Quantum Dot Labelling of Adenovirus Allows Highly Sensitive Single Cell Flow and Imaging Cytometry

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Supporting Information

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Experimental Section

Viruses and Biotinylation

AdGFP (ΔE1/E3 CMV_{IE}GFP) is a replication defective Ad5 vector, encoding eGFP under control of the CMV immediate early promoter. Wildtype (WT) Ad11 was a kind gift from Professor Gavin Wilkinson (University of Cardiff). AdpIX-GFP was a kind gift from Dr Robin Parks (Ottawa Health Research Institute) and expresses a pIX-GFP fusion protein for fluorescent tracking. AdDM-1/E2F and AdDM-1/E2F-RGD are Ad5 based gene therapy vectors with the E1A promoter replaced by an E2F promoter/Kozak sequence downstream of a SV40 late poly(A) signal and a DM-1 insulator element (generated using plasmids kindly donated by Dr Alemany). Both viruses have a splice acceptor-linked eGFP gene inserted downstream of the protein IV (fiber) gene. AdDM-1/E2F-RGD contains a cyclic RGD sequence inserted into the HI loop of the fibre gene. AdGFP and AdpIX-GFP were grown in HEK293 cells whilst Ad5, Ad11, AdDM-1/E2F and AdDM-1E2F-RGD were grown in A549 cells, all grown in Dulbecco’s Modified Eagle Medium (DMEM) supplemented with 2% foetal calf serum (FCS) and GlutaMAX-1 (Invitrogen). All viruses were CsCl banded\textsuperscript{56}, titered by Picogreen assay for particle number and plaque assayed for infectivity before and after biotinylation.
For biotinylation, viruses were incubated with 10 μg/ml NHS-Biotin (Sigma, Pool, UK) for 4 hours at 20°C, unless otherwise stated. Excess NHS-Biotin was removed by dialysis against storage buffer (PBS, 10% glycerol (v/v), 500 μM MgCl₂, 900 μM CaCl₂) for 16 hours at 4°C.

**Cell Preparation and Qdot Labelling**

A549, HEK293 and SKOV3 cells were obtained from the ATCC (Manassas, VA, USA). A9 and A9-CAR cells were kindly donated by Dr Paul Freimuth (Brookhaven National Laboratory). SKOV-CAR cells were generated by transfection with the pXLNC-hCAR retrovirus as has been described previously. All cells were grown in DMEM supplemented with 10% foetal calf serum and GlutaMAX-1 (Invitrogen).

For virus Qdot labelling, cells were trypsinised, trypsin neutralised by addition of excess full growth media, washed (PBS supplemented with 5% FCS) before the cell suspension was incubated with excess streptavidin, washed and incubated with excess biotin (Endogenous Biotin Blocking kit, Invitrogen, Paisley, UK). Cells were washed and each sample resuspended in 50μl PBS supplemented with 5% FCS. Virus was bound to cells on ice for 90 minutes, washed, re-suspended in 50 μl PBS + 5% FCS containing 1 μl of Qdot655-Streptavidin conjugate (Invitrogen). Cells were incubated for 30 minutes on ice, washed and either analysed directly or co-stained with antibodies.

**Confocal Microscopy**

For confocal microscopy cells were grown on glass cover slips and prepared as above. Cells were grown on cover slips (Fisher Scientific, Loughborough, UK) and prepared as above but without cell detachment by trypsinisation. Cells were stained with 5 μg anti-CAR mAb RmCb (Cancer Research UK, UK) (20 minutes at 4°C), visualised with secondary anti-mouse Qdot525 (Invitrogen) and counter-stained with 0.5 μg/ml DAPI (Sigma) (10 minutes
at 4°C). Cells were mounted in Clarion Mounting Media (Sigma) and visualised using a Zeiss LSM510 meta confocal microscope.

**Flow Cytometry**

Where indicated cells in suspension were co-stained with 5 μg anti-CAR mAb RmCb (20 minutes at 4°C) and detected using anti-mouse FITC secondary antibody (Invitrogen), following manufacturer’s instructions. Data was collected using a LSR II flow cytometer (BD Bioscience, San Jose, CA, USA) equipped using a 488nm laser 530/30nm band-pass filter (for FITC and GFP) and a 405nm laser 655/8nm band-pass filter (for Qdot655). Data was analysed using FlowJo software (Tree Star, Ashland, OR, USA). Statistical analysis was performed using Graph Pad Prism 6, using paired T Test and significance indicated as *P<0.05 , **P<0.005, ***P<0.0005.

**Imaging Cytometry**

Cells were suspended at 10^6/ml in PBS + 5% FCS and data acquired using an Image Stream 100 (Amnis, Seattle, US) with excitation at 488 nm (30 mW) and 405 nm (350 mW). Analysis was performed on single cell in focus events, identified by brightfield aspect ratio/area analysis and brightfield gradient RMS. To measure internalisation of Qdot labelled virus, default total cell masks were used to calculate total cell fluorescence and the area erode tool (brightfield channel) used to identify cell interiors. Internalisation index (defined by the percentage of interior cell fluorescence:total cell fluorescence) was calculated and expressed as percentage of maximum internalisation. Data analysis was performed with Ideas Software (Amnis, Seattle, WA, USA).