Clinical Immunology

Five Things to Consider when Developing Clinical Biomarkers

Developing cellular biomarkers is particularly challenging when it comes to biomarker validation, because flow cytometry has considerable limitations with respect to sample stability and multiplexing. Moreover, the development of highly multiplex flow cytometry assays is time-consuming, and expanding existing panels can be quite cumbersome. Chipcytometry is an imaging-based cytometry platform bridging the gap between the discovery and validation of clinical biomarkers through flexible "sequential singleplexing". Contrary to flow cytometry, the cells are immobilized inside a microfluidic channel, thus allowing the stepwise quantitative analysis of unlimited numbers of biomarkers on the same cells with very little hands-on time [1, 2, 3, 4]. Here are five things to consider when developing clinical biomarkers:

1. Long-term access to precious clinical samples

Although several new techniques of immune cell stabilization have been published in recent years, marker stability on and in the cells remains very limited (maximum 12-72 hours after sampling). Conventional flow cytometry requires fresh samples for biomarker development, and the cryopreservation of immune cells leads to significant changes in biomarker signatures. In Chipcytometry, the cells are fixed with 4% paraformaldehyde on coated glass surfaces, which yields remarkable long-term stability of the surface and intracellular biomarkers (minimum 24 months after sampling). Before designing a project on biomarker development, you can now easily prepare a small "disease biobank" (e.g. 20-50 samples; Fig.1) of high clinical relevance, with more than 1 year to study the biomarker signatures of these precious samples.

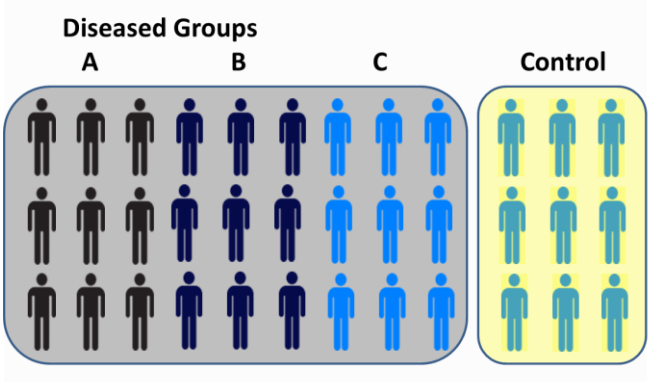

Figure 1: Typical "disease biobank" including healthy control and disease [mild (A), severe (B) or relapsing (C)] samples.

2. Low sample consumption maximizes the information you get out of each clinical sample

During conventional flow cytometry, the samples are lost with the flowing solution. One could split a sample and run several multiplex panels. But even after investing several months of lab work in panel development (e.g. four 8-plex assays), the number of potential biomarker candidates would still be limited. In Chipcytometry, the original samples are stored on small microfluidic chips, where they can be assayed over and over again (Fig. 2).

Figure 2: The ZellSafe™ Cell (left) and ZellSafe™ Rare (right) microfluidic biorepositories for long-term sample storage. Whereas ZellSafe™ Cell stores 250,000 cells, ZellSafe™ Rare stores about 1,000,000 cells for the investigation of rare events (< 0.02%).

During each assay cycle, the cells are stained with fluorescent-labeled antibodies, followed by image recording, and then fluorescence bleaching. Because the cells are not destroyed or lost during the assays, multiple sequential singleplex assays can be performed on the same cells, allowing you to stain virtually an unlimited number of biomarker candidates.

3. Incurred sample reanalysis (ISR) in cytometry

The implementation of standardized cytometry-based biomarker assays in clinical trials remains a challenge due to the limited stability of clinical specimens and inter-instrument variations. As such, the FDA implemented the ISR as a standard requirement for immunoassay-based clinical sample analysis [5]. Standard ISR protocols reanalyze randomly selected samples 3-6 months after the initial analysis in order to verify the consistency of the method throughout a clinical trial, and to prevent assay or instrument drift. However, sequential analysis of the same sample is impossible with conventional flow cytometry because the cells are lost during the initial assay. In contrast, Chipcytometry accommodates ISR protocols because the samples are preserved after the initial assay. Thus, clinical trials using Chipcytometry are compliant with the current FDA regulations and provide an additional layer of confidence into the analyses.

4. Biomarker development: from the concept to the results

Zellkraftwerk recently conducted a case study on a rare disease comparing the flow cytometry and Chipcytometry analyses of 4 cell types in samples of peripheral blood mononuclear cells (PBMC). The study was initiated with 18 biomarker candidates and 13 clinical samples (10 diseased and 3 controls). Suitable commercially available clones and positive/negative controls were easily obtained for all 18 biomarker candidates. Flow cytometry required 81 days, compared to only 42 days for Chipcytometry (Table 1).

Table 1: Biomarker development: from concept to results comparing Chipcytometry and flow cytometry

Platform	Assay Formats	Panel Set-up (days)	Non-GLP Validation (days)	Sample Analysis (days)	Data Analysis (days)	Total time: Sample to result (days)
flow cytometry	three 6- plex assays	67	11	2	1	81
ChipCytometry	eighteen singleplex assays	9	12	20	1	42

Chipcytometry instruments can run 5-plex multicolor-assays in parallel. Nonetheless, we selected the "sequential singleplex" mode because singleplexed assays avoid fluorescence spillover, color compensation, and variations in dye-induced antibody affinity. This strategy reduces the assay development time by more than 85% and provides the highest flexibility to add more biomarkers to the panel during the study. Overall, this study demonstrates that Chipcytometry is approximately twice as fast as flow cytometry and offers more design flexibility. At the beginning of a project, we use the manual system (ZellScanner ONE™) for its high flexibility and the possibility to immediately adapt the protocol to the new findings. Then, we use the fully automated Chipcytometry instrument (CYTOBOT™) to support the routine sample analyses of clinical trials (Fig. 3).

Figure 3: Cytobot™; Chipcytometry system for fully automated Chipcytometry analysis and data processing in 24/7 operation.

5. From biomarker development to clinical trial support

Once the list of 18 biomarker candidates was narrowed down to 11 biomarkers (3 cell types), we used the Chipcytometry 5-plex mode to conduct high-throughput analysis of the phase I clinical trial samples. At that point, we could have used flow cytometry, but we choose Chipcytometry for the long-term stability of the samples. In our laboratory, Chipcytometry has been validated for a wide variety of clinical specimens including PBMS. The stability of cell surface and intracellular biomarkers has been validated for a period of 24 months (data not shown).

About

Jan Detmers is the Head of Clinical Services at Zellkraftwerk. His service laboratory conducts exploratory ("translational") projects and provides clinical trial support (phase I-III). All Chipcytometry instruments and software comply with the CFR part 11 regulations (GLP). Zellkraftwerk has 95 human assays validated (Table 2).

Table 2: Validated 95-plex immune cell phenotyping panel

CD3	CD31	CD81	CD195 (CCR5)	IgA
CD4	CD32	CD86	CD196 (CCR6)	IgD
CD5	CD34	CD90	CD197 (CCR7)	IgG
CD8	CD38	CD95	CD206	IgM
CD10	CD39	CD105	CD244	IL1b
CD11b	CD40	CD115	CD257 (BAFF)	IL8
CD11c	CD45	CD123	CD273 (PD-L2)	IL10
CD14	CD45RA	CD127	CD274 (PD-L1)	IL12
CD15	CD45RO	CD138	CD278 (ICOS)	IL17A
CD16	CD54	CD141	CD279 (PD-1)	IL17F
CD19	CD56	CD152 (CTLA-4)	CD319 (CRACC)	Ki-67
CD20	CD57	CD161	CD326 (EpCAM)	LC (κ)
CD21	CD64	CD163	AIOLOS (IKZF3)	LC (λ)
CD24	CD66b	CD172a/b	FoxP3	pan Cytokeratin
CD25	CD68	CD183 (CXCR3)	GM-CSF	RORγ(t)
CD27	CD69	CD184 (CXCR4)	Granzyme B	T-bet
CD28	CD71	CD185 (CXCR5)	Helios	TNFα
CD29	CD73	CD193 (CCR3)	HLA-DR	Vimentin
CD30	CD80	CD194 (CCR4)	IFNγ	Zap-70

Literature

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Contact

Jan Detmers, Ph.D., Head of Clinical Services
E-Mail: detmers@zellkraftwerk.com
From USA (617) 500-8221; From Europe +49 441 3508 2402