Video Article

The Use of Fluorescent Target Arrays for Assessment of T Cell Responses *In vivo*

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Abstract

The ability to monitor T cell responses *in vivo* is important for the development of our understanding of the immune response and the design of immunotherapies. Here we describe the use of fluorescent target array (FTA) technology, which utilizes vital dyes such as carboxyfluorescein succinimidyl ester (CFSE), violet laser excitable dyes (CellTrace Violet: CTV) and red laser excitable dyes (Cell Proliferation Dye eFluor 670: CPD) to combinatorially label mouse lymphocytes into >250 discernable fluorescent cell clusters. Cell clusters within these FTAs can be pulsed with major histocompatibility (MHC) class-I and MHC class-II binding peptides and thereby act as target cells for $CD8^+$ and $CD4^+$ T cells, respectively. These FTA cells remain viable and fully functional, and can therefore be administered into mice to allow assessment of $CD8^+$ T cell-mediated killing of FTA target cells and $CD4^+$ T cell-meditated help of FTA B cell target cells in real time *in vivo* by flow cytometry. Since >250 target cells can be assessed at once, the technique allows the monitoring of T cell responses against several antigen epitopes at several concentrations and in multiple replicates. As such, the technique can measure T cell responses at both a quantitative (*e.g.* the cumulative magnitude of the response) and a qualitative (*e.g.* functional avidity and epitope-cross reactivity of the response) level. Herein, we describe how these FTAs are constructed and give an example of how they can be applied to assess T cell responses induced by a recombinant pox virus vaccine.

Video Link

The video component of this article can be found at https://www.jove.com/video/51627/

Introduction

T cells play a central role in the adaptive immune response and are often targeted for manipulation in immunotherapy. $CD4^+$ effector T cells respond to foreign antigen by secreting cytokines that regulate many aspects of immunity and can also directly help B cells to manufacture antibodies. $CD8^+$ cytotoxic T cells (CTLs) can also respond to foreign antigen by secreting cytokines as well as playing a central role in directly killing cells expressing a foreign antigen. The fundamental interaction that initiates these T cell effector functions involves the interaction of the T cell receptor (TCR) with foreign peptides displayed on MHC molecules on the surface of cells. $CD4^+$ T cells recognize peptides displayed on MHC class-II molecules on antigen presenting cells and $CD8^+$ T cells recognize peptides displayed on MHC class-I molecules that are typically displayed on microbe infected cells.

In order to assess the role T cells play in an immune response, it is essential that their effector functions are measured by reliable and sensitive techniques. Common methods for T cell response assessment include; MHC class-I/II/peptide tetramer reactivity; cytokine production by ELISPOT and intracellular cytokine staining; and killing capacity by ^{51}Cr-release assays. These assays, however, are typically performed *ex vivo* with *in vitro* stimulation, or provide limited insight into T cell function. Ideally, when measuring T cell responses it would be beneficial to assess them *in situ*, *in vivo* as they occur, with no manipulation of T cells so as to avoid changes in functional parameters that may occur through *in vitro* stimulation. Some of the most commonly used *in vivo* T cell functional assays are based on measuring CTL mediated killing of target cells pulsed with MHC class I-binding peptides, that are enumerated *in vivo* via their detection through fluorescent labeling with vital dyes such as CFSE. While these types of assays can monitor CTL mediated killing of targets when they happen *in vivo*, they have previously had a relatively limited capacity to assess killing of multiple targets presenting different concentrations and different types of peptide epitopes, which is required to allow qualitative parameters such as functional avidity and epitope variant cross-reactivity to be assessed. These assays also do not provide any information on $CD4^+$ T cell mediated responses.

To overcome many of the limitations with current methods used to assess T cell responses, we have recently developed a multiplex assay based on fluorescent target arrays (FTAs), which allows the monitoring of T cell responses against >250 target cells simultaneously in one animal by flow cytometry [1,2]. FTAs are comprised of lymphocytes labeled with several concentrations and combinations of vital dyes like CFSE, CTV and CPD allowing >250 cell clusters of unique fluorescence to be generated. Since these cells remain viable and fully functional, they can be injected into animals to allow monitoring of their interaction with effector T cells *in vivo*[3]. For example, the FTA cell clusters can be pulsed with MHC class-I-binding peptides to allow assessment of antigen-specific CTL mediated killing of target cells[1]. In addition, the FTA cell clusters can

also be pulsed with MHC class-II-binding peptides, allowing assessment of antigen specific T helper cell (T_H) activity by assessing activation (by assessment of activation markers such as CD69, CD44 and/or CD62L) of B cells within the FTA bearing cognate peptide[2]. Since more than 250 targets can be detected simultaneously, it is possible to measure CTL and T_H responses against many target cell clusters pulsed with numerous peptides at different concentrations and the inclusion of many replicates. The FTA assay therefore provides an unprecedented level of T cell effector response assessment *in vivo*.

Here we describe in detail the construction of a FTA and show how they can be applied to assessing T cell responses *in vivo*. The procedure describes the construction of a FTA comprised of 252 discernable cell clusters through the use of three vital dyes, comprised of 6 repeats of 42 cell clusters pulsed with MHC class-I and II-binding peptides. The labeling of 42 cell clusters occurs in 10 ml conical bottomed tubes and it is helpful to lay these out in a tube rack as depicted in **Table 1**. This method can be adjusted for smaller numbers of discernable clusters as required by reducing the amount of labeling of each dye performed[1].

We highlight the utility of the assay by showing how it can measure responses generated by recombinant pox -virus vaccination against multiple epitopes in a small cohort of mice. This shows how the FTA assay can be used to measure cumulative responses and functional avidity through, respectively, the use of area under curve (AUC) assessments and measurement of effective peptide concentration required to generate half maximal responses (EC_{50}).

Protocol

Note: Mice used under this protocol were handled according to the guidelines of the Australian National University Animal Experimentation Ethics Committee and mice were euthanized by cervical dislocation.

1. Dye and Peptide Preparation

1. Dye preparation
   Note: Dyes are prediluted at different concentrations to allow labeling of cells at discrete fluorescence intensities. CFSE is used at seven concentrations made through 3.5-fold serial dilutions, and CTV and CPD are used at six different concentrations made through 3.7-fold serial dilutions (see **Tables 2-4**). The stock concentrations of dyes listed in **Tables 2-4** will be used to label target cells at the final dye concentrations listed in **Tables 2-4**. Dyes react upon exposure to aqueous solution. It is therefore important that vials be equilibrated to RT prior to opening to minimize exposure of dyes to condensation.
   1. CFSE
      Note: CFSE is purchased as carboxyfluorescein diacetate succinimidyl ester (CFDA, SE; MW 557.47), typically at 25 mg/vial.
      1. Resuspend 25 mg of CFSE in 4.48 ml DMSO to obtain a 10 mM stock solution.
      2. Serially dilute CFSE: Add 35 µl of 10 mM CFSE stock into 87.5 µl of DMSO and repeat this with diluted stock solution to give each dye concentration in **Table 3**.
   2. CTV
      Note: CTV is typically purchased in packs of 9 vials, each of which can be reconstituted with 10 µl of DMSO to generate a 10 mM solution of the dye.
      1. Reconstitute and pool all 9 vials of CTV with 90 µl of DMSO to give a 10 mM solution.
      2. Serially dilute CTV: Add 11 µl of 10 mM CTV stock into 29.7 µl of DMSO and repeat this with diluted stock solution to give each dye concentration in **Table 2**.
   3. CPD
      Note: CPD (MW 792.6) is typically purchased as 0.5 mg/vial.
      1. Resuspend 0.5 mg of CPD in 63 ml of DMSO to get a 10 mM solution.
      2. Serially dilute CPD: Add 11 µl of 10 mM CPD stock into 29.7 µl of DMSO and repeat this with diluted stock solution to give each dye concentration in **Table 4**.
         Note: Dye stocks can be stored at -20 °C for several months and can be thawed and refrozen several times without significant loss in function.

2. Peptide preparation
   Note: MHC class-I-binding peptides are generally used to pulse target cells at 6 different concentrations made using 10 fold dilutions from a starting concentration of 1 µM (**Table 5**). MHC class II-binding peptides are generally used to pulse target cells at 6 different concentrations made using 3 fold dilutions from a starting concentration of 400 µM (**Table 5**). Each peptide is made at 2x final concentration in PBS such that every peptide concentration has a minimum volume of 250 µl. Peptide stocks can be prepared prior to FTA construction and stored at -20 °C without significant loss in function.
   1. Preparing stock MHC class-I-binding peptides
   2. Prepare the highest concentration of each peptide epitope to 2 µM stock solutions (2x 1 µM)
   3. Serially dilute MHC class-I-binding peptides: add 44.4 µl of 2 µM peptide stock solution into 400 µl of PBS. Repeat this with diluted stock solution to give each peptide concentration in **Table 5**.
   4. Preparing stock MHC class-II-binding peptides
   5. Prepare the highest concentration of each peptide epitope to 800 µM stock solutions (2x 400 µM).
   6. Serially dilute MHC class-II-binding peptides: add 150 µl of 800 µM peptide solution into 300 µl of PBS. Repeat this with diluted stock solution to give each peptide concentration in **Table 5**.

2. FTA Preparation

Note: The procedure below outlines the construction of a FTA comprised of cells pulsed with 7 different peptide epitopes at 6 different concentrations (*i.e.* 42 cell clusters) repeated 6 times to generate 252 discernable cell clusters. Within each repeat, a cell cluster not pulsed with any epitope (Nil), is included as a control. It is helpful for FTAs to have a CD45 allotype difference from host animals to allow their discrimination from recipient cells by antibody labeling at the time of analysis. Otherwise FTAs can be labeled with other dyes such as PKH-26 for this purpose (described in step 2.6). Typically, the FTA preparation procedure is carried out from start to finish during one sitting.

1. Label 42, 10 ml conical bottomed plastic tubes 1-42 (as in **Table 1** for example).
2. Preparation of cells
    1. Isolate spleen and/or lymphnodes from mice. Prepare single cell suspension from tissues by mashing through a 70 µm pored sieve with a 5 ml syringe plunger and count cells using a hemocytometer.
    2. Resuspend lymphocytes at up to 200 x 10^6 cells/ml in 11.5 ml of Rochester Park Memorial Institute 1640 (RPMI, or equivalent) containing 5% fetal calf serum (FCS). Note: It is important to use a buffer with high amine content to minimize dye toxicity to cells[3].
3. CTV labeling
   Note: Cells are initially labeled with 6 concentrations of CTV.
    1. Thoroughly resuspend the cells by inverting the tube several times. Add 1.9 ml of cell suspension to 6 of the 10 ml tubes labeled 37-42, taking care not to wet the top half of the tubes.
    2. To label cells with CTV, remove the tube cap and lay the tube horizontally.
    3. Add 83 µl of PBS to the non wetted portion at the top of the tube, and to this add 17 µl of stock CTV (see **Table 2** for which stock solution is assigned to which tube). Note: A non wetted tube is important to prevent cell suspension movement and premature mixing of the cell solution with the dye solution.
    4. Cap the tube and mix the cell suspension with the dye quickly and thoroughly by vortexing.
    5. Repeat steps 2.3.2-2.3.4 for the stock solutions of CTV in the designated tubes described in **Table 2**.
    6. Incubate cells for a minimum of 5 min at RT (20 °C).
4. CFSE labeling
    1. After CTV labeling, add 5 ml of RPMI containing 5% FCS to each tube and thoroughly resuspend the cells by vortexing.
        1. From tube 37 transfer 1 ml of cell suspension to tubes 31, 25, 19, 13, 7, and 1.
        2. From tube 38 transfer 1 ml of cell suspension to tubes 32, 26, 20, 14, 8, and 2.
        3. From tube 39 transfer 1 ml of cell suspension to tubes 33, 27, 21, 15, 9, and 3.
        4. From tube 40 transfer 1 ml of cell suspension to tubes 34, 28, 22, 16, 10, and 4.
        5. From tube 41 transfer 1 ml of cell suspension to tubes 35, 29, 23, 17, 11, and 5.
        6. From tube 42 transfer 1 ml of cell suspension to tubes 36, 30, 24, 18, 12, and 6
           Note: Take care not to wet the top half of the tubes during steps 2.4.1.1-2.4.1.6.
    2. To label cells with CFSE, remove the tube cap and lay the tube horizontally.
    3. Add 103 ml of PBS to the non wetted portion at the top of the tube, and to this add 7 ml of stock CFSE (see **Table 3** for which stock solution is assigned to which tube).
    4. Cap the tube and mix the cell suspension with the dye quickly and thoroughly by vortexing.
    5. Do steps 2.4.2-2.4.4 for the stock solutions of CFSE in the designated tubes described in **Table 3**.
    6. Incubate cells for a minimum of 5 min at RT (20 °C).
    7. Wash cells: Dilute cell suspension with 9 ml of 20 °C RPMI containing 5% FCS, sediment by centrifugation at 300 x g for 10 min at 20 °C and remove supernatants by aspiration with a transfer pipette.
5. Peptide pulsing and cell washing
   Note: After cells have been labeled with CTV and CFSE, they are pulsed with MHC class-I and/or MHC class-II binding peptides (prepared in 1.2). One of the cell populations must also not be pulsed with peptide (*e.g.* Nil in **Table 1**) and used as a negative control for calculation of T cell responses.
    1. Peptide pulsing
        1. Resuspend cells in a total volume of 250 µl of RPMI containing 5% FCS. Note: Typically 50 µl of cell suspension remains after aspiration of the supernatant at the end of step 2.4.7, therefore add 200 µl of medium to cell pellet.
        2. Add 250 µl of pre prepared peptide stocks (as in **Table 5**) to appropriately designated tubes (as in **Table 1**) and be sure to include a control tube of PBS added alone without peptide as a Nil control.
        3. Mix cell suspensions with a vortex. Note: It is critical that each peptide epitope and each peptide concentration is assigned to a single tube and this recorded clearly based on the expected fluorescence of the cells in this tube, since the fluorescence signature of this cluster will define this peptide (as in **Table 1** for example).
        4. Incubate the cells at 37 °C for 1 hr.
    2. Cell washing
        1. Add 5 ml of ice cold (4 °C) RPMI containing 5% FCS to the cell suspension and resuspend cells by inverting tube. Carefully underlay the cell suspension with 3 ml of ice cold (4 °C) FCS.
        2. Sediment the cells by centrifugation at 300 x g for 10 min at 4 °C. Use slow acceleration and braking to ensure the interface of FCS and the cell suspension solution is maintained.
        3. Carefully aspirate off the RPMI and then the FCS with a transfer pipette, leaving the washed cell pellets undisturbed. Note: The use of a FCS underlay to wash cells helps to ensure as much peptide solution is removed from the cells as possible and thereby limiting exposure of cell populations to multiple free peptides when cells are pooled.
        4. Wash cells again: Resuspend the cell pellets in 10 ml of 4 °C RPMI containing 5% FCS. Sediment the cells by centrifugation at 300 x g for 10 min at 4 °C. Pour off supernatants.

5. Pool all cell populations together into a single tube with a pipette using 6 ml of 4 °C RPMI containing 5% FCS. Sediment pooled cells by centrifugation at 300 x g for 10 min at 4 °C. Aspirate off supernatant with a transfer pipette.

6. CPD Cell labeling
   Note: At this point six intra assay replicates can be generated by labeling peptide pulsed cells with 6 different concentrations of CPD.
   1. Add 11.4 ml of 20 °C RPMI containing 5% FCS to the pooled cell pellet and resuspend thoroughly using a pipette.
   2. Add 1.9 ml of cell suspension to 6, 10 ml tubes labeled A-F, taking care not to wet the top half of the tubes.
   3. To label cells with CPD, remove the tube cap and lay the tube horizontally.
   4. Add 92 µl of PBS to the non wetted portion at the top of the tube, and to this add stock CPD (see **Table 4** for the quantity of stock solution assigned to each tube).
   5. Cap the tube and mix the cell suspension with the dye quickly and thoroughly by vortexing.
   6. Do steps 2.6.3-2.6.5 for the stock solutions of CPD in the designated tubes described in **Table 4**. Note: Unlike CFSE and CTV, the CPD labelling concentration is not precisely linearly related to the resulting fluorescence intensity of labelled cells, and so the labelling concentration used to obtain equidistant fluorescent peaks of several labelled populations has been determined empirically (see **Table 4**).
   7. Incubate cells for a minimum of 5 min at RT (20 °C).
   8. Wash cells twice: Resuspend cells to 10 ml with 20 °C RPMI containing 5% FCS. Sediment the cells by centrifugation at 300 x g for 10 min at 20 °C. Aspirate off supernatant with a transfer pipette. Repeat.
   9. Pool all cells together into a single tube using 8 ml 4 °C RPMI containing 5% FCS with a pipette. Sediment pooled cells by centrifugation at 300 x g for 10 min at 4 °C and aspirate off the supernatant with a transfer pipette.

7. (Optional) PKH-26 Cell labeling
   Note: If FTAs cannot be constructed with cells expressing a CD45 allotypic difference to host mice, they can be labeled with PKH-26 to allow their discrimination from recipient cells.
   1. Add 2.9 ml of 20 °C PBS to the pooled cell pellet and resuspend thoroughly with a pipette.
   2. Add cell suspension to a non wetted 10 ml tube, taking care not to wet the top half of the tube.
   3. Remove the tube cap and lay the tube horizontally.
   4. Add 58 µl of diluent C (in the PKH-26 dye kit) to the non wetted portion at the top of the tube, and to this add 42 µl of 1 mM stock PKH-26.
   5. Cap the tube and mix cell solution with the dye thoroughly by vortexing.
   6. Incubate cells for a minimum of 10 min at RT (20 °C).
   7. Wash cells twice: Resuspend cells to 10 ml with 20 °C RPMI containing 5% FCS. Sediment the cells by centrifugation at 300 x g for 10 min at 20 °C. Aspirate off supernatant with a transfer pipette. Repeat.

8. Injecting FTAs into host animals
   Note: To measure T cell responses *in vivo*, FTAs are injected into animals that have an active immune response and left *in situ* for up to 24 hr . It is critical that the FTAs are also injected into a naïve animal as a negative control.
   1. Count and resuspend cells at 2.5 x 10^8 cells per ml in PBS. Inject 200 µl of cells intravenously into host mice, including a naïve animal as a control.

3. Flow Cytometry

1. 18-24 hr after FTA injection harvest blood or spleen (or other tissues of interest) from host mice.
2. Prepare single cell suspension from tissues by mashing through a 70 µm pored sieve with a 5 ml syringe plunger.
3. Resuspend cells at up to 65 x 10^6 cells/ml in PBS containing 0.1% BSA.
4. Dispense 100 µl aliquots of cell suspension into wells of a microtitre plate for antibody labeling.
5. Label cells with fluorochrome labeled antibodies and fluorescent viability probes with spectrally compatible fluorescence to CFSE, CTV and CPD. Note: Antibodies to B cell markers such as B220 and activation markers such as CD69 are essential to measure B cell activation if using the FTA to measure T_H cell responses[2]. Include an antibody to CD45.1 and/or CD45.2 if the FTAs and host mice have a CD45 allotypic difference.
6. Add 100 µl of 2x stock solution of antibodies/viability dyes (in PBS containing 0.1% BSA) to 100 µl aliquots of cells, mix well and incubate on ice (4 °C) for 30 min.
7. Wash cells: Sediment cells by centrifugation at 300 x g for 5 min at 4 °C and remove supernatant. Resuspend cells in 200 µl of PBS containing 0.1% BSA, sediment cells by centrifugation at 300 x g for 5 min at 4 °C and remove supernatant.
8. Resuspend cells in a total volume of 400 µl of PBS containing 0.1% BSA, filter cells through a 70 µm mesh and analyze by flow cytometry in a flow cytometer capable of detecting the relevant fluorescent dyes and conjugates. Collect up to 3 x 10^6 lymphocyte events in order to resolve each FTA cell cluster and gain enough cells for statistical analysis. Note: Ensure all the typical controls (such as single stained controls) for flow cytometry are employed. Typically, CFSE requires excitation from a blue laser source (typically at 488 nm) and detection with band pass filters centered over 520 nm; CTV requires excitation from a violet laser source (typically at 405 nm) and detection with band pass filters centered over 450 nm; and CPD requires excitation from a red laser source (typically at 633 nm or 640 nm) and detection with band pass filters centered over 670 nm.

4. Data Analysis

1. Analyze flow cytometry data using standard flow cytometry software (see representative result for an example of the type of gating strategy employed).

2. For % specific killing, calculate the number of cells in each FTA cell cluster pulsed with MHC class-I-binding peptides and the FTA clusters that were not peptide pulsed ("Nil") and using the following formula to calculate % Specific Killing.

$$\text{\% Specific Killing} = \left[1 - \left(\frac{\text{Targets}^{+\text{peptide}}_{\text{primed}} \,/\, \text{Targets}^{+\text{nil}}_{\text{primed}}}{\text{Targets}^{+\text{peptide}}_{\text{naive}} \,/\, \text{Targets}^{+\text{nil}}_{\text{naive}}} \right) \right] \times 100$$

Note: In the above formula "primed" refers to targets from animals that are thought to have an immune response against the target peptides, "naïve" refers to targets from naïve animals, "peptide" refers to targets pulsed with peptides and "nil" refers to targets not pulsed with any peptides.

3. For B cell activation as a measure of T_H activity, calculate the geometric mean fluorescence intensity (GMFI) of CD69 antibody fluorescence on FTA B cells pulsed with MHC class-II-binding peptides in "primed" animals and from this subtract the GMFI of CD69 expression on the corresponding FTA B cells in "naïve" animals to give a measure of T_H activity.
4. From the statistics generated in steps 4.2 and 4.3, use mathematical spreadsheet software such as GraphPad Prism to calculate other quantitative and qualitative parameters such as area under curve and EC_{50} (an in depth description of how these calculations are performed for AUC can be found at http://graphpad.com/guides/prism/6/statistics/index.htm?stat_area_under_the_curve.htm, and for EC50: http://www.graphpad.com/guides/prism/6/curve-fitting/index.htm?reg_the_ec50.htm).

Representative Results

As an example of the use of the FTA assay, a BALB/c mouse was immunized with recombinant vaccinia virus (VV) expressing HIV-I epitopes (VV-HIV) and responses to the HIV-I CTL epitopes, Gag, Gag mut, Env and Pol, the VV CTL epitopes F2L and F2L mut, and the HIV-I T_H cell epitope, Gag Th (as described in[2]) were assessed using a 252 parameter FTA assay (**Figure 1B**). Epitope variants of Gag (Gag mut) and F2L (F2L mut) are not expressed in the VV-HIV vector and therefore responses against these epitopes are reflective of epitope variant cross reactive T cell responses. Naïve mice were also injected with the FTA as a negative control. The FTA was discriminated from host mouse cells by PKH-26 labeling and FTA B cells discriminated by B220 antibody staining via flow cytometry (**Figures 1A** and **1B**). Each of the 6 intra animal replicates was gated based on CPD fluorescence (**Figure 1C**) and FTA targets expressing the various concentrations of epitopes gated based on CFSE and CTV fluorescence (**Figure 1D**). % Specific Killing of each replicate was assessed by comparing FTA cluster cell death in primed animals relative to corresponding FTA clusters in naïve animals (**Figure 1D**). T_H activity was assessed by comparing FTA B cell upregulation of CD69 in primed animals relative to corresponding FTA B cell clusters in naïve animals (**Figure 1E**).

From this analysis, VV-HIV infection generated strong CTL responses against the immunodominant VV epitope F2L, with 100% of FTA target cells pulsed with 0.001 mM or more of the epitope being removed from the spleen (**Figure 2A**). There was also a strong response generated against the variant form of F2L, F2L mut, with 100% of FTA target cells pulsed with 0.01 mM or more of the epitope being removed from the spleen. Since F2L mut is not present in the infecting virus, this response indicates an epitope variant cross-reactive response. There was also a relatively moderate response generated against targets expressing the HIV Gag epitope and a slight cross reactive response against the variant form of this epitope, HIV Gag mut. There appeared to be negligible responses generated to the HIV Pol and HIV Env CTL epitopes.

In addition to CTL responses, the FTA example assay shown also measured T_H responses by assessing activation of FTA B cell expressing the HIV MHC class-II-binding epitope HIV Gag Th (**Figure 2B**). This showed antigen specific B cell activation occurred in primed animals suggesting generation of HIV Gag Th-specific T_H cell effectors.

These measures of T cell response magnitude can be summarized by generating area under curve values (AUC) for each of the epitopes (**Figure 2C**) that measures the cumulative magnitude of the response.

In addition to magnitude of antigen specific and epitope variant cross-reactive responses, the FTA assay allows functional avidity measurements to be made. For example, the effective peptide concentrations used to pulse FTA target cells required to give half maximal responses (EC_{50}), is shown for the CTL effectors (**Figure 2D**). This showed the functional avidity to the epitopes F2L mut, HIV Gag and HIV Gag mut, were, approximately 10, 100 and 4,000 fold lower, respectively, than responses generated to the dominant VV epitope F2L.

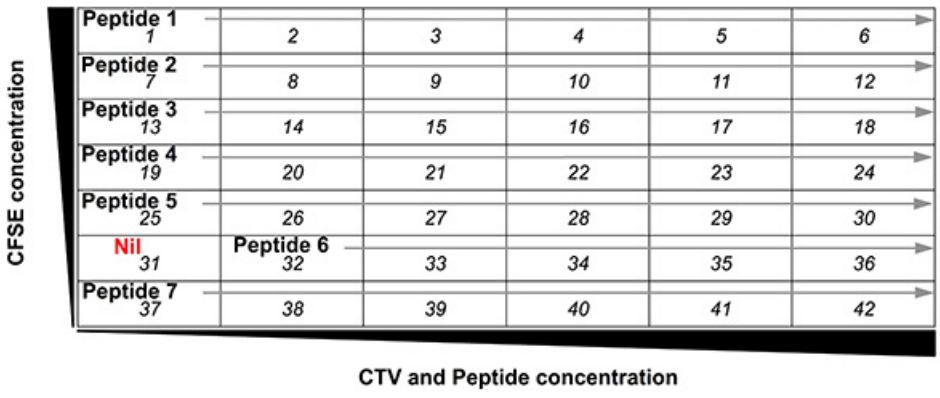

Table 1. Layout of a 252 parameter FTA. Typical layout of one replicate of a 252 FTA comprised of 42 samples/tubes pulsed with 7 different peptide epitopes at 6 different concentrations and including an un pulsed (Nil) sample. Please click here to view a larger version of this figure.

Tube Number	Volume (µl)	Dye stock (mM CTV)	Final concentration (µM)
37	0	0	0
38	17	0.053	0.451
39	17	0.197	1.48
40	17	0.73	6.21
41	17	2.7	23
42	17	10	85

Table 2. CTV stocks used to label cells. Volume of listed CTV stock used to label cells in 2 ml to give the final labeling dye concentration.

Tube Number	Volume (µl)	Dye stock (mM CFSE)	Final concentration (µM)
37 - 42	0	0	0
31 - 36	7	0.022	0.139
25 - 30	7	0.078	0.492
19 - 24	7	0.23	1.45
13 - 18	7	0.82	5.17
7 - 12	7	2.9	18.3
1 - 6	7	10	63.1

Table 3. CFSE stocks used to label cells. Volume of listed CFSE stock used to label cells in 1.11 ml to give the final labeling dye concentration.

Tube Number	Volume (µl)	Dye stock (mM CPD)	Final concentration (µM)
A	0	0	0
B	4	0.053	0.106
C	6.3	0.197	0.62
D	7.5	0.73	2.74
E	7.3	2.7	9.86
F	7.7	10	38.5

Table 4. CPD stocks used to label cells. Volume of listed CPD stock used to label cells in 2 ml to give the final labeling dye concentration.

	Peptide concentration (µM)						
MHC class I-binding peptides	0	0.00002	0.0002	0.002	0.012	0.2	2
MHC class II-binding peptides	0	3.29	9.88	29.6	88.9	266	800

Table 5. MHC-class I and II-binding peptide stock concentrations. Typical stock concentrations[1] of peptide epitopes used to construct a FTA[1]. These concentrations are 2x the final concentrations used to pulse target cells.

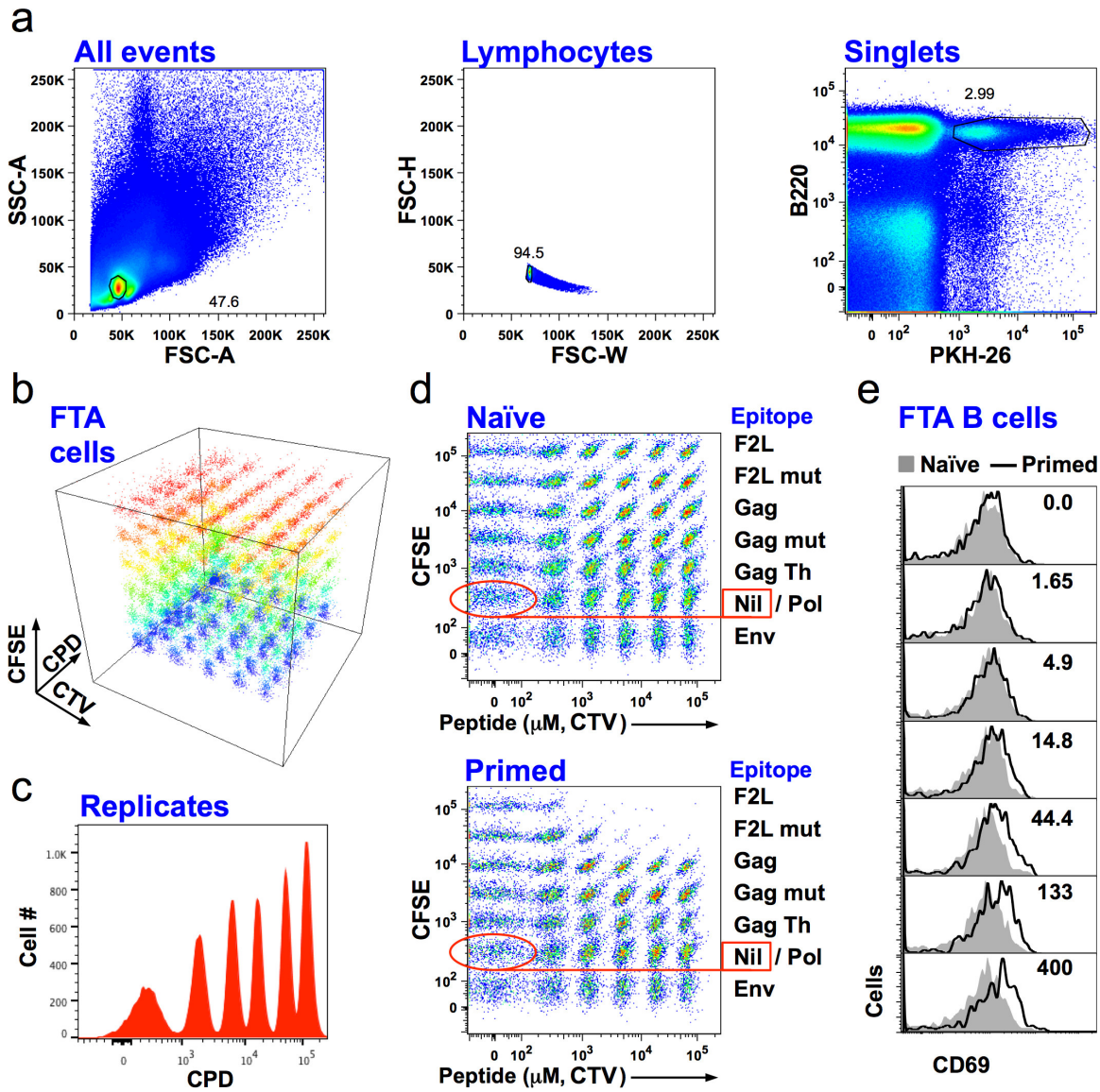

Figure 1. Typical flow cytometry analysis of FTAs. Splenocytes from BALB/c mice were used to construct a 252 parameter FTA as described in the text. FTA clusters were pulsed with 6 concentrations (as listed in **Table 5**) of 7 different viral epitopes, including the MHC class-I-binding epitopes, F2L (SPYAAGYDL, an L^d-restricted vaccinia virus (VV) epitope); F2L mut (SPGAAGYDL, a variant of F2L), Gag (AMQMLKETI, a K^d-restricted HIV Gag epitope[4]), Gag mut (AMQMLKDTI, a variant of HIV Gag[5]), Pol (VGPTPVNII, a D^d-restricted HIV pol epitope[6]), Env (RGPGRAFVTI, a D^d-restricted HIV env epitope[7]); and the MHC class-II-binding peptide Gag T_H (PVGEIYKRWIILGLN, a H-2^d-restricted HIV Gag epitope[4]). FTAs were injected *i.v.* into a BALB/c mouse that had been infected intranasally (*i.n.*) 6 days earlier with recombinant VV expressing HIV epitopes (VV-HIV, primed animal). The FTA was also injected into naïve mice as a control. After 18 hr *in vivo*, FTA cells present in splenocyte suspensions from host mice were analyzed by flow cytometry. **A)** Typical progressive gating strategy to identify FTA cells showing gates for lymphocytes, singlets and PKH-26^+ FTA cells (it is also helpful to resolve live cells using a viability dye like Hoechst 33258, not shown). **B)** 3D plot showing all FTA clusters from naïve host mouse. **C)** Histogram plots showing FTA CPD fluorescence marking each of the 6 intra animal replicates. **D)** 2D plots showing one of the 6 intra-animal FTA B cell replicates presenting the different types and concentrations of epitopes from the naïve and primed animal. This shows the absence of FTA cells in the primed animal relative to the naïve animal, revealing "killing events" by CTLs that form the basis of the FTA killing assay[1]. **E)** Histogram analysis of FTA B cell expression of the activation marker CD69 in primed animals relative to naïve animals, which forms the bases of the FTA T helper assay[2]. Please click here to view a larger version of this figure.

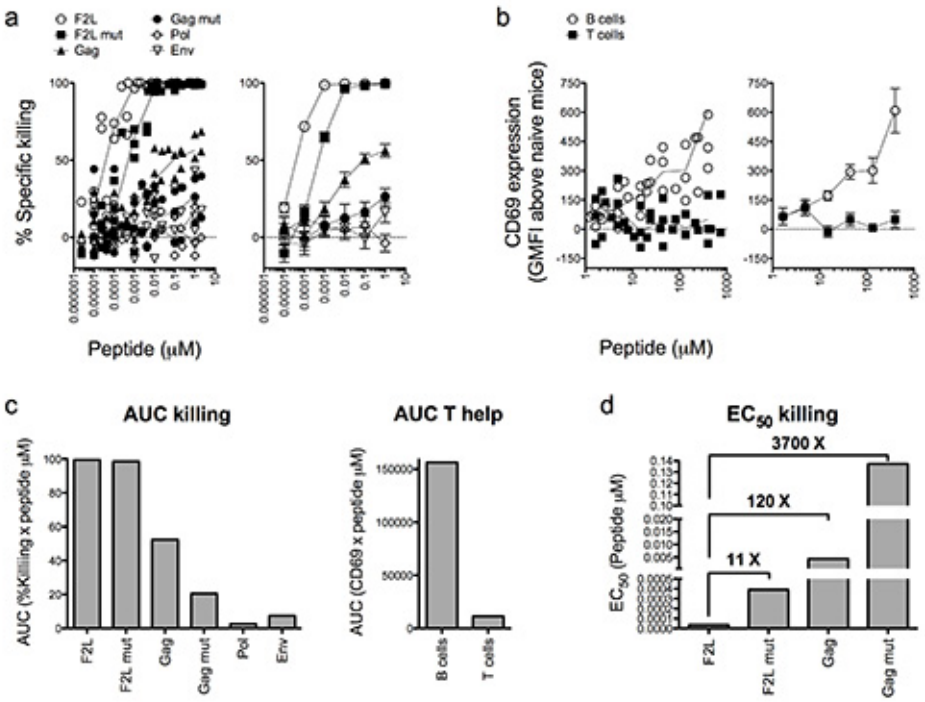

Figure 2. FTAs enable the measurement of the magnitude and avidity of T cell responses *in vivo*. A 252 parameter FTA was used to assess T cell responses in mice infected with VV-HIV as described in **Figure 1**. **A)** % Specific killing was calculated for all CTL epitopes and results from each of the 6 replicates shown (left panel) as well as means and standard error of means (right panel) shown. **B)** T_H response was assessed by measuring activation of FTA B cell presenting the HIV Gag Th epitope for each of the 6 intra animal replicates (left panel) and means and standard error of means (right panel) calculated. **C)** AUC measurements for % specific killing (a) and T helper responses (b) was calculated as a measure of cumulative magnitude. **D)** EC_{50} measurements were calculated for % Specific killing data (a) as a measure of functional avidity. Please click here to view a larger version of this figure.

Discussion

The advantage of FTA-based assays is that they allow the discrimination of >250 viable and fully functional target cell populations from a single host animal by flow cytometry. This provides a level of complexity to *in vivo* flow cytometry based assays that has not been possible before. This is highlighted in the 2 animal experiments shown above, where responses to 7 distinct viral epitopes at 6 concentrations could be monitored in replicates of 6 simultaneously in a single animal allowing parameters such as AUC and EC_{50} to be determined for T cell responses *in vivo*.

In addition to measuring T cell responses *in vivo*, the FTA-based assays can be modified to measure responses *in vitro*[1]. This essentially involves adding FTAs to T cells in culture to measure responses over several hours *in vitro*. One potential limitation to *in vitro*-based assays using FTAs is the tendency for the labeled cells within the FTA to transfer the labeling dye to surrounding cells[1,3]. This can result in decreased resolution of FTA cell clusters as their fluorescence becomes less distinct between each population. This is more apparent in *in vitro* assays compared to *in vivo* assays because the cells are in close proximity for the longer periods of time in *in vitro* assays. This loss in resolution of FTA cell populations can be minimized in *in vitro* assays by reducing the culture time of FTAs with effector T cells and by using FTAs that have a less target cell populations that have a greater amount of “fluorescent space” between them so that dye transfer has less impact.

We have also noted loss in resolution of FTA cell populations when FTAs were placed *in vivo* for 48 hr[1]. This appeared to be the result of target B cells within the FTA proliferating and thereby reducing dye fluorescence intensity. This is likely a result of B cells presenting cognate antigen to antigen-specific effector $CD4^+$ T cells that resulted in B cell stimulation. It is therefore recommended that the assay be limited to ~24 hr or less when B cell targets are being monitored.

The ability to label multiple (>96) unique cell clusters fluorescently has been reported previously using fixed non-viable cells[8]. The staining of live cells, however, has been more problematic with the availability of compatible vital dyes, and only 8-12 viable cells clusters has been achieved previously[9]. The ability to generate >250 uniquely fluorescently labeled viable and functioning cells reported here, relies on the properties of CFSE-like vital dyes. Several of these dyes, such as CTV and CPD, have only recently become available. These dyes have the characteristics of being capable of labeling cells with a high fluorescence intensity, that is long lived and of low variance[3]. These properties coupled with the fact that there is a linear relationship (particularly in the case of CFSE and CTV) between the concentration of the dye used for labeling and the fluorescence intensity of the labeled cells, means that cells can be labeled with several (up to 7 shown here) intensities of each dye that can be easily distinguished by flow cytometry. Furthermore, since the fluorescence emission of CFSE, CPD and CTV have minimal spectral overlap, they can be used in combination to label cells, thus allowing up to >250 fluorescent cell signatures to be detected by flow cytometry. It should be noted that in order to achieve labeling of cells with this number of discernable fluorescent signatures, it is important that cell labeling is performed rapidly[10,11] (to generate low fluorescence variance) and in an appropriate buffer containing free amines[3] (to buffer dye toxicity which can otherwise occur with these dyes at high concentrations). It is also critical that during acquisition of FTAs by flow cytometry that erroneous fluctuations in event fluorescence are monitored (for example by measuring CFSE, CTV and/or CPD fluorescence intensity over time). This is

important because such fluctuations in event fluorescence that are due to machine introduced errors may result in misinterpretation of correct target cell cluster positioning that in turn may result in errors in the final measures of T cell effector function. Such flow cytometer introduced errors can be limited by ensuring high quality samples are prepared (particularly paying attention to filtering cells through a 70 µm mesh to minimize occlusion of fluidic lines in the flow cytometer by large cell aggregates) and that the flow cytometer is maintained to a high standard.

Measurement of T cell responses *in vivo* has been performed for many years using vital dyes such as CFSE to monitor target cells death *in vivo*[12]. Typically these assays, however, have been only capable of monitoring up to 7-8 different target cells at once[13] and so provide a limited capacity to assess killing of targets expressing multiple concentrations of multiple epitopes that are normally required to generate a detailed assessment of parameters such as the functional avidity of T cell responses. In this respect, tetramer dissociation assays can be used to provide a measure of T cell avidity from freshly isolated effector T cells[14-16]. This technique, however, measures MHC/TCR binding avidity, and so is not a complete reflection of functional avidity, which can also depend on changes in the structure of the immunological synapse and the state of T cell signaling components[17]. Therefore, ideally, functional avidity requires dose-response experiments to be performed. This has been accomplished previously using *in vitro* techniques such as the[51] Cr-release assay, the intracellular cytokine staining assay[18,19] or the ELISPOT assay[20,21]. These techniques, however, often require the effector T cells to be stimulated *in vitro*, which can alter the overall functional avidity of the population[22]. The FTA assays, therefore, offers an improvement over existing techniques, measuring $CD4^+$ T cell and $CD8^+$ T cell dose-responses *in situ*, in real time against multiple epitopes simultaneously, and so providing a valuable addition to existing techniques to measure T cell effector function. We anticipate that the use of FTA technology may become valuable in screening immunotherapy strategies designed to generate high quality T cell responses, such as vaccines directed against HIV-1 and hepatitis C.

Disclosures

The authors declare that they have no competing financial interests.

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