.981622**
NAK	12.56%	16.04%	Rc	15.61%	2.64%	Rc	29.38%	25.68%	Rc	10.18%	23.25%	Rc	22.23%	90.22%	Rc	18.34%	19.43%	Rc
SMA	19.13%	11.02%	0.938518	15.29%	22.53%	0.567358	15.16%	11.84%	0.775184	12.73%	21.47%	0.559305	13.56%	7.97%	0.739375	19.57%	5.72%	0.978895
SURV	79.93%	60.88%		72.29%	58.14%		56.04%	94.24%		35.60%	90.89%		81.38%	90.81%		73.70%	73.42%	
VIM	40.04%	35.97%		37.49%	54.62%		40.19%	64.24%		25.09%	54.63%		48.39%	49.19%		47.66%	42.47%	

Figure 3: Individual and across all biomarker concordance between batches. To measure single biomarker concordance across all slides, we turned to pearson correlation coefficicient as a measure of between batches.

Questions?
Contact CellDIVE@leica-microsystems.com.
Contact Cellsignal.com or rprioli@cellsignal.com or lisa.arvidson@cellsignal.com.