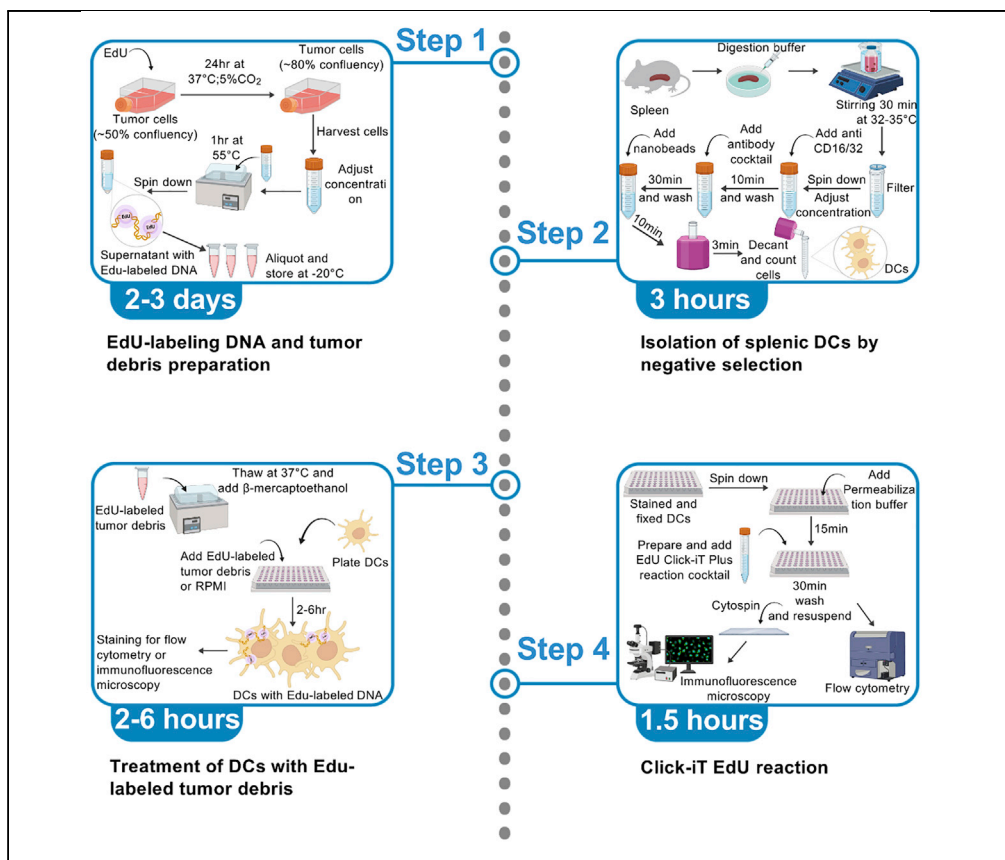


Protocol

Detection of exogenous DNA uptake by murine dendritic cells



This protocol has been developed to measure exogenous DNA uptake by murine dendritic cells (DCs) using supernatant containing cellular debris, which allows for DNA uptake in the absence of transfection reagents. Inhibitors or antibodies that alter the process can be added, and either flow cytometry or fluorescent microscopy can be used to measure DNA uptake. This is intended to mimic the exposure of DCs to dying cells in the tumor microenvironment or other pathological conditions of high cellular death.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

In vitro assay to measure exogenous DNA uptake by primary murine dendritic cells

Designed to mimic conditions of high cell death such as those within tumors

DNA uptake occurs spontaneously without the use of transfection reagents

Useful for studying DNA uptake and searching for activators or inhibitors

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101464
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<https://doi.org/10.1016/j.xpro.2022.101464>



Protocol

Detection of exogenous DNA uptake by murine dendritic cells

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<https://doi.org/10.1016/j.xpro.2022.101464>

SUMMARY

This protocol has been developed to measure exogenous DNA uptake by murine dendritic cells (DCs) using supernatant containing cellular debris, which allows for DNA uptake in the absence of transfection reagents. Inhibitors or antibodies that alter the process can be added, and either flow cytometry or fluorescent microscopy can be used to measure DNA uptake. This is intended to mimic the exposure of DCs to dying cells in the tumor microenvironment or other pathological conditions of high cellular death.

For complete details on the use and execution of this protocol, please refer to de Mingo Pulido et al. (2021).

BEFORE YOU BEGIN

Institutional permissions

Animal studies were performed in accordance with the Institutional Animal Care and Use Committee (IACUC) at the University of South Florida. Users will require approval from their institution for the breeding of animals and the harvesting of tissues post mortem.

EdU-labeling DNA and cell debris preparation

⌚ Timing: 2–3 days

This portion of the protocol will generate supernatant containing EdU-labeled DNA for tracking uptake by DCs in the subsequent steps. Using unpurified DNA allows for uptake to occur without a transfection step as a result of DNA-binding proteins such as HMGB1, thereby better mimicking the *in vivo* process. The EdU labeling portion of the procedure allows for fluorescent staining to be performed in fixed cells at the end of the protocol.

1. Plate tumor cells in 75 cm² flask in 20 mL of complete medium (DMEM supplemented with 10% fetal bovine serum and 1% Penicillin/Streptomycin).

Note: This portion of the protocol was optimized using PyMT-B6 murine mammary cancer cells, and has been validated with MDA-MB-231 human breast cancer cells. Other cell lines are expected to produce similar results, but different doubling times may require changes to seeding density, etc.



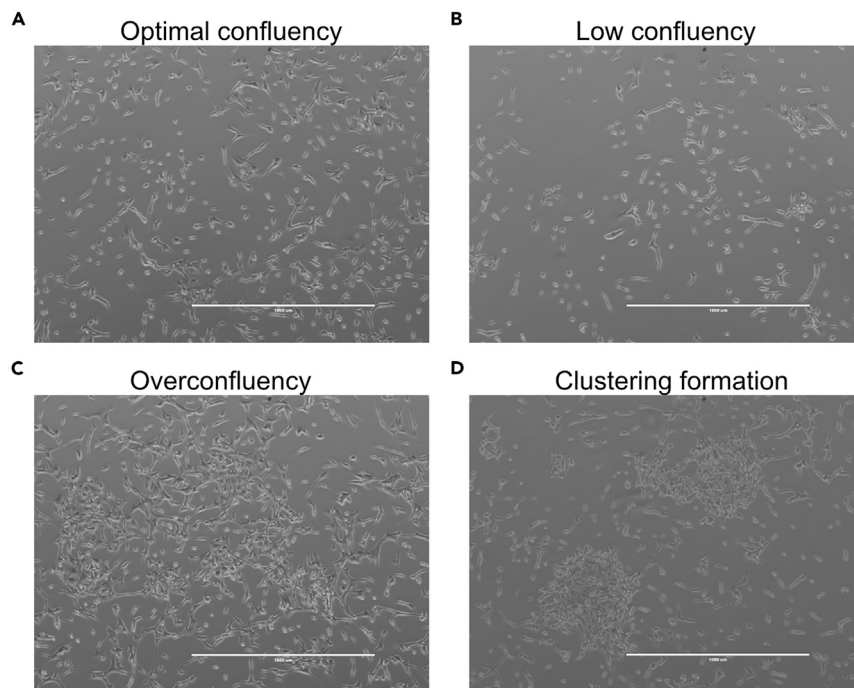


Figure 1. Confluency of PyMT-B6 to prepare tumor debris

An optimal confluency of pymt cells is necessary to achieve an efficient incorporation of EdU and therefore, an adequate level of EdU-labeled tumor DNA (A).

A low confluency may translate into an insufficient concentration of EdU-labeled DNA for later use in cultures of DCs (B).

An overconfluency prior to the addition of EdU, may reduce the rate of cell proliferation, decreasing the incorporation of EdU (C).

Cluster formation could also affect proliferation and consequently the incorporation of EdU into cells. This may reflect limited trypsin digestion when the cells were originally plated (D).

Scale bar: 1000 μm .

Note: With 1 million starting PyMT-B6 tumor cells, confluency will be reached in approximately 2 days. However, this is cell line specific and needs to be optimized by the user.

- Once the cells reach 50% confluency (Figure 1) add EdU stock solution (10 mM; obtained from Click-iT™ Plus Flow Cytometry Assay Kits) for a final concentration of 10 μM and incubate for 24 h at 37°C and 5% CO_2 .

Note: EdU is used here in place of BrdU as it allows for a chemical reaction to be used for labeling. This avoids the DNA denaturing step normally required to detect BrdU with an antibody, and increases the versatility of the protocol.

Note: When adding EdU, it is recommended to take 10 mL old media from the flask and add 10 mL of fresh media containing 20 μM EdU to increase cell viability.

Note: Check cells before the 24 h time point. Confluency should not exceed 80% at the time of harvest. If high confluency or cell death is observed, proceed with the harvest early to avoid a reduction in yield. However, a minimum of 16 h of EdU incubation time is recommended.

- Wash the cells with 1 \times PBS and add 5 mL of trypsin to the cells. Incubate for 5 min and check if cells are detached.

- Add 5 mL of complete DMEM to the flask to neutralize trypsin and harvest the cells into a 15 mL tube. Spin down at 400 g for 5 min at 4°C.
- Resuspend the cells in 5 mL of RPMI (without FBS) and count the cells to adjust to a final concentration of 2×10^6 cells/mL in a 15 mL tube.

Note: Lower volumes of RPMI can be used to generate a concentrated version that can increase the amount of DNA uptake observed. The concentrated version can also be diluted after thawing so the user can optimize the assay.

- Incubate cells in a water bath at 55°C for one hour, with agitation every 15 min.
- Spin down at 300 g for 10 min at 4°C and dispense the supernatant into 1 mL aliquots.

▣▣ Pause point: Store at –20°C until use. For measuring DNA uptake these aliquots are stable for 3 months.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Anti-mouse CD8 alpha clone 53.6-7 BUV800 (1/800)	BD	564920 RRID: AB_2716856
Anti-mouse MHCII M5/114.15.2 BV421 (1/800)	BD	562564 RRID: AB_2716857
Anti-mouse CD11c clone N418 BV605 (1/400)	BioLegend	117334 RRID: AB_2562415
Anti-mouse/human CD11b M1/70 BV711 (1/400)	BD	563168 RRID: AB_2716860
Anti-mouse CD45 30-F11 BV785 (1/800)	BD	564225 RRID: AB_2716861
Anti-mouse CD103 clone 2E7 PE-Cy7 (1/200)	BioLegend	121426 RRID: AB_2563691
TruStain FcX™ PLUS (anti-mouse CD16/32) (1/2000)	BioLegend	156603 RRID: AB_2783137
Anti-mouse CD3 epsilon clone 145-2C11 biotin (1/25)	BioLegend	100304 RRID: AB_312669
Anti-mouse/human B220 clone RA3-6B2 biotin (1/25)	BioLegend	103204 RRID: AB_312989
Anti-mouse Ly6G clone 1A8 biotin (1/33)	BioLegend	127604 RRID: AB_1186108
Anti-mouse CD49b clone DX5 biotin (1/50)	BioLegend	108904 RRID: AB_313411
Anti-mouse Ter119 clone TER-119 biotin (1/50)	BioLegend	116204 RRID: AB_313705
Anti-mouse Ly6C clone HK1.4 biotin (1/100)	BioLegend	128004 RRID: AB_1236553
Anti-mouse GAPDH Polyclonal Antibody (1/200)	Thermo Fisher Scientific	PA1-16777 RRID: AB_568552
Donkey anti-Rabbit IgG (H+L) Highly Cross-Adsorbed Secondary Antibody, Alexa Fluor 488 (1/200)	Thermo Fisher Scientific	A-21206 RRID: AB_2535792
Chemicals, peptides, and recombinant proteins		
Zombie NIR Fixable Viability Kit	BioLegend	423105
Collagenase A	Sigma-Aldrich	11088793001
DNase I	Roche	10104159001
DMEM, high glucose	Thermo Fisher Scientific	11965118
Penicillin/Streptomycin	Thermo Fisher Scientific	15140-122
RPMI 1640 Medium, HEPES	Thermo Fisher Scientific	22400105
DPBS	Thermo Fisher Scientific	14190144

(Continued on next page)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
HBSS, no calcium, no magnesium, no phenol red	Thermo Fisher Scientific	14175103
BD Cytotfix	BD Biosciences	554655
Fetal Bovine Serum United States Heat inactivated	Peak Serum	PS-FB1
TrypLE Express Enzyme (1 x), No Phenol Red	Thermo Fisher Scientific	12-604-021
2-Mercaptoethanol (55 mM)	Thermo Fisher Scientific	21985023
Hoechst 33342 Solution	Thermo Fisher Scientific	62249
Triton X-100	Thermo Fisher Scientific	BP151-500
Copper (II) sulfate pentahydrate	Sigma-Aldrich	31293
Sulfo-Cyanine 5 azide	Lumiprobe	B3330
Ethylenediaminetetraacetic Acid (0.5 M Solution/pH 8.0)	Fisher BioReagents	BP2482100
Bovine Serum Albumin, Heat Shock Treated	Fisher BioReagents	BP1600-100
Critical commercial assays		
Click-iT™ Plus EdU Alexa Fluor™ 594 Flow Cytometry Assay Kit	Thermo Fisher Scientific	C10646
Experimental models: Cell lines		
PyMT-B6 (any passage number)	David G. DeNardo, Washington University	Meyer et al. Nat. Commun. 2018 PMID: 29593283
Experimental models: Organisms/strains		
Mouse: C57BL/6J (M/F, 8–16 weeks of age)	The Jackson Laboratory	JAX: 000664
Software and algorithms		
FlowJo	FlowJo LLC	https://www.flowjo.com/
Prism	GraphPad	https://www.graphpad.com/scientific-software/prism/
Other		
MojoSort Streptavidin Nanobeads	BioLegend	480016
EasySep™ Magnet	STEMCELL	18000
Corning™ Costar™ 96-Well, Cell Culture-Treated, U-Shaped-Bottom Microplate	Fisher Scientific	0720095
T75 Cell Culture Vented Flask	Sarstedt	83.3911.002
StatSpin Technologies CytoFuge	StatSpin Technologies	ML9225
BD LSR II™ flow cytometer	BD Biosciences	N/A

MATERIALS AND EQUIPMENT

FACS Buffer

Reagent	Final concentration	Amount
PBS	n/a	494 mL
BSA (100 mg/mL)	1%	5 mL
EDTA (1 M)	2 mM	1 mL
Total	n/a	500 mL

Note: Store at 4°C for up to 1 year.

STEP-BY-STEP METHOD DETAILS

Isolation of splenic DCs by negative selection

⌚ **Timing:** 3 h

This step enriches the presence of DCs to minimize interference from other immune cell subsets present in the spleen. Despite negative selection producing less purity than positive selection, we have observed that negative selection results in surface marker expression more consistent with untouched DCs analyzed directly from the spleen. Note that a commercial splenic DC purification

kit can also be used here. DNA uptake assays can also be performed with bone marrow-derived DCs (BMDCs, see below).

1. Harvest 2 mouse spleens in RPMI and keep on ice.
2. Inject 50 μ L of digestion buffer (1 mg/mL Collagenase A and 50 U/mL DNase I in RPMI into each side of the spleen and incubate for 1 min at room temperature (20°C–25°C).
3. Mince the spleens and digest with 10 mL of digestion buffer for 30 min at 32°C–35°C with gentle stirring using a magnet.

Note: Add EDTA for a final concentration of 1 mM during the last 5 min of digestion in step 3 to minimize cell clumping.

4. Filter through a 70 μ m cell strainer. Spin down cells at 300 g for 10 min at 4°C.

Note: Wash the cell strainer with 5 mL of HBSS in the 50 mL tube before filtering the cells to reduce the impact of cells landing in the bottom of the tube.

5. Resuspend cells in 1 mL FACS buffer. Count cells and adjust to 1×10^8 cells/mL in FACS buffer.
6. Add TruStain FcX™ PLUS (i.e., anti-CD16/CD32) at 1:2000 dilution and incubate 10 min on ice.
7. Prepare the mixture of biotin conjugated antibodies (10 μ L Ly6C, 20 μ L Ter119, 20 μ L CD49b, 30 μ L Ly6G and 40 μ L of CD3e and B220 in 1000 μ L total volume of FACS buffer).

Note: The total volume of antibody needs to match that in step 5 to maintain the cell concentration for step 8 (i.e., if there are 1.5×10^8 cells in 1.5 mL in step 5, then scale up to prepare 1.5 mL of total antibody mixture).

8. Spin down cells at 300 g for 10 min at 4°C. Add biotin antibody mixture to splenocytes. Mix well. Incubate 20 min on ice.
9. Spin down at 300 g for 10 min at 4°C. Wash 1 \times with 2 mL cold FACS buffer.
10. Vortex the MojoSort Nanoparticles. Prepare a working dilution by adding 7.5 μ L–992.5 μ L FACS buffer. Scale appropriately so that the final cell concentration matches that in step 5.
11. Add pre-mixed MojoSort beads to splenocytes. Mix well. Incubate 10 min on ice.
12. Add FACS buffer to reach 3 mL total volume. Transfer cells to a 5 mL polystyrene tube and place in a magnet designed to hold the tube for 3 min.

Note: Either the BioLegend MojoSort (#480019) or the STEMCELL EasySep Magnet (#18000) can be used during this step.

13. Decant supernatant containing dendritic cells. Count cells and resuspend at 10^6 /mL in RPMI supplemented with 1% fetal bovine serum. Proceed to plate cells as in step 15.

Note: Do not invert the tube more than once in order to maximize purity of the DCs.

Note: Approximately 1×10^6 cells will be recovered from 10^8 total splenocytes, with 40%–60% purity for CD11c⁺MHCII⁺ DCs. If higher purity is required then fluorescence activated cell sorting is recommended.

Treatment of DCs with EdU-labeled tumor debris

⌚ Timing: 2 h for DNA uptake; 6 h for cell activation

This in vitro assay has been designed to track the tumor DNA uptake by DCs. Cells are stimulated with tumor debris to mimic the tumor microenvironment interactions. This protocol can also be

adapted for use with BMDCs: (i) Flt3L-FC BMDCs differentiated following Brasel et al. (Brasel et al., 2000) or (ii) CD103+ BMDCs differentiated following Mayer et al. (Mayer et al., 2014).

14. Quickly thaw frozen EdU-labeled tumor debris at 37°C. Once thawed, add β -mercaptoethanol to 55 μ M final concentration.
15. Plate DCs from step 13 in 96-well round-bottom plate. Recommend using between 1×10^5 to 2×10^5 cells per well. Spin down at 300 g for 10 min at 4°C.
16. Remove supernatant and resuspend in 100 μ L of EdU-labeled tumor debris (prepared in step 14).

Note: It is suggested to use RPMI supplemented with β -mercaptoethanol (55 μ M) as a staining control. Alternatively tumor cell debris could be generated in the absence of EdU.

17. Culture at 37°C and 5% CO₂ for up to 2 h before proceeding to the next step. Alternatively, cells can be incubated for 4–6 h to evaluate DC activation. Incubation over 6 h is not recommended with splenic DCs due to reductions in cell viability. For intracellular cytokine detection, monensin and brefeldin A should be added during the last 4 h of culture.

Note: Incubation times can vary, since DNA uptake has been observed as early as 15 min and splenic DC activation as early as 2 h. BMDCs may require longer incubation times if interested in measuring activation (up to 24 h).

Note: Tumor cell debris can be supplemented with 1% FBS for incubation times longer than 2 h to improve cell viability. Higher levels of FBS can reduce the amount of activation observed.

Optional: Antibodies or other reagents can be added to the tumor cell debris in step 14 to evaluate their ability to regulate DNA uptake or DC activation. For example, DNA uptake in splenic DCs and CD103⁺ BMDCs is increased by the addition of anti-TIM-3 (clone RMT3-23) and reduced by the addition of anti-HMGB1 (clone 3E8) (de Mingo Pulido et al., 2021).

Surface staining for flow cytometry

⌚ **Timing:** 2 h

This step will label the cells with the specific flow cytometry antibodies necessary to identify the cells of interest.

18. Spin down plate at 300 g for 10 min at 4°C and wash once with 200 μ L cold FACS buffer.
19. Create a mixture of Live/Dead Zombie NIR dye (1:1000 dilution) and TruStain FcX (1:2000 dilution) in PBS (no protein). Add 200 μ L of the mixture to the cells and incubate for 30 min on ice protected from light.
20. Prepare the surface antibody mixture (200 μ L per sample) in cold FACS buffer during the incubation period in step 19. See the gating strategy used in Figure 2.

Note: Suggested markers are listed below. See the resource table for recommended antibodies and dilution factors.

- a. Splenic DCs: CD45⁺, CD11c⁺, MHCII⁺ and CD11b⁺ (cDC2) or CD8a⁺ (cDC1).
- b. CD103⁺ BMDCs: CD45⁺, CD11c⁺, MHCII⁺, CD11b⁺, CD103⁺
- c. Flt3L-FC BMDCs: CD45⁺, CD11c⁺, MHCII⁺, CD11b⁺

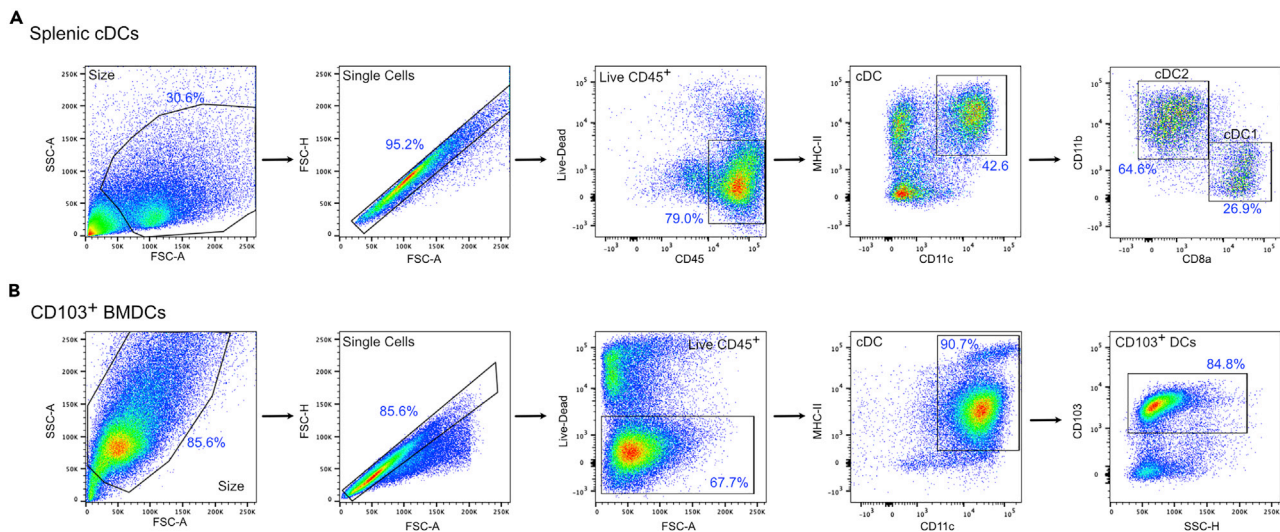


Figure 2. Gating strategies for DCs

(A and B) A representative gating strategy illustrating the purity of splenic DCs (A) and BMDCs generated with FLT-3L and GM-CSF (i.e., iCD103⁺ BMDCs) (B).

21. After step 19 is complete, spin down at 300 g for 10 min and wash with 200 μ L ice cold FACS buffer.
22. Add 200 μ L of antibody mixture to cells and resuspend gently by pipetting up and down. Then, incubate for 30 min on ice protected from light.
23. Spin down at 300 g for 10 min and wash with 200 μ L ice cold FACS buffer.
24. Resuspend the pellet gently in 100 μ L BD Cytofix Fixation Buffer and incubate 15 min on ice protected from light. If doing the Click-iT reaction, it is recommended to use the Click-iT fixative included in the kit, otherwise BD Cytofix Fixation Buffer can be used.
25. Spin down at 500 g for 5 min and discard supernatant.
26. Store cells in FACS buffer or proceed to Click-iT EdU reaction.

Pause point: After fixation, samples can be stored at 4°C and protected from light in order to continue staining the next day for the Click-iT EdU reaction or intracellular proteins. If interested in evaluating cytokine expression, follow the standard intracellular staining protocol for flow cytometry. Using the same sample to detect DNA uptake and cytokine production is not recommended.

Immunofluorescent staining for confocal microscopy

⌚ Timing: 3 h

As an alternative to flow cytometry, BMDCs can be analyzed by immunofluorescence microscopy. Using specific cytoplasmic markers (such as GAPDH) or nuclear markers (such as Hoechst 33342), the internalized DNA and its subcellular location can be determined.

27. Harvest BMDCs and distribute 10^6 per well of a 96-well plate.
28. Spin down plate at 300 g for 5 min at 4°C and wash once with 200 μ L cold FACS buffer.
29. Fix cells for 10–15 min at room temperature using 200 μ L BD Cytofix or 4% paraformaldehyde in 1 \times PBS.
30. Permeabilize and block cells for 10 min at room temperature using 200 μ L of perm/block buffer (0.2% Triton X-100, 2% BSA, in 1 \times PBS).

31. Spin down plate at 300 g for 5 min at 4°C and wash once with 200 µL FACS buffer.
32. Primary antibody incubation – incubate with 200 µL of a solution of anti-mouse GAPDH in FACS buffer (1:200) for 1 h at room temperature.

▮▮ Pause point: Samples can be stored at 4°C any point after fixation in order to continue staining the next day.

33. Spin down plate at 300 g for 5 min at 4°C and wash twice with 200 µL FACS buffer.
34. Secondary antibody incubation: incubate with 200 µL of AF488 anti-rabbit secondary antibody in FACS buffer (1:200) for 1 h at room temperature.
35. Spin down plate at 300 g for 5 min at 4°C and wash twice with 200 µL FACS buffer.
36. Nuclear staining: incubate with 200 µL of Hoechst 33342 in FACS buffer (1:1000) for 10 min.
37. Proceed to Click-iT EdU reaction.

Click-iT EdU reaction

⌚ **Timing:** 1.5 h

This step labels the DNA with the fluorophore of choice using the Click-iT reaction. The Click-iT EdU reaction provides a more sensitive and robust assay to detect the uptake of exogenous DNA by DCs (as compared to BrdU labeling), as it does not require denaturation of the DNA.

38. Using the splenic DCs (from step 26) or BMDCs (from step 37), spin down the plate at 300 g for 10 min at room temperature and remove the supernatant.
39. Add 40 µL Click-iT-Saponin based permeabilization buffer and resuspend the DCs. Incubate for 15 min at room temperature protected from light. This buffer needs to be prepared from 10× stock solution by diluting in deionized water.
40. Prepare the EdU Click-iT Plus reaction cocktail as follows:
 - a. Prepare 1× Click-iT EdU reaction buffer additive by diluting the 10× stock solution in deionized water. Volume required will depend on the number of reactions. Refer to the recipe below ([as per the manufacturer's directions](#)).
 - b. Prepare Click-iT Plus reaction cocktail in the following order (200 µL per sample/well will be used).
 - i. PBS or DPBS 175 µL.
 - ii. Copper protectant 4 µL.
 - iii. Fluorescent dye 1 µL.
 - iv. Reaction buffer additive (1×) 20 µL.

Note: Use Click-iT Plus reaction cocktail within 15 min.

Note: Copper protectant can be replaced by 400 mM CuSO₄ solution in deionized water.

Note: Fluorescent dye can be replaced by 4 mM Sulfo-Cy5-Azide in deionized water.

41. Add 200 µL Click-iT Plus reaction cocktail per sample directly onto the 40 µL Click-iT-Saponin and gently pipette up and down 2–3 times for mixing. Incubate for 30 min at room temperature, protected from light.
42. Spin down plate at 500 g for 5 min and wash with 1× Click-iT-Saponin based permeabilization buffer.
43. Spin down and repeat washing step with Click-iT-Saponin based permeabilization buffer.
44. Resuspend in FACS buffer and analyze by flow or image cytometry. For immunofluorescent microscopy, cells can be immobilized on glass slides by cytospin centrifugation (1000 g for 10 min) and can be stored under coverslips using Prolong Gold Mountant (up to 7 days at 4°C).

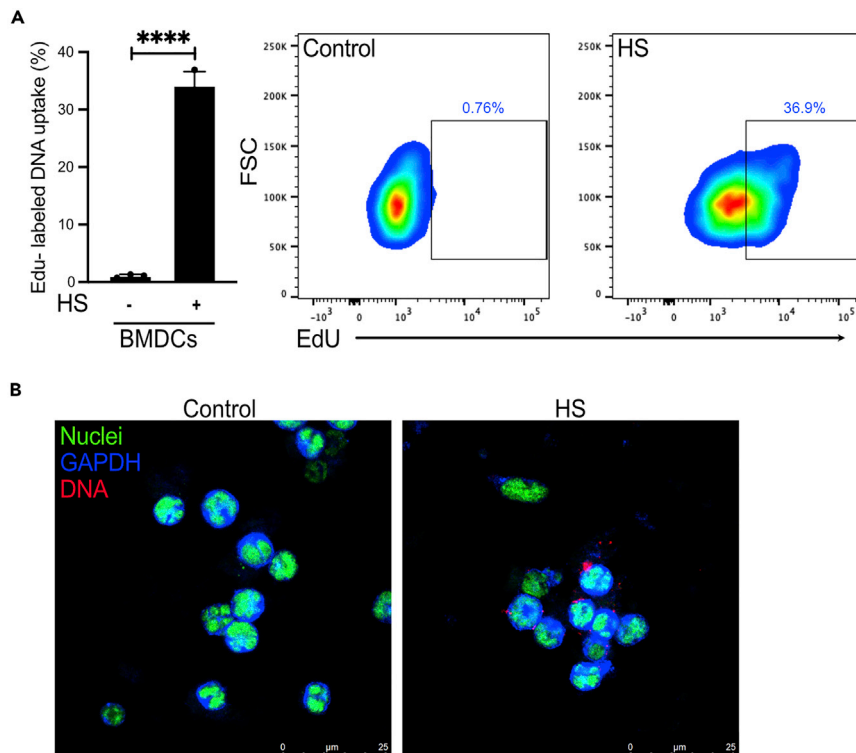


Figure 3. DNA uptake by DCs analyzed by flow cytometry and confocal microscopy

(A) Intracellular flow cytometric detection of tumor DNA (EdU-labeled) within FLT-3L BMDCs after a 2 h incubation with tumor debris generated by heat shock (HS). Data shown as mean \pm SD, with **** $p < 0.0001$ determined by Student's *t* test.

(B) Stacked confocal microscopy images displaying EdU-labeled exogenous DNA (red), nuclei (green) and GAPDH (blue) in FLT-3L BMDCs treated with tumor debris generated by heat shock (HS). Scale bar: 25 μ m.

EXPECTED OUTCOMES

This protocol has been developed to track exogenous DNA uptake by DCs. The use of supernatant from dead cells allows for uptake to occur in the presence of DNA-bound proteins, which in the case of HMGB1, is important for uptake by DCs. DNA-bound proteins may also alter intracellular localization and cellular responses, but these have not been evaluated. Although originally designed to mimic the tumor microenvironment, it is possible that this assay may be applicable for studying other pathological conditions where extracellular DNA accumulates.

Although the use of murine splenic DCs is described here, we have used a similar approach with human peripheral blood DCs isolated by negative selection and incubated with tumor debris from a human breast cancer cell line (de Mingo Pulido et al., *Immunity*, 2021). In addition, the assay has been used to measure extracellular DNA uptake by murine bone marrow-derived macrophages. Thus, the protocol described herein appears to be broadly applicable for use in different species and cells.

The use of EdU labeling for the exogenous DNA allows for the use of the Click-iT reaction, which is compatible with antibody binding and data acquisition techniques, including flow cytometry (Figures 3A and 3B) and fluorescent microscopy (Figure 3C). For flow cytometry, data can be displayed as either the total fluorescence intensity or the percentage of positive cells (Figure 3A). However, since the reaction does can produce background fluorescence, a control sample that does not include EdU needs to be included as a control for selecting a positive gate (Figure 3B). Since flow

cytometry cannot distinguish between extracellular and intracellular staining, image cytometry can be used as an alternative to quantify DNA uptake in different cell populations, such as the spleen. This was reported in our recent publication (de Mingo Pulido et al., *Immunity*, 2021).

Fluorescent confocal microscopy offers an alternative to cytometry techniques and permits the inclusion of additional antibodies to mark subcellular localizations, such as the cytoplasm (Figure 3C). Images can be analyzed for total DNA fluorescence, fluorescence on a single cell basis, or co-localization with intracellular markers. Because the Click-iT reaction requires fixation and permeabilization, the protocol does not permit live imaging of the DNA uptake process. Thus, as endocytosis occurs rapidly, it may require the analysis of a large number of cells to capture DNA in compartments other than the cytoplasm.

LIMITATIONS

The protocol is limited by the lack of other components of the tumor microenvironment, including the stroma and other immune populations. Similarly, BMDCs or splenic DCs may not reflect all aspects of the biology of DCs within the tissues being modeled in the assay. The use of splenic DC negative selection only provides around 40%–60% purity, so it is possible that changes in DC activation are a result of other immune subsets responding to the experimental conditions. Confirmation of direct effects on DCs can be achieved by cell sorting or using BMDCs to achieve over 90% population purity.

The other major limitation is that the protocol directly exposes DCs to DNA and other cellular debris from cancer cells, but the concentration of DNA and form of DNA is unclear. It may include soluble DNA, as well as DNA packaged within cell bodies or exosomes. Therefore, there may be multiple pathways for DNA uptake with different processes engaged depending on the cell type and conditions. Finally, the use of fixed and permeabilized cells for the Click-iT reaction prevents the use of live imaging. For this reason the protocol can be used to measure total DNA uptake by cells, but does not allow for tracking of DNA uptake or for a detailed analysis of the kinetics by which this occurs.

TROUBLESHOOTING

Problem 1

You see poor enrichment for DCs (refers to steps 7 or 13 in ‘[isolation of splenic DCs by negative selection](#)’).

Potential solutions

During negative selection, do not try to recover all of the liquid from the tube in the magnet. Also do not invert the tube more than once.

This protocol has been optimized for C57/BL6 mice, and certain markers are differentially expressed between strains. Specifically, Ly6C was not compatible with the FvBN strain due to higher expression on splenic DCs. In addition to trying to optimize the antibody combination, there are commercial kits available that may provide higher purity.

Problem 2

You do not see changes in activation (refers ‘[isolation of splenic DCs by negative selection](#)’ or ‘[EdU-labeling DNA and cell debris preparation](#)’).

Potential solutions

For negative selection is important to check purity, but also that the molecule of interest is not altered from the purification process. It is recommended to save an aliquot pre-sort and post-sort to check purity and expression.

DCs can be activated through sheer stress. It is recommended that the cells are pipetted carefully, and to not go beyond 300 g during centrifugation of live cells.

FBS can impact the baseline level of DC stimulation. High FBS levels modify DC activation.

The FBS source and batch can alter BMDCs generation. We have observed differences in regards to the cell number, viability and the expression of DC markers (CD11c, MHCII and CD103).

The tumor debris preparation may have expired or not been generated correctly. Create a new batch for testing. Consider creating a more concentration version of the tumor cell debris by using less RPMI during the heat shock process.

Problem 3

You do not see DNA uptake (refers to '[EdU-labeling DNA and cell debris preparation](#)').

Potential solutions

EdU may have been added to tumor cells that were not proliferating robustly, preventing sufficient incorporation. Ensure that your cells are not overgrown (>60% confluency) and are actively cycling.

EdU labeling may differ between tumor debris batches. Incorporation can be confirmed by staining the cell line prior to cell killing. If the problem persists, consider creating a more concentration version of the tumor cell debris by using less RPMI during the heat shock process.

The tumor debris may have become degraded during storage. Storage beyond 3 months is not recommended as a loss of signal has been observed.

Problem 4

Your signal for DNA is too bright (refers to steps 14 or 17 in '[treatment of DCs with EdU-labeled tumor debris](#)').

Potential solutions

There may be residual cell fragments from the tumor cells that are staining brightly for EdU. Repeat the centrifugation step at 500 g for 10 min.

DNA can adhere to the cell surface. To reduce background levels and to ensure that only intracellular DNA is being measured, DNAase (50 U/mL) can be added to the cells during the last 5 min of incubation.

Problem 5

Your negative control is too bright (refers to step 44 in [click-iT EdU reaction](#)).

Potential solutions

The click reaction causes autofluorescence. Your negative staining control should be a sample treated with the click reaction, but that does not contain EdU. Set your flow cytometry settings using this staining control.

The compensation on the flow cytometer may not be optimal. Try using compensation beads (e.g., BD CompBeads) stained with a secondary antibody conjugated to the Click-iT fluorophore used in the protocol (e.g., Alexa Fluor 594).

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Brian Ruffell (brian.ruffell@moffitt.org).

Materials availability

The study did not generate new unique reagents.

Data and code availability

The study did not generate new code.

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AUTHOR CONTRIBUTIONS

Conceptualization, A.D.P., B.R.; Funding acquisition, B.R.; Methodology, A.D.P., D.C.; Supervision, B.R.; Validation, A.D.P., D.C., B.R.; Writing, A.D.P., D.C., B.R.

DECLARATION OF INTERESTS

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