Macpherson and McCoy laboratories
2013

Protocol 601_FACS staining of live bacteria

Reagents

Sterile (0.2µm filtered) PBS
Sterile PBS/ 2% BSA (+Azide 0.02%, sterile filter after adding BSA!)
Bacteria
Serum
FITC/PE/PerCP/APC-anti-isotypes
BD FACS-staining 96 well plates
FACS library tubes and racks
2% PFA/PBS – sterile filtered

Protocol

1. Make a 3ml overnight culture of relevant bacteria from single colony, (without shaking gives better antibody targets!)
2. Recover the bacteria and spin for 2mins at 7000rpm in eppendorf minifuge to pellet
3. Wash twice in sterile-filtered PBS/2% BSA/AZIDE
4. Resuspend in 1ml sterile-filtered PBS/2% BSA/AZIDE, measure OD and dilute to approx 10^7 per ml in sterile-filtered PBS/2% BSA/AZIDE. Either measure conventionally using a spectrophotometer, or follow the protocol below for the ELISA reader (useful if you have many strains!).
    a. to use ELISA-reader, aliquot 100µl buffer per well of U-bottom 96 well plate (about 6 wells per bacterial strain analysed). Serially dilute 100µl of bacterial suspension across the plate (i.e. 2-fold serial dilutions). Avoid blowing bubbles as this affects the OD dramatically!
    b. Read A570 on ELISA plate reader (no blanks, no standards). The dilution which give an OD of approx 0.1 corresponds to approx 10^8 bacteria per ml. Dilute 10-fold from this. EG if the 3rd serial dilution gives a reading of 0.098, this is your original suspension diluted 2^3=8 fold. Therefore you need to dilute 1:80 to reach approx 10^7/ml
    c. The plate method is obviously unconventional and not super-accurate, but it is accurate enough, and over hundreds of assays has been found sufficient to calculate the optimal dilution for most bacteria tested.

5. Aliquot 25µl per well of your final bacterial suspension into a BD FACS plate (or any other V-bottom 96 well plate than survives centrifugation at 4000rpm in large centrifuges) to give approx 2.5x10^5 bacteria per well
6. In a separate plate, make 3-fold dilutions of serum starting at 1:10, or intestinal washes, neat. details:
    a. Aliquot sufficient serum or intestinal wash into an eppendorf tube and heat to 56ºC for 30mins to inactive complement (pre-dilute serum 1:10 if very small volume!)
    b. Centrifuge serum/intestinal wash samples at 13000rpm in minifuge for 5mins to sediment any bacterial-sized contaminants that may produce artefacts in the FACS
    c. Make up 3-fold serial dilutions of serum starting at 1:10 or of intestinal wash starting at neat in sterile PBS/BSA
7. Add 25µl serum/wash serial dilutions to plated bugs

   a. NB: If you are experienced with this, you can make up the serial dilutions in the V-bottom plate and add the bacteria 2nd, to waste a bit less plastic!
8. Incubate on ice/at 4ºC for 30mins (up to 2hrs).
9. Wash pelleted bacteria twice with 200µl PBS/BSA per well, spinning for 10mins at 4000rpm (large eppendorf centrifuge with plate spinners) between each wash (NB – no need to resuspend bacteria during washes)
10. Add 25µl PBS/BSA containing relevant secondary reagent to each well

    a. FACS/Immunofluorescence (all BD Pharmingen antibodies)
        i. FITC-anti-IgG2a 1:100
        ii. FITC-anti-IgG3 1:100
        iii. FITC-anti-IgG1 1:100
        iv. FITC-anti-IgG2b 1:100
        v. FITC-anti-IgA 1:50
        vi. APC-anti-IgM 1:100
        vii. PerCP-anti-IgG2a+b 1:25 (very dilute antibody!)
        viii. PE-anti-IgG1 1:100

11. Resuspend bacteria by pipetting
12. Incubate in secondary reagent for 30mins at 4ºC

13. Wash once in PBS/BSA 300µl

14. resuspend cells 2%PFA/PBS (also sterile-filtered) for the FACSCalibur, or just add 200µl without resuspension for the FACSArray (use multichannel!)

15. Set up the FACSArray in "bacterial FACS mode". Optimal settings are:

    a. Thresholds: FSc -200 AND SSc-200
    b. Parameters:
        i. FSC – 300
        ii. SSc – 350
        iii. Green – 500
        iv. Yellow – 500
        v. NIR – not using so leave at 80
        vi. Red – 350

Bacteria are FSC-W-low

After you ran the experiment, please export the FCS 2.0 file. The FCS 3.0 file will give very high geometric mean.

NOTES
In principal this should work with any bacterial strain

Using low numbers of bacteria dramatically increases the sensitivity of the assay. 10^5 per well is optimal in my hands to produce a positive signal whilst still being

able to distinguish bacteria from dust and bubbles in the FACS. The FSC-W parameter is a better measure of size than FSC-A for bacteria in digital cytometers.

Heat-inactivating the serum makes a difference to agglutination of bacteria by serum (visible as increased Forward and sideward scatter in the FACS). You will still see small changes in Fsc/Ssc profiles of the bacteria with large amounts of specific IgM even with HI serum, but otherwise changes should be tiny/absent.

Using APC and FITC on the FACS avoids the need to compensate between colours. Compensation controls can however be produced by staining bacteria with positive control serum then mixing unstained and stained bacteria, aliquoting into separate wells and staining each with a separate secondary reagent. This normally yields a clear positive and negative population within each control to allow set-up

IMPORTANT: THIS TECHNIQUE USES A LOT OF PIPETTE TIPS. DO NOT USE THE ONES SOMEBODY ELSE HAS SPENT HOURS RACKING – DO THIS YOURSELF!

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IgA surface staining of E cloacae using intestinal washes from mice serially gavaged PBS or E cloacae

cloacae