ELECTROPHORETIC CYTOMETRY: SINGLE-CELL SEPARATIONS ON MICROPARTICLES TO ELUCIDATE BIOLOGICAL VARIATION

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ABSTRACT

Single-cell immunoassays fail to incorporate selective probing with high-throughput readout for quantitative analysis. We report for the first time on a microfluidic approach to conduct protein isoform cytometry in releasable individual hydrogel microparticles (µGELs) made of polyacrylamide with 40-µm thickness. We fabricate thousands of µGELs in an array to facilitate single cell isolation and lysis, followed by protein mass separation, immobilization and probing. Releasable µGELs enable subgrouping of individual bioassays for selective probing reproducibly. Using µGELs, we demonstrate that increasing cell-confluency increases truncated-estrogen receptor isoform expression, playing role in hormone resistance development in estrogen sensitive breast cancer cell lines.

KEYWORDS: Hydrogel particles, proteomics, single-cell analysis, electrophoretic cytometry, breast cancer.

INTRODUCTION

The past decade has seen remarkable advances in single-cell resolution genomics (DNA) and transcriptomics (RNA). Measuring cell-to-cell variation and identifying rare-cell subpopulations boots our ability to understand and, ultimately, treat human disease.[1] An exciting emerging area of cytometry (single-cell measurements) is single-cell proteomics, with tremendous focus on immunoassays (i.e., flow cytometry, ELISAs, mass cytometry/CyTOF).[2] Yet, thousands-to-millions of protein targets are not detectable by immunoassays. The gap is that suitably selective antibody probes simply do not exist. This protein 'blind spot' is comprised of proteoforms (or isoforms). Isoforms are chemically diverse, dynamic, and each can play a major role in disease development, progression, and response to treatment. As another key challenge, there is a trade-off between satisfactory assay sensitivity and assay throughput in immunoassay-based approaches, although measuring cell-to-cell variability in large populations require thousands of concurrent single-cell immunoassays.[3-5] Releasable µGELs surmount measurement challenges where (i) immunoassays are insufficient owing to antibody probes with insufficient selectivity, and (ii) powerful mass spectrometry tools lack analytical sensitivity and throughput.

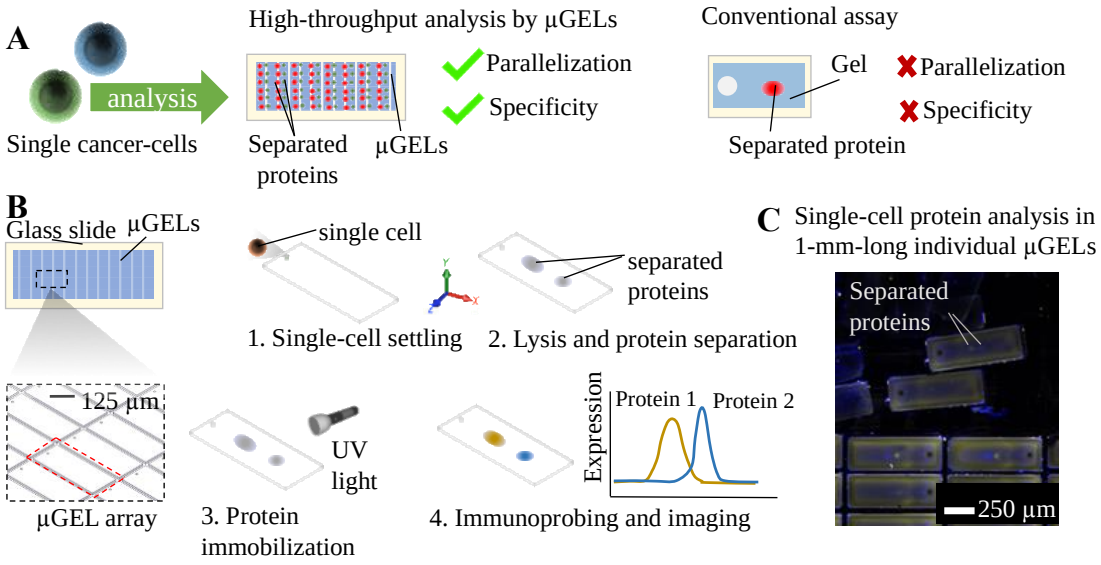

Figure 1: µGELs - Hydrogel microparticles support single-cell protein electrophoresis. *(A) µGELs confer target selectivity and large-scale single-cell processing. (B) A microfluidic array of hydrogel particles (40 µm deep, 1 mm long, 250 µm wide) fabricated on a glass slide via micromolding. µGEL particles parallelize electrophoretic cytometry. (C) Anchored and released µGEL formats facilitate high-throughput protein analysis.*

EXPERIMENTAL

We first devised a multi-step micromolding and release process to fabricate thousands of µGELs (250×1000×40-µm) from a 35×24-mm mini-slab polyacrylamide gel (Figure 1A). The polyacrylamide gel (8%T, 2.6%C) was layered on a glass slide, with 50-µm wide perforations defining each µGEL particle. We followed the single-cell western blotting procedure developed by our group[6] to separate proteins (Figure 1B). Depending on the imaging technique, we either left µGELs on the glass slide in array format, or released µGELs from the glass slide by shearing with a razor blade. The perforations between individual µGELs allowed for releasing individual particles without sticking to each other. Fluorescence readout was performed along the separation (long) axis of each µGEL (Figure 1C), allowing quantitation of µGEL morphology (shrinking), protein isoforms abundance, and separation resolution – in either hydrated or dehydrated states.

RESULTS AND DISCUSSION

Thanks to the perforations, individual particles can be released with minimal (2%) damage from the glass slide (n=5.2K particles). The hydration state affected the number of damaged µGELs after the release. When compared to released dehydrated µGELs, released hydrated particles showed 0.7% less damage; and this difference was found to be statistically significant ($p = 0.0001$, $n = 2483$) (Figure 2A). Upon dehydration the µGELs shrink isotropically by 24-32% (Figure 2B-2D); however, measured protein levels (GAPDH and β-Tubulin from MCF 7 cells, $p<0.05$ for both states) and electrophoresis resolving power did not significantly change (Figure 2C, n = 2.5K particles, for all paired comparisons $p<0.05$). Particularly, separation resolution between β-Tubulin and GAPDH proteins was found to be 0.72 (CV = 22.86, n = 16) for released/hydrated, 0.72 (CV = 21.43, n = 62) for released/dehydrated, 0.70 (CV = 25.00, n = 16) for attached/hydrated, and 0.70 (CV = 23.61, n = 62) for attached/dehydrated µGELs. These results imply that the difference in R_s between hydrated/dehydrated/attached/detached states was not statistically significant, two sample t-test $p = 0.69$.

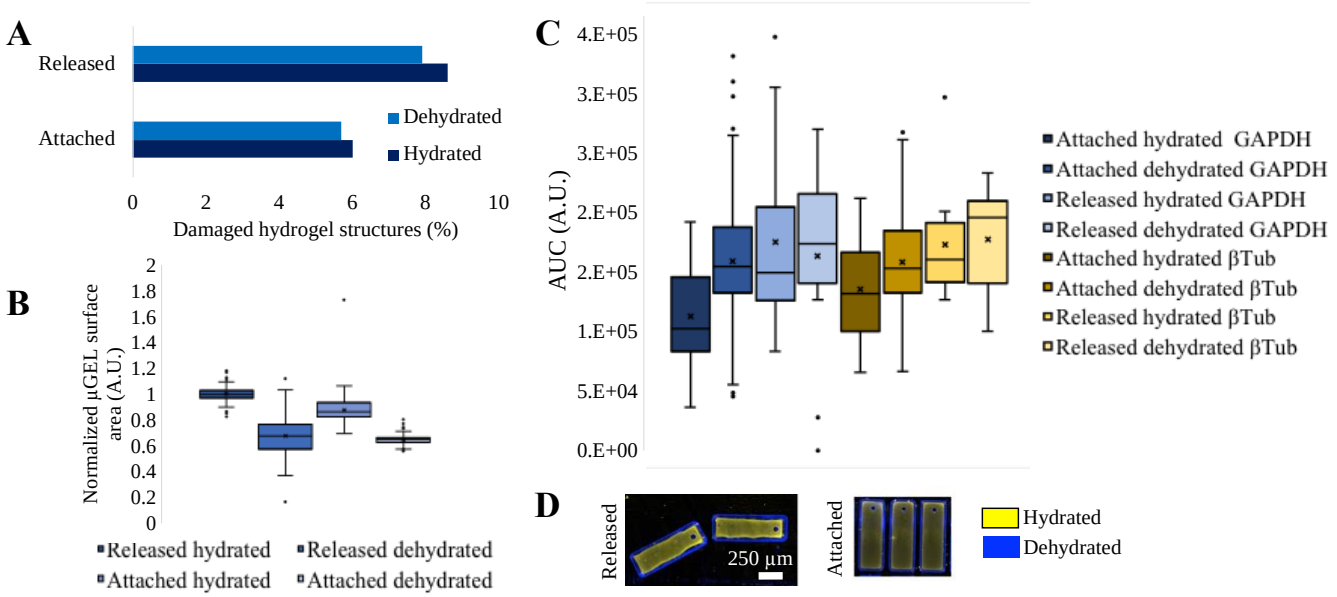

Figure 2: Fabrication and release of mGELs. *(A) Perforations created during micromolding facilitate particle release. (B) Released µGELs shrink isotropically by 7.%. (C) Protein expression of GAPDH and β-Tubulin from U251 cells is invariant. (D) False-colored micrographs of released and attached µGELs at hydrated and dehydrated states.*

Next, we applied the µGELs to measure breast cancer cell-to-cell variability in expression of the Estrogen Receptor (ER) protein isoforms: Erα46 and Erα66 in MCF 7 cell line. Specifically, we studied confluency-dependent variation in the isoform expression with the µGELs.[7] The two isoforms were baseline resolved, $R_s = 1.7$ (Figure 3A) in the µGELs. We detected a 2.8 fold increase in truncated estrogen receptor ERα isoform and 6.4 fold decrease in full-length isoform in single estrogen-sensitive breast cancer cells over a 14-day period ($n = 478$ cells) (Figure 3B). Slab gel western blot results were in accordance with the measurement obtained by µGELs

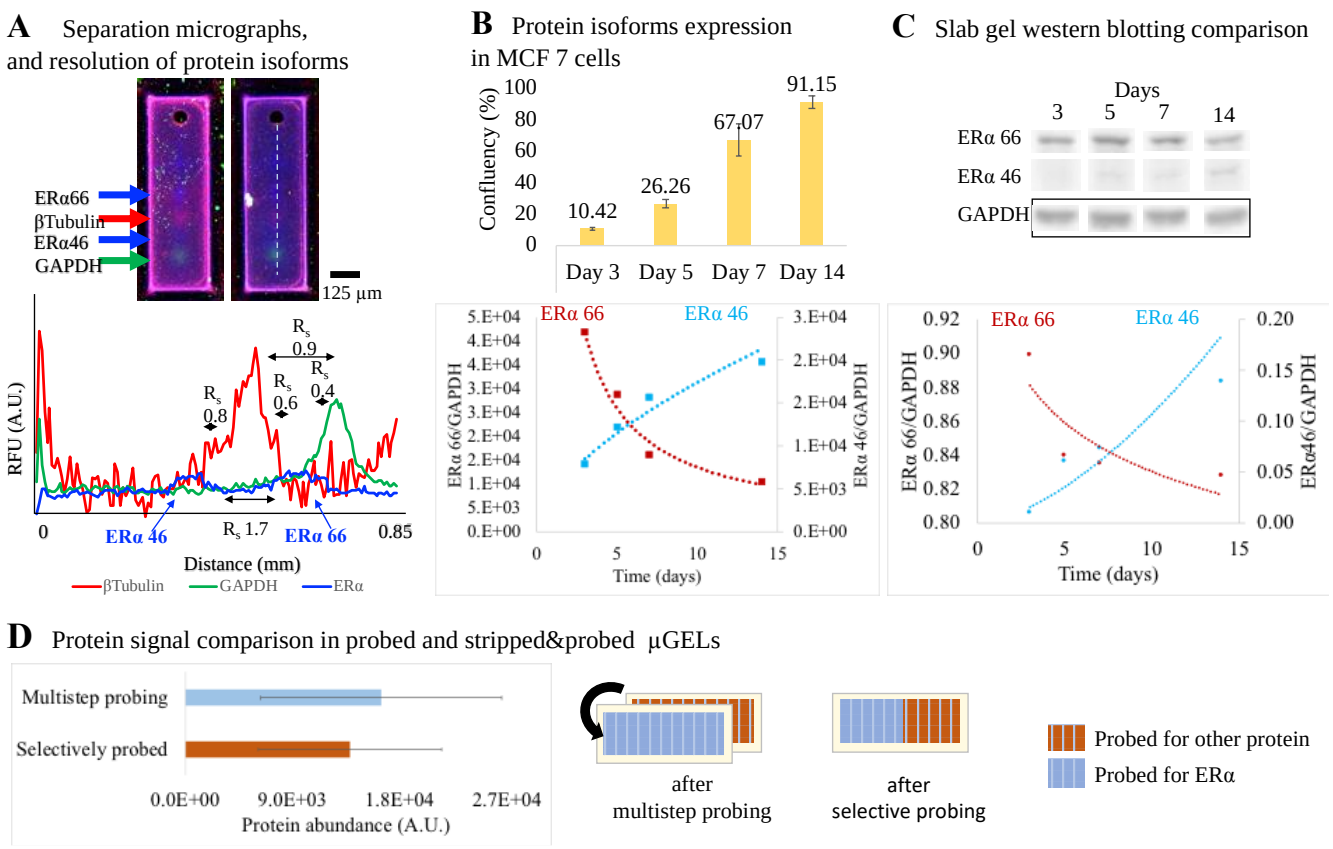

Figure 3: *Variation in MCF 7 breast cancer cells (Era66 vs. Era46 isoform expression) as reported on µGELs. We observe* ***confluency-dependent heterogeneity cell populations expressing ERα46 and ERα66 isoforms****. (A) False-colored images of the µGELs demonstrating baseline separation of housekeeping and target proteins from single cells (n=2.5K particles). Dashed line represents the region of interest for the fluorescence intensity graph. (B) Single-cell western blotting monitors expression over day 3 to 14 time course, while (C) slab-gel western blot failed to detect ERα46 in day 3. (D) Protein signal changes in directly probed vs. stripped (for other protein targets) and probed µGELs (p=0.36, n=54 particles). Selective immunoprobing in grouped µGELs gains importance in sensitive measurements where stripping & reprobing cannot be tolerated.*

(Figure 3C). This finding is an important observation to show that the increased cell-confluency may increase hormone resistance in estrogen sensitive breast cancer cell lines.

In contrast to conventional single-cell analysis tools, releasable µGELs allowed us to harness selective probing and multiplexing by subgrouping and isolation of individual micro-bioassays. In immunoprobing, antibodies of interest might have overlapping types, hampering the multiplexed probing. As an approach, multiple probing cycles have been ran in the majority of immunoasssays, which led to decreased protein signal after the first cycle. In our work, µGELs yielded 15.8% higher signal intensity of ERα in the first probing cycle as compared to non-grouped µGELs probed multiple cycles. This strategy also helps to limit consumption of precious diagnostic samples and costly immunoprobing reagents. Figure 3D shows changes in ERα expression from the same single-cell group when multiple probing cycles were applied on non-grouped µGELs and selective probing was applied on grouped µGELs.

CONCLUSION

We showed that the use of µGELs (i) confer selectivity suitable for isoform detection for protein immunoblotting, in contrast to immunoassays, and (ii) confer throughput on par with flow cytometry, in contrast to slab-gel western blotting. Additionally, we incorporated incorporates releasable hydrogel particles with single-cell western blotting to provide with control over subgrouping of the particles for selective immunoprobing. This quantitative analysis tool presents a key component to guide cancer screening for personalized therapies.

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