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Application note

Accelerate your cell engager discovery with high throughput measurements of cell avidity

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Keywords:
Bispecific engager, Cell avidity, Cell engager, High throughput, Titration, Screening

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Abstract

T cells play a pivotal role in tumor immunosurveillance. Multispecific cell engagers (CEs) have been adopted in the field of immuno-oncology to redirect T cells toward cancer cells, thereby unleashing the anti-tumor potential of the patient's immune system. CE-mediated cell binding induces T cell activation and the formation of an immunological synapse, which is a prerequisite for effective tumor cell lysis.

The strength of the initial binding events between a T cell and a tumor cell dictates the efficiency of the anti-tumor response. Assessing cell avidity, i.e. the total intercellular interaction strength between two cells, gives crucial insights into the efficacy of CEs as anti-tumor therapeutic agents.

Here, we deploy LUMICKS' high throughput avidity measurement (HTAM) technology to measure CE-induced cell avidity in a high throughput manner. We demonstrate the assay performance characteristics, i.e. specificity, precision, and range, via CE titration experiments in the context of a Jurkat T cell model system. We find that the HTAM CA assay is suitable for candidate screening in high throughput, with high sensitivity and precision.

Introduction

Cell Engagers in cancer immunotherapy:

The mammalian immune system has evolved to combat pathogenic infections and clear transformed cells. Due to its ability to induce an immune response and clear cells based on targeting specific antigens, the immune system has a tremendous potential to be used as a therapeutic to cancer.

CEs are multispecific molecules that are used as a linker between tumor cells and cells of the immune system. This way, they force immune cells and tumor cells to connect, which leads to the activation of the immune cell and the killing of the tumor cell. Classically, these molecules are antibody (fragment)-based molecules, but in recent years other formats have been developed as well (Ma et al.).

The main advantage of the use of CEs in comparison to cell therapies is that CEs are cheaper to produce and can be used off-the-shelf, whereas most cell therapies are autologous and require GMP-grade cell manufacturing facilities to produce, making them extremely costly. In addition, when off-tumor effects are an issue, treatments like antibody therapy are transient in nature.

While the field of CEs is growing, many issues remain unresolved. The workflow for CE development (CE development pipeline) is tedious and costly and includes many optimization iterations before getting to a final product as often the *in vitro* assays employed (*in vitro* cytotoxicity or cytokine secretion) do not accurately predict *in vivo* efficacy.

Cell Avidity and the HTAM assay:

Cell avidity is defined as the total interaction strength of cell-cell interaction. Cell avidity measurements have recently shown promise as an accurate predictor of CAR T and CAR NK *in vivo* functionality (Leick et al. and Chockley et al.). As such, we propose that cell avidity measurements can also be used to assess the functionality of CEs.

To probe cell avidity using multispecific CEs we employed LUMICKS' high throughput avidity measurement (HTAM) technology (**Figure 1**). This system allows for highly controlled force application and simultaneous, high throughput analysis of cell avidity between effector cells and target cells in the presence of CEs.

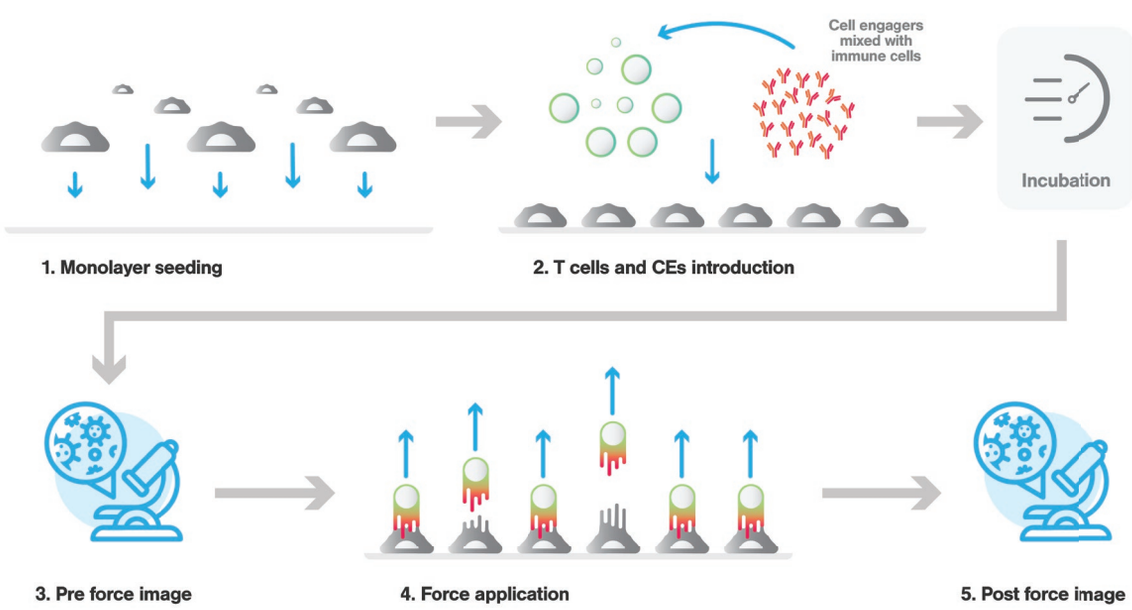

Figure 1 HTAM workflow – Antigen expressing target cells are seeded to form a confluent monolayer in a microfluidic chip (1) and T cells are fluorescently labelled and co-injected in the chip with CE at the indicated concentration (2) after which the T cells, CE and target cells are co-incubated at the indicated time intervals (3). Following a pre-force image (4), force is applied to the chip, separating unbound T cells from the target-cell monolayer (5). Finally, a post-force image is generated (6) followed by image analysis to determine the overall avidity of the sample.

Model system and HTAM Assay Validation

Blinatumomab (Blincyto®) was used as the CE in this validation study. Blinatumomab is a well-characterized FDA- and EMA-approved Bi-specific T-cell engager (BiTE) used to treat B cell precursor acute lymphoblastic leukemia (B-ALL) (Mack et al. and Topp et al.). Blinatumomab targets the tumor-associated antigen (TAA) CD19 and the T cell receptor/CD3 complex (Reusch et al.).

The T cell lymphoblastic cell line, Jurkat E6.1 was selected as effector cells. One of the drawbacks of experiments utilizing primary human materials is that these models are inherently heterogenic, thus creating intrinsic assay variance. To circumvent this, we chose to perform this validation study using a Jurkat E6.1 model system instead of primary human T cells. Jurkat E6.1 is a T cell leukemia-derived cell line that expresses the TCR-CD3 complex and signaling machinery and has the capability of conveying antigenic TCR-CD3 stimuli (Hartl et al. and Nika et al.).

The lymphocyte-like, B cell leukemia cell line, NALM6, was selected as the accompanying tumor target. Several key attributes led to this target cell line selection. As a well-established, CD19-expressing tumor model system frequently employed in immune-oncology (IO) research and IO drug development, selecting NALM6 as the tumor target helps ensure the relevance of the assay validation. Similar to the Jurkat E6.1 model system, the inherent stability of the NALM6 cell line reduces unwanted biological noise, safeguarding the quality of the validation results.

HTAM assay validation approach

In a pre-validation step, we characterized the impact of the effector and target cell incubation time and mapped out the assay response with respect to CE concentration. Based on the results we established the assay's dynamic range and chose an incubation time for optimized assay performance characteristics.

Next, we conducted assay validation to assess the performance characteristics (ICH Guideline Q2), quantifying among others specificity, precision, and range in intra and inter day experiments.

Results

Pre-Validation

To optimize HTAM assay performance, we characterized the assay response with regards to the two key assay inputs, specifically effector and target cells incubation time and CE concentration. To do so, we performed a blinatumomab titration ranging over 9 orders of magnitude of concentrations across 5 different incubation times using the Jurkat E6.1 model described above (for further details please refer to the materials and methods section).

We observed a blinatumomab dose-dependent response in cell avidity, confirming the applicability of HTAM to investigate bispecific antibody-mediated cell avidity (**Figure 2A**). **Figures 2B** and **C** show blinatumomab-induced cell avidity, or avidity signal defined as the cell avidity value in absence of CE subtracted from the cell avidity value in presence of CE. We observed a CE-specific avidity signal at a blinatumomab concentration of 200 pM and an incubation time of 5 min, showcasing the sensitivity of the approach. Increasing incubation time resulted in an ~8-fold increase in the avidity signal, demonstrating the time-dependent behaviour of CE-specific cell engagement.

The highest cell avidity signal was observed at a CE concentration of 20 nM, for all the incubation times tested. At higher concentrations, the avidity signal remained stable or decreased, a trend also observed in legacy assays (Betts and van der Graaf). This is likely the result of blinatumomab saturation of both CD19 and CD3, preventing the formation of a trimeric complex. To form a trimeric complex either a target-cell-blinatumomab dimer must find a non-decorated T cell or a T cell-blinatumomab dimer must find a non-decorated target cell. This further substantiates the claim that the avidity signal is the result of bridging T-cells to the target cells via the formation of a TAA-CE-CD3 trimeric complex.

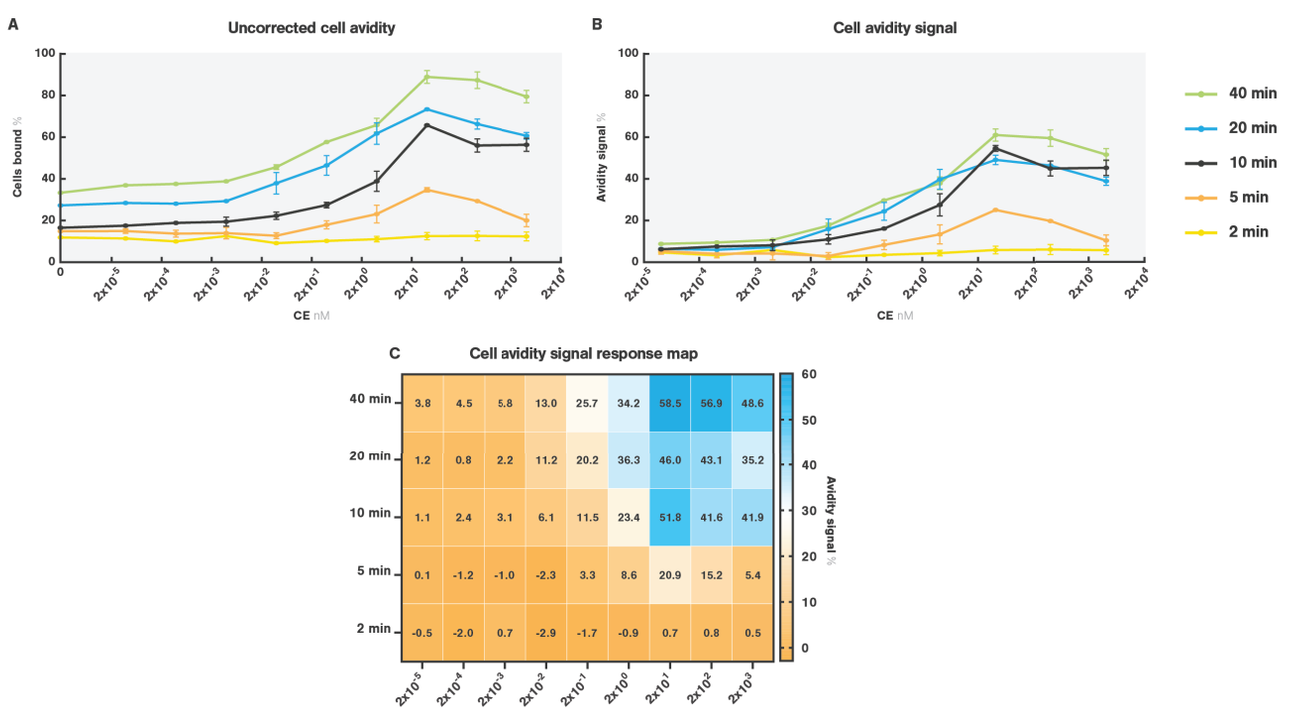

Figure 2 HTAM response – Nine concentrations (10-fold serial dilution ranging from 20 fM to 2 µM) over five incubation times were tested for the cell avidity input variable mapping. The uncorrected cell avidity in % cells bound **(A)** and the avidity signal **(B)** are shown. The combined avidity signal response map **(C)** shows the assay response as a function of incubation time and CE concentration. Points represent replicate means ± SD.

Technical validation of HTAM assay

Based on the pre-validation results, we selected a testable CE concentration range of three orders of magnitude from 200 pM to 20 nM with an incubation time of 10 min. While 40 min incubation time yielded a higher CE-induced cell avidity signal, we opted for a 10 min incubation time to optimize assay throughput.

To determine the HTAM assay performance characteristics including precision, specificity, and range, we performed duplicate experiments over 3 days and by 3 different users. Each experiment comprised of a titration of 4 different blinatumomab concentrations, a no-blinatumomab control and a negative control BiTE (see Materials and Methods) (**Figure 3A**). All experiments were done at 1000 g of force.

Precision, defined as intra day repeatability, resulted in a standard deviation of 3.1% at the midrange CE concentration value (2 nM) using the pooled variance. Intermediate precision, defined as inter user and inter day reproducibility, was determined by comparing all technical repeats across 3 different days and 3 different users and showed a standard deviation of 6.4% at midrange CE concentration (**Figure 3B**).

Using the averaged CE-induced cell avidity signal of 24.1% ± 6.4% at 2 nM, we find signal-to-noise (SNR) values of SNR = 7.8 and SNR = 3.8 for technical repeatability and inter-day user-to-user reproducibility respectively. Both values are well above the detection limit, defined as 3:1, proving specificity in line with ICH Guideline Q2.

To assess the assay intrinsic response, averaged technical repeats show a strong linear response with respect to log concentration ranging from 200pM to 20nM with R^2 of >0.95 via non-linear regression (**Figure 3C**). The CE induced cell avidity signal showed a SNR above 3:1 (SNR = 3.2) at 200 pM based on intraday repeatability identifying the lower range limit of the assay.

Within technical repeats, we typically find a good linear response with a mean R^2 = 0.94 ± 0.12 (min/max 0.52 and 0.99) (**Figure 3C**). The intrinsic variability seen day-to-day and user-to-user indicates room for further improvements in automation.

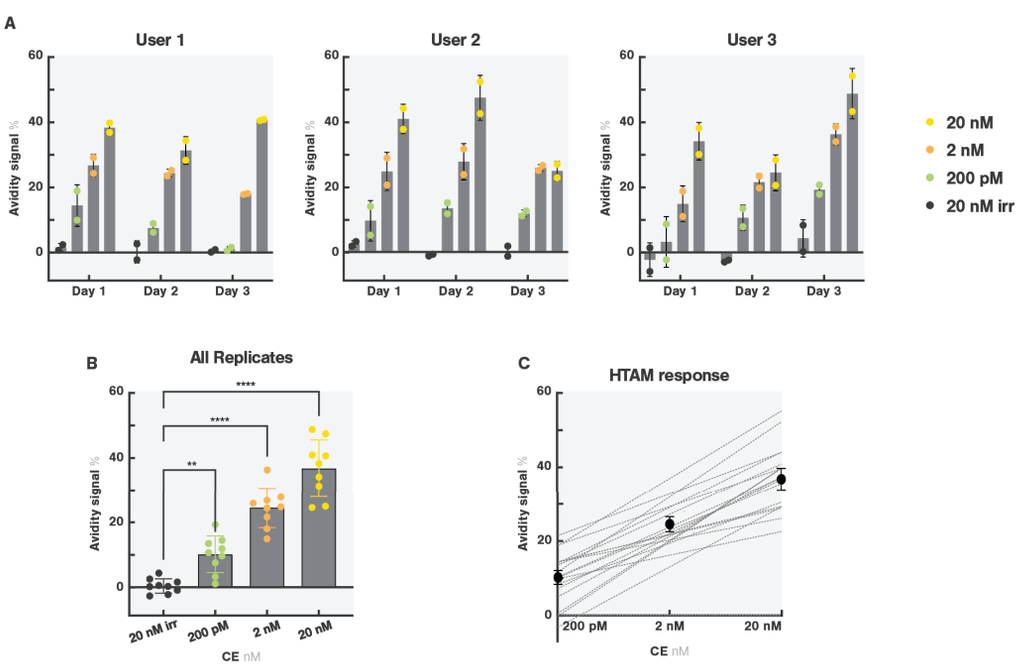

Figure 3 HTAM assay validation **(A)** Inter-user (n=3), inter-day (n=3) avidity signal replicates and **(B)** combined technical replicates of HTAM experiments performed over one irrelevant bispecific concentration and four blinatumomab concentrations using an incubation time of 10 minutes. **(C)** Linearity of avidity signal in-day replicates (grey) and assay intrinsic response over all technical repeats (black) with linear regression fits (lines) over three CE concentration steps. **(A+B)** Bars represent replicate means ± SD **(C)** Grey lines represent linear regression of individual technical repeats, points represent mean of all technical replicates ± SEM.

Conclusions

Here we established the performance characteristics of the LUMICKS HTAM assay for CE-mediated cell avidity between T cells and target cells. We demonstrate that HTAM can determine CE-specific avidity with high precision, sensitivity and reproducibility over a wide range of CE concentrations.

We show that the HTAM assay displays a CE concentration-dependent log-linear response over 3 orders of magnitude with a precision capable of resolving orders of magnitude differences in CE concentration. User-to-user and day-to-day reproducibility proved to be sufficiently robust to allow for implementation in a laboratory environment with multiple operators (**Figure 4**).

Thus, the HTAM assay demonstrates significant potential as a fast and high throughput cell avidity assay to facilitate CE therapeutic candidate selection.

Characteristic	Definition	Result
Reportable range	Lower and upper limit: Of CE concentration meeting specificity, response and precision criteria	0.2 – 20 nM
	Assay intrinsic and technical repeat response: Log-linear response assessed via non-linear regression over reportable range	$R^2 > 0.95$ $R^2 = 0.94 \pm 0.12$
Precision	Repeatability: SNR at mid value of the reportable range (2nM) assessed via pooled variance of intraday technical repeats	SNR = 7.8 (24.1% ± 3.1%)
	Reproducibility: SNR at mid value of the reportable range (2nM) assessed via mean and SD of technical repeats across ≥3 days by ≥3 operators	SNR = 3.8 (24.1% ± 6.4%)
Specificity	Specificity: SNR over reportable range assessed via mean and SD of technical repeats across ≥3 days by ≥3 operators	SNR > 3

Figure 4 HTAM Assay Performance Characteristics – Definition of and determined values for HTAM assay performance characteristics.

Materials and Methods:

Biological Model

To determine the technical validity of the HTAM assay we employed the T cell-like cell line Jurkat E6.1. The lymphocyte-like, B cell leukemia cell line, NALM6, was used as a tumor target model system. As a multispecific CE, we used the well-characterized FDA- and EMA-approved bispecific antibody blinatumomab (Blincyto®). Negative control BiTE was used containing an identical CD3-targeting scFv as blinatumomab but with a target arm directed against bacteria-derived β-galactosidase protein, which is absent on Nalm6.

Avidity Assay Workflow

All measurements were performed using 6-channel plastic flow cells with a channel volume of 30 µL and a surface area of 0.6 cm^2. To promote monolayer cell adhesion, channels were coated with 0.02 mg/mL of Poly-L-Lysine.

Target monolayers were created by introducing NALM6 cell suspensions ($30x10^6$/mL) into each channel of the multi-channel flow cell and incubated at 37°C for 60 minutes to allow the monolayer to adhere to the flow cell surface. Immediately before introducing effector cells and CE, monolayers were force tested and each channel was washed to remove any detached monolayer cells.

Jurkat E6.1 effector cells were stained for imaging using the fluorescent dye, CellTrace FarRed, according to the manufacturer's protocol. After staining, effector cells were resuspended in culture media at 0.5 $x10^6$/mL and placed at 37˚C.

Immediately before use, effector cells were mixed with the appropriate CE dilution and co-injected into the monolayer containing channels of the 6-channel flow cell and incubated for 10 minutes, or the indicated incubation time.

After incubation, the flow cells were placed on a fluorescent microscope. Pre-force brightfield and fluorescence images were acquired for each channel.

After pre-force image collection force was applied. Brightfield and fluorescence images were acquired after each round of force application.

The percentage of cells bound after each force application was quantified by comparing the number of cells present in the pre vs post-force application fluorescence images for all channels. Fluorescence images were analyzed using custom in-house cell detection image analysis software.

The avidity value was calculated as the channel averaged, fraction of stuck cells bound to the monolayer after force application. The CE-induced cell avidity signal was determined by subtracting the bound fraction of the no CE control channel from the bound fraction of each CE-containing channel.

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