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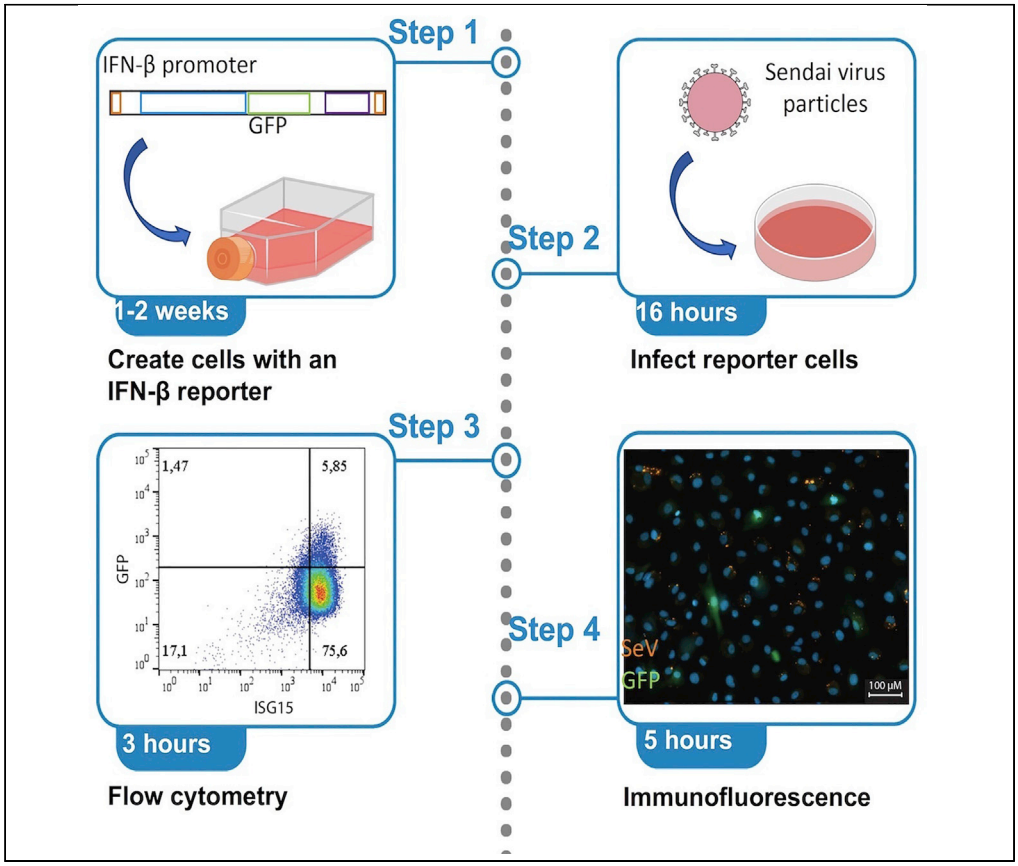
Detecting single cell interferon-beta production using a fluorescent reporter telomerase-immortalized human fibroblast cell line



Recent data suggest that cells respond to infection by upregulating the antiviral cytokine interferon-beta in a fraction of infected cells. Approaches are thus needed to study these responses on a single-cell level rather than bulk population. Here, we describe a protocol to analyze the IFN response of individual cells using flow cytometry and immunofluorescence microscopy. We show the heterogeneous IFN response to inactivated Sendai virus and human cytomegalovirus, but this protocol can be adapted to other viruses.

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Highlights

Single-cell assays are needed to measure IFN production in virus-infected cells

Immortalized THF cells with IFN-b reporter are used to measure IFN production

Individual cells are analyzed via flow cytometry and fluorescence microscopy

Simple single-cell assays can uncover data obscured in bulk population measurements

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Detecting single cell interferon-beta production using a fluorescent reporter telomerase-immortalized human fibroblast cell line

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SUMMARY

Recent data suggest that cells respond to infection by upregulating the antiviral cytokine interferon-beta (IFN-ß) in a fraction of infected cells. Approaches are thus needed to study these responses on a single-cell level rather than bulk population. Here, we describe a protocol to analyze the IFN-ß response of individual cells using flow cytometry and immunofluorescence microscopy. We show the heterogeneous IFN-ß response to inactivated Sendai virus and human cytomegalovirus, but this protocol can be adapted to other viruses.

For complete details on the use and execution of this protocol, please refer to Hare et al. (2020).

BEFORE YOU BEGIN

The protocol below was used to generate fluorescent reporter cell lines and measure production of the antiviral cytokine interferon-beta (IFN-ß) in individual cells by flow cytometry and fluorescence microscopy. While cytokines are normally secreted and difficult to detect with fluorophore-conjugated antibodies, reporter-driven fluorophores accumulate in cells over time providing more reliable detection.

Before beginning this protocol, researchers must clone or otherwise obtain fluorescence reporter constructs for their gene of interest and identify a cell type they seek to examine. This protocol uses IFN beta-GFP reporter lentivirus (Cellomics) encoding green fluorescent protein (GFP) downstream of the *IFNB1* promoter sequence. The IFN beta-GFP reporter lentivirus was used to transduce telomerase-immortalized human fibroblasts (THF). THF cells were chosen because they were immortalized in a way that preserves innate antiviral signaling (Hare et al., 2016).

Transduction and selection for IFN-GFP reporter cells

Timing: 1–2 weeks

1. Titrate antibiotic selection medium
   a. Add 5 × 10^4 THF cells in 0.5 mL per well to a 24-well plate and incubate 16–24 h

   Note: THF cells should reach ~90% confluency the next day
   b. Create a range of puromycin dilutions in culture medium from 0.1–10 μg/mL
   c. Add puromycin containing medium to wells in duplicate

d. Observe cells over the next 72 h and select a puromycin concentration and incubation time that effectively kills 100% of cells

Note: This concentration of puromycin will be used later to select for lentivirus transduced cells expressing puromycin resistance by killing all non-resistant cells. We found that 2–5 ug/mL of puromycin killed 100% of THF cells.

2. Transduce cells
   a. Add 2 × 10^5 THF cells in 2 mL per well to a 6-well plate and incubate 16–24 h

   Note: THF cells should reach ~70% confluence the next day
   b. Thaw IFN beta-GFP reporter lentivirus and dilute to 1 transduction unit (TU)/cell in 2 mL of culture medium containing 8 μg/mL polybrene
   c. Remove medium from adherent cells and add 2 mL/well of medium containing lentivirus and incubate for 6–24 h to allow transduction
   d. Dilute puromycin at the pre-optimized concentration in culture medium
   e. Dissociate cells with trypsin-EDTA and re-seed cells to a T75 flask in medium containing the previously determine concentration of puromycin
   f. Incubate cells for 24–72 h or until selection is complete

   Note: Culture your cells in media containing reduced puromycin (~50% of selection) for subsequent steps to maintain reporter expression

3. Test reporter activity using the dsRNA mimetic poly I:C
   a. Add 1 × 10^5 THF cells in 1 mL per well to a 12-well plate and incubate 16–24 h

   Note: THF cells should reach ~90% confluency the next day
   b. Create a range of poly I:C dilutions (10–1000 ng) in 0.1 mL of OptiMEM and dilute 10 μL of lipofectamine 3000 in 1 mL of OptiMEM
   c. Combine equal volumes poly I:C and lipofectamine and incubate for 5 min at 23°C before adding the mixture to cells dropwise and incubating for 16–24 h
   d. Measure GFP fluorescence in transfected cells using a fluorescence scanner, plate reader or fluorescence microscope and select an amount of poly I:C that results in enhanced green fluorescence with little or no toxicity

   Note: The concentration of poly I:C needed to observe GFP fluorescence is dependent on the cell type and the poly I:C formulation. We found that 100 ng of poly I:C triggered detectable GFP fluorescence in 1 × 10^5 THF cells.

   Note: If cells don't sufficiently upregulate GFP and/or IFN-ß refer to troubleshooting 1 - Cells don't upregulate IFN.

4. Sort a GFP-high population from poly I:C transfected cells
   a. Add 1.5 × 10^5 transduced THF cells in 10 mL to each of 2 10 cm plates and incubate 16–24 h

   Note: THF cells should reach ~90% confluency the next day
   b. Dilute 15 times the previously determined amount of poly I:C in 1 mL of OptiMEM and 15 μL of lipofectamine 3000 in 1 mL of OptiMEM
   c. Combine poly I:C and lipofectamine solutions, gently vortex before and after to mix, and incubate for 5 min at 23°C before adding the transfection mixture dropwise to one of the 2 plates and incubating cells for 16–24 h

d. Dissociate cells with trypsin-EDTA, resuspend cell pellet in 1 mL of FACS buffer, such that cell concentration is no more than 1 × 10^6 cells/mL, and filter samples using a 35 μm nylon mesh cell strainer
e. Prior to sorting GFP+ cells on a Beckman Coulter MoFlo XDP (or similar cell sorter), set the FSC/SSC gate and negative gate for GFP levels using non-transfected cells cultured under the same conditions
f. Sort poly I:C transfected cells with high GFP fluorescence (GFP-high) into a 5-mL polypropylene tube with 3 mL of collection medium making note of the total cell count from the sorter

Note: If cell numbers are reduced prior to or during sorting refer to troubleshooting 2 - Low cell count.

g .Pellet cells at 100 × *g* and 4°C for 10 min, resuspend in 2 mL of culture medium and add 10^5 cells in 2 mL to a 6-well plate
h .Expand cell population and freeze down vials of low passage cells in freezing medium for future experiments

Note: If antibiotic selection or flow sorting are not possible, either method alone can be used to select for transduced cells. To maximize the proportion of cells containing functional reporter constructs, it is best to use both methods.

⚠ CRITICAL: Care should be taken at each step to avoid contaminating cells containing the reporter construct. Use of antibiotics during cell maintenance should be avoided as they can alter cell biology and hide the presence of certain contaminating bacteria.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Rabbit anti-GFP IgG, Alexa Fluor 488	Invitrogen	Cat# A-21311
Mouse anti-ISG15 IgG (Clone 2.1)	Dr. Ernest Borden/PBL Assay Science	(D'Cunha et al., 1996) Cat# 21900-1
Rabbit anti-SeV IgG	MBL	Cat# PD029
Rat anti-mouse IgG, APC	BioLegend	Cat# 406609
Goat anti-rabbit IgG, Alexa Fluor 594	Invitrogen	Cat# A-11012
Bacterial and virus strains		
Sendai virus (Cantell strain)	Charles River	Cat# 10100774
Human cytomegalovirus (AD169 strain)	Dr. Theresa Compton/ATCC	(Juckem et al., 2008) Cat# VR-538
IFN beta-GFP reporter lentivirus	Cellomics	Cat# PLV-10145-200
Biological samples		
Poly I:C	InvivoGen	Cat# tlrl-pic
Chemicals, peptides, and recombinant proteins		
Puromycin	Gibco	Cat# A1113803
Polybrene (hexadimethrine bromide)	Sigma-Aldrich	Cat# H9268-5G
Hoechst 33342	Thermo Scientific	Cat# 62249
Critical commercial assays		
Cytofix/Cytoperm intracellular staining kit	BD Biosciences	Cat# 554714
Lipofectamine 3000	Invitrogen	Cat# L3000015
Experimental models: cell lines		
Telomerase-immortalized human fibroblasts (THF)	Dr. Victor DeFilippis	(Bresnahan et al., 2000)

MATERIALS AND EQUIPMENT

Culture medium	Final concentration	Amount
40 mM L-glutamine	0.4 mM	5 mL
Fetal bovine serum (FBS)	10%	50 mL
Dulbecco's modified Eagle's medium (DMEM)	n/a	445 mL
Total	**n/a**	**500 mL**

FACS buffer	Final concentration	Amount
0.5 M EDTA	5 mM	5 mL
Bovine serum albumin (BSA)	1%	5 g
Phosphate buffered saline (PBS)	n/a	495 mL
Total	**n/a**	**500 mL**

Note: Filter sterilize and store FACS buffer at 4°C

Collection medium	Final concentration	Amount
40 mM L-glutamine	0.4 mM	0.2 mL
Fetal bovine serum (FBS)	30%	6 mL
Dulbecco's modified Eagle's medium (DMEM)	n/a	13.8 mL
Total	**n/a**	**20 mL**

Note: Maintain sterile and store collection medium at 4°C

Freezing Medium	Final concentration	Amount
DMSO	10%	1 mL
Fetal bovine serum (FBS)	90%	9 mL
Total	**n/a**	**10 mL**

Note: Make up freezing medium fresh before freezing cells

IF Blocking Buffer	Final concentration	Amount
Bovine serum albumin (BSA)	3%	3 mL
Goat serum	3%	3 mL
Tween-20	0.2%	0.2 mL
Phosphate buffered saline (PBS)	n/a	96 mL
Total	**n/a**	**100 mL**

Note: Filter sterilize and store IF blocking buffer at 4°C

Note: Some antibodies may require alternatives to goat serum for blocking

Mounting Medium	Final concentration	Amount
Glycerol	85%	8.5 mL
P-phenylenediamine in 10× PBS	1 mg/mL	0.5 mL
0.2M carbonate-bicarbonate buffer pH 9.2	pH to 8.0	~0.5 mL
Total	n/a	10 mL

Note: Resuspend P-phenylenediamine at 20 mg/mL in 10× PBS, aliquot and freeze at −70°C

Note: Aliquot mounting medium, protect from light and store at −70°C

Note: Mounting medium should be orange-yellow and discarded after turning brown

⚠ CRITICAL: P-phenylenediamine is toxic, so avoid contact or inhalation

Alternatives: many types of commercial mounting media are available

STEP-BY-STEP METHOD DETAILS

Virus infection

Timing: 36 h

Cells respond to microbe-associated molecular patterns by upregulating IFN and IFN-stimulated genes (ISGs). Particles of Sendai virus (SeV) and human cytomegalovirus (HCMV) trigger an IFN response in the absence of virus replication and have been used extensively to study innate antiviral defense pathways. To examine the proportion of infected cells that upregulate IFN-ß in response to virus particles, we infected IFN-GFP THF cells with live or non-replicating UV-inactivated SeV (SeV-UV) or HCMV (HCMV-UV) particles.

1. Seed cells for immunofluorescence and flow cytometry
   a. Using sterile forceps, transfer glass coverslips soaked in 70% ethanol to a 12-well plate and wash the coverslips with PBS before adding cell suspension
   b. Add 1.5 × 10^6 THF IFN-GFP cells in 10 mL to 10 cm dishes and 1 × 10^5 THF IFN GFP cells in 1 mL per well to coverslips in 12-well plates and incubate 16–24 h

   Note: THF IFN-GFP cells should reach ~90% confluency the next day
   c. Remember to seed an extra well and an extra plate for cell counting the next day

   Note: Coverslips may be included in count wells if the coverslip is thought to affect cell count

   Note: If cells do not adhere well to glass coverslips refer to troubleshooting 3 - Cells fail to adhere.

2. Infect cells with live or inactivated SeV and HCMV
   a. Dissociate and re-suspend cells in the count well with trypsin-EDTA and count cells using a glass hemocytometer
   b. Dilute SeV to 2 plaque-forming units (pfu)/cell and HCMV to 0.3 pfu/cell in minimal volume of serum-free medium (0.3 mL/well for 12-well plates and 2 mL/dish for 10 cm dishes)
   c. Add 0.5 mL of diluted virus per well to a 12-well plate and expose virus to a pre-optimized amount of UV-radiation in a stratolinker UV cross-linker
   d. Remove medium from cells and add live or UV-inactivated virus
   e. Incubate cells at 37°C for 1 h, rocking plates periodically (10–15 min) to prevent cells from drying out

f. Replace medium containing inactivated virus with 1 mL/well and 10 mL/plate maintenance medium (2% FBS DMEM) and incubate for 16–24 h while GFP is expressed and accumulates in cells.

Note: UV-inactivation should be pre-optimized to reduce infectious titer by $>10^5$ fold, or to where there is <1 plaque-forming unit per treatment condition. If you encounter difficulty with UV-inactivation refer to troubleshooting 4 - Ineffective UV-inactivation.

Note: We have included a sample calculation for diluting SeV

$$\frac{Multiplicity\ of\ infection\ (pfu/cell)}{SeV\ titre\ (pfu/ml)} \times Cell\ count\left(\frac{cells}{well}\right) \times \frac{Dilution\ volume\ (ml)}{Infection\ volume\ (ml/well)}$$

$$\frac{2\ pfu/cell}{1.2 \times 10^7\ pfu/ml} \times 10^5 cells \Big/ well \times \frac{1\ ml}{0.3\ ml/well}$$

Detecting IFN production by flow cytometry

Timing: 3 h

Flow cytometry can be used to easily quantify the level and frequency of IFN producing cells in a population. To quantify upregulation of IFN and ISG15 in individual cells we used flow cytometry of fixed cells with intracellular staining.

3. Fix and permeabilize cells
   a. Wash THF cells in 10 cm dishes with PBS and incubate with 2 mL of trypsin-EDTA at 37°C until thoroughly dissociated
   b. Resuspend dissociated cells in sorting buffer and pellet at 500 × *g* and 4°C for 5 min
   c. Resuspend cell pellet in 100 µL of Cytofix/Cytoperm solution, transfer to a round bottom 96-well plate and incubate at 4°C for 20 min to fix
   d. Pellet cells at 500 × *g* and 4°C for 5 min and wash twice in 100 µL of Perm/Wash buffer

Note: Make sure to dilute 10× Perm/Wash buffer to a 1× solution in distilled water

4. Stain cells for expression of ISG15
   a. Pellet cells at 500 × *g* and 4°C for 5 min, resuspend in 50 µL of mouse anti-ISG15 diluted 1:10 in Perm/Wash buffer and incubate at 4°C for 30 min
   b. Pellet cells at 500 × *g* and 4°C for 5 min and wash twice with 100 µL of Perm/Wash buffer
   c. Pellet cells at 500 × *g* and 4°C for 5 min and resuspend in 50 µL of APC-conjugated anti-mouse diluted 1:100 in Perm/Wash buffer and incubate at 4°C for 30 min in the dark
   d. Pellet cells at 500 × *g* and 4°C for 5 min, wash twice with 100 µL of Perm/Wash buffer and re-suspend cells in 1 mL of sorting buffer

Note: Antibody dilutions should be optimized by the user to minimize background staining

5. Measure single cell fluorescence using Beckman Coulter MoFlo XDP (or similar flow analyzer)
   a. Set FSC/SSC gate and the negative gate for ISG15 expression and GFP using mock treated cells
   b. Run cells stained with anti-ISG15 and APC-conjugated anti-mouse to quantify expression of ISG15 and GFP in fixed and permeabilized cells

Note: If cell numbers are reduced prior to or during flow cytometry refer to troubleshooting 2 - Low cell count. If GFP fluorescence is too low or background fluorescence too high to detect changes in fluorescence refer to troubleshooting 5 - Low GFP fluorescence or troubleshooting 6 - High background fluorescence.

⚠ CRITICAL: Be careful to avoid exposing fixed cells to light as GFP and APC-conjugated antibody are light sensitive

Detecting IFN production by fluorescence microscopy

Timing: 5 h

Fluorescence microscopy is useful to visualize production of IFN and other molecules in an intact cell monolayer or for intracellular localization. To see whether IFN-producing cells cluster or are associated with higher levels of SeV infection, we used fluorescence microscopy of stained coverslips.

6. Formalin fix and permeabilize cells
   a. Wash cells with PBS, add 1 mL of 10% formalin per well and incubate for 10 min at 23°C away from direct light
   b. Remove formalin and wash off any excess formalin with PBS
   c. Add 1 mL of 0.2% Tx-100 in PBS per well and incubate for 10 min at 23°C away from direct light
   d. Wash cells with PBS, add 1 mL of IF blocking buffer per well and incubate 1 h at 4°C in the dark

   Pause point: Permeabilized coverslips can be left overnight in blocking buffer at 4°C in the dark

7. Stain cells with SeV- and ISG15-specific antibodies
   a. Dilute mouse anti-SeV 1:2000 and mouse anti-ISG15 1:50 seperately in IF blocking buffer
   b. Remove IF blocking buffer and use forceps to center coverslips within the well
   c. Add just enough diluted antibody to cover the coverslip (~30 µL, surface tension should keep it from running off) and incubate for 1 h in the dark
   d. Dilute Alexa Fluor 594-conjugated anti-mouse IgG (Invitrogen) 1:500 and Alexa Fluor 488-conjugated anti-GFP (invitrogen) 1:1000 together in IF blocking buffer
   e. Wash 3 times with PBS and once with IF blocking buffer
   i. The IF blocking buffer wash is necessary to keep the secondary antibody on the coverslip
   f. Center the coverslip, add just enough secondary antibody to cover the coverslip (~30 ul) and incubate 1 h in the dark
   g. Dilute Hoechst 33342 1:5000 in PBS
   h. Wash with PBS, add 0.5 mL of Hoechst per well and incubate 10 min in the dark

   Note: Antibody dilutions should be optimized by the user to minimize background staining

8. Mount coverslips on slides
   a. Discard Hoechst, wash 3 times with PBS and once with water to avoid salt crystalizing

   Note: Make sure to properly dispose of Hoechst and the first PBS wash
   b. Add ~3 µL of mounting medium to slides where the coverslips will be placed
   c. Using forceps carefully lift the coverslips, blot excess liquid from the edges and lower the coverslips onto the mounting medium so as to avoid trapping air bubbles as much as possible

   Pause point: With proper anti-fade mounting media, coverslips can be kept at 4°C and should maintain fluorescence for at least a few days.

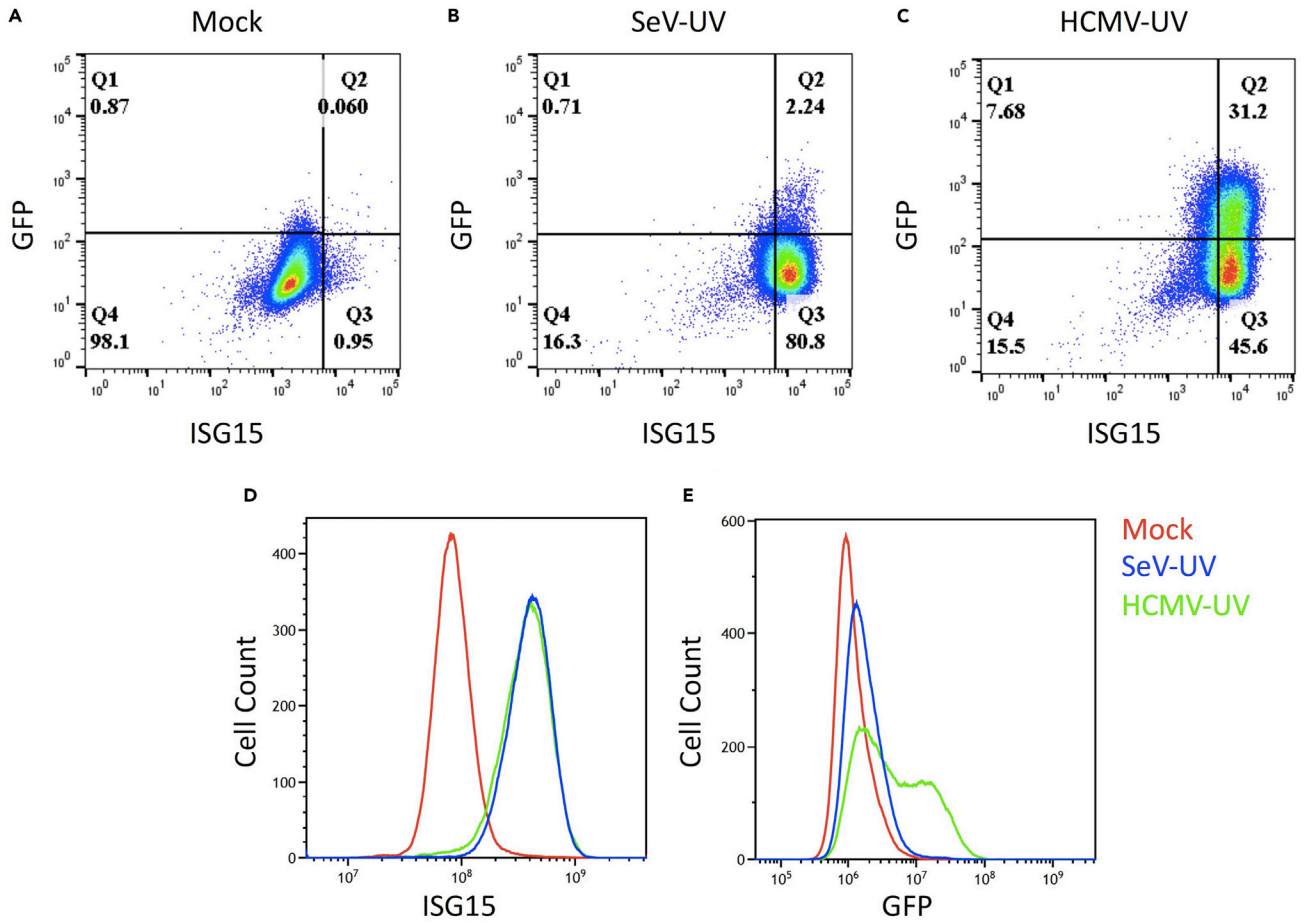

Figure 1. Detecting IFN production by flow cytometry

THF IFN-GFP cells were mock infected, infected with UV-inactivated Sendai virus (SeV-UV) or infected with UV-inactivated HCMV (HCMV-UV). 16 h later cells were dissociated, fixed, and stained for ISG15 and an APC-conjugated secondary antibody. Individual cell fluorescence was measured using MoFlo XDP. Flow-plots from mock, SeV-UV, or HCMV-UV infected THF IFN-GFP are shown (A–C). Histograms for APC-stained ISG-15 and IFN-GFP reporter are shown (D and E). Figure re-printed with permission from the authors (Hare et al., 2020).

9. Visualize cells with a fluorescence microscope

Note: If GFP fluorescence is too low or background fluorescence too high to detect changes in fluorescence refer to troubleshooting 5 - Low GFP fluorescence or troubleshooting 6 - High background fluorescence.

⚠ CRITICAL: Be careful to avoid exposing fixed cells to light during incubations as GFP and other fluorophores are light sensitive.

EXPECTED OUTCOMES

Infection of human fibroblasts with inactivated SeV (Cantell strain) or HCMV (AD169 strain) should lead to detectable upregulation of ISG15 and GFP by flow cytometry and immunofluorescence microscopy. Infection with 0.3 pfu/cell of HCMV-UV triggered IFN-ß upregulation in the majority of cells while 2 pfu/cell of SeV-UV triggered IFN-ß upregulation in a small subset (Figure 1). IFN-ß production in this subset of SeV-UV infected cells did not correlate with higher detectable SeV protein (Figure 2) or SeV genomes (Hare et al., 2020) and has been suggested to result from stochastic cellular signaling events (Zhao et al., 2012). Stochastic or heterocellular IFN production has been

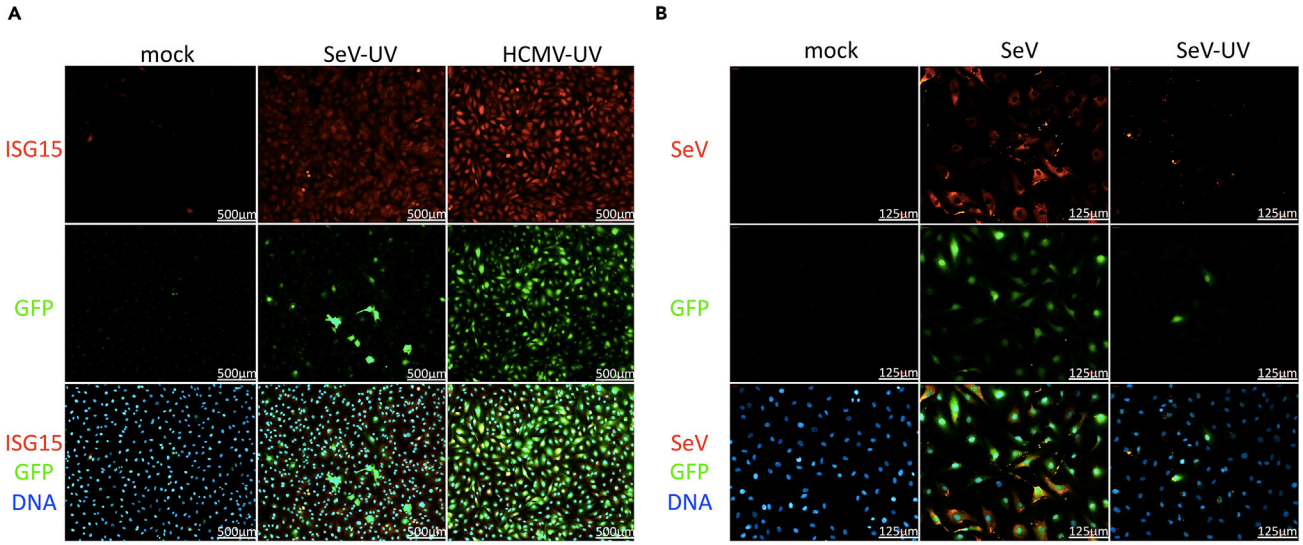

Figure 2. Detecting IFN production by fluorescence microscopy

THF IFN-GFP cells were mock infected, infected with UV-inactivated SeV (SeV-UV), UV-inactivated HCMV (HCMV-UV) or live SeV. Cells were fixed 16 h later and stained with ISG15 (red) to show ISG upregulation (A), pan-SeV antibody (red) to show SeV infection (B), GFP antibody (green) to boost GFP fluorescence and Hoechst (blue) to show nuclei (A and B). Fluorescence images were taken using a Zeiss Axio Vert microscope. Scale bars of 500 μm (A) and 125 μm (B) are included in the lower right corner. Figure re-printed with permission from the authors (Hare et al., 2020).

described for several RNA viruses (Chen et al., 2010), although the underlying mechanism is not completely understood.

QUANTIFICATION AND STATISTICAL ANALYSIS

Flow cytometry analysis was used by designating the negative region to 98% of mock treated cells and measuring the proportion of cells in treatment groups with fluorescence levels outside those gates. These data are presented as flow plots allowing cells to be categorized into groups based on whether they contain higher levels of GFP and other markers being analyzed. The proportion of cells falling into each group can then be compared by analysis of variance (ANOVA) and post-tests to determine whether the proportions in treated groups differ from mock treated controls.

While measures of central tendency (i.e., mean or median fluorescence) can be used to compare groups, this does not capture the heterogeneous response of individual cells to infection. When examining the response to SeV-UV (Hare et al., 2020) or other viruses (Chen et al., 2010; Zhao et al., 2012), bulk measurements or measures of central tendency obscure the robust response of a small subset of cells.

LIMITATIONS

The system we have described involves introduction of a fluorescent reporter into an immortalized fibroblast model. Some cell types can be difficult to transduce or culture long term, but lentiviral transduction and flow cytometric sorting can quickly enrich for cells containing the IFN-GFP reporter. Reporter mice already exist with luciferase or yellow fluorescence protein (YFP) under control of the *IFNB1* promoter and these mice could be used in conjunction with microscopy or flow cytometry for *in vivo* studies (Lienenklaus et al., 2009; Scheu et al., 2008).

We found that the IFN-GFP reporter was less sensitive to detecting low levels of IFN-ß production than bulk assays (VSV restriction or luciferase-based reporters). In some cases, GFP-specific antibodies are required to boost the signal from the IFN-GFP reporter. While THF cells produce IFN

2–6 h post-infection, fluorescence from the IFN-GFP reporter peaks near 16 h post-infection. This kinetic difference presents a challenge to looking at early times post-infection.

TROUBLESHOOTING

Problem 1 - Cells don't upregulate IFN

Encountered in transduction and selection for IFN-GFP reporter cells.

While we found that primary and immortalized human fibroblasts reliably upregulate IFN in response to infection with HCMV or SeV particles, other cell types may not respond similarly. Many cancer-derived cell lines lack antiviral signaling molecules critical for recognition of DNA or RNA virus particles (Hare et al., 2016). Some cell types differentially express surface receptors necessary for virus entry or signaling molecules necessary for virus recognition. Therefore, it is possible that different cell types respond differently or not at all to virus infection.

Potential solution 1

Before going to the trouble of making reporter cells it is important to confirm that the cell type is capable of detecting mis-localized nucleic acid and producing IFN. This can be done by transfecting cells with the dsRNA mimetic poly I:C or dsDNA and measuring upregulation of antiviral IFN-stimulated genes.

Problem 2 - Low cell count

Encountered in transduction and selection for IFN-GFP reporter cells and detecting IFN production by flow cytometry.

Low cell counts during sorting or during flow cytometry sometimes mean the experiment needs to be repeated. There are a number of possible explanations for low cell counts during flow cytometry experiments.

Potential solution 2

To determine where cells are being lost it is important to check cell number at different steps during the protocol. It is important to check viability by visible inspection before treatment and before fixing or sorting. Virus infection or transfected nucleic acid sometimes activate cell death pathways and you could be losing cells over the course of the experiment. If this is a problem, decreasing the stimulus or choosing an earlier timepoint may be necessary.

Fibroblasts are particularly prone to aggregating during flow cytometry, which reduces the overall number of cells once these aggregates are excluded. If you detect a large number of aggregates during flow cytometry you may need to further dilute your samples in FACS buffer or better dissociate cells using trypsin-EDTA. It may be also necessary to re-filter cells using a 35 μm nylon mesh cell strainer.

Problem 3 - Cells fail to adhere

Encountered in virus infection.

Adherence to coverslips during cell culture and after fixing is important for high resolution immunofluorescence microscopy. Sometimes cells fail to attach or are washed off during the staining process.

Potential solution 3

Once adhered to coverslips, cells should maintain a similar morphology to cells in culture. Fibroblasts adhere readily to uncoated glass coverslips, but other cell types may require pre-coated coverslips for proper adherence. Adherence to glass coverslips takes a little longer than adherence to plastic tissue culture dishes, so ample time should be allowed for cells to settle. Cell stress is

particularly evident when seeding to coverslips, so avoid stressing cells as much as possible prior to and during cell seeding.

If cells are lifting off coverslips after fixing ensure that you are using fresh fixative and allowing sufficient time for cell fixing. Avoid any unnecessary shear stress to cells during staining by pipetting liquid onto the side of the well rather than directly onto coverslips.

Problem 4 - Ineffective UV-inactivation

Encountered in virus infection.

If you use UV-inactivated virus to stimulate cells it is important you have properly optimized inactivation conditions for each virus preparation. A common sign that your virus is not completely inactivated is when you observe cell rounding or other signs of toxicity in your infected cells. Alternatively, excess UV-radiation can damage the virus particle and prevent virus entry and upregulation of IFN.

Potential solution 4

When inactivating virus, it is important not to deviate too far from optimized inactivation conditions. UV-light does not penetrate as far as visual light, so UV-inactivation is very sensitive to the depth of the inactivation volume, the concentration of virus and cellular debris and any plastic between the virus and the UV-bulbs. Therefore, it is important to maintain optimized conditions in order to avoid altering the degree of inactivation.

While heat or formaldehyde are sometimes used to inactivate virus, these treatments damage virus particles making them incapable of entry, which is important for the IFN response in fibroblasts. In contrast, UV-inactivation damages the viral genome leaving the particle mostly intact.

Certain viruses are difficult to properly inactivate. For instance, inactivated vaccinia virus can trigger apoptosis because the immediate-early gene products are toxic and standard UV-inactivation does not uniformly inactivate the genome. In this case, addition of psoralen and long wave UV-A radiation more evenly inactivates viral genomes.

Problem 5 - Low GFP fluorescence

Encountered in detecting IFN production by flow cytometry and detecting IFN production by fluorescence microscopy.

It is possible that under certain experimental conditions it is difficult to detect GFP upregulation. This issue may result from insufficient sensitivity as mentioned above. There are ways of enhancing GFP fluorescence while avoiding signal loss or enhanced background fluorescence.

Potential solution 5

There are several ways to increase signal by altering experimental conditions (increasing the stimuli, selecting later time-points, etc.). Another approach is to enhance signal in the green channel using fluorophore-conjugated anti-GFP antibodies. However, care must be taken to properly titrate these antibodies to avoid background fluorescence.

Certain fixatives may reduce GFP fluorescence by directly inactivating GFP or allowing it to escape after fixing cells. We recommend using 10% formalin (3.6% formaldehyde) to fix cells despite a slight reduction in fluorescence. Other fixatives like methanol will drastically reduce GFP fluorescence. While working with GFP or other fluorophores, care must be taken to avoid light exposure which will impair fluorescence intensity.

Low GFP fluorescence may also be due to poor reporter expression in your cells. Some reporter constructs contain synthetic or virus-derived enhancers adjacent to the promoter insert which can boost inducible expression levels. Additionally, enhanced GFP (EGFP) variants with greater fluorescence or stability are commonly used. Altering the reporter construct to incorporate these features may enhance fluorescence signal and reporter sensitivity.

Reporter expression may decline following transfection/transduction. Therefore, it is important to limit the number of passages between transfection/transduction and experiments. Expression from non-integrating vectors, such as plasmids, will be rapidly lost as cells divide and the non-integrated DNA is lost. Expression from integrating vectors, such as lentiviral vectors, is more stable but may still decline over time due to epigenetic silencing or *in vitro* selection pressures.

Problem 6 - High background fluorescence:
Encountered in detecting IFN production by flow cytometry and detecting IFN production by fluorescence microscopy.

High background fluorescence reduces sensitivity by lowering the signal to background ratio. This can be especially problematic for GFP as media components and cells themselves sometimes autofluoresce at this wavelength.

Potential solution 6
If you are observing particularly high background while measuring fluorescence first make sure the instrument you are using is set up properly. If autofluorescence from medium is a problem, you can replace the medium with PBS or a low-fluorescence media alternative. Cellular autofluorescence increases when cells undergo apoptosis so one way to improve imaging is to improve cell viability. Non-specific antibody binding can cause high background florescence. This can be improved by using fresh blocking buffer, increasing the blocking time or reducing the staining concentration of antibody.

Another possibility is GFP expression from the reporter construct in unstimulated cells. This could be because the cells are more or less transcriptionally active or the reporter is activated in response to stressors or microbial contamination. These factors must be taken into account when trying to optimize the signal to background ratio.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, Dr. Karen Mossman (mossk@mcmaster.ca).

Materials availability

THF IFN-GFP reporter cells are available upon request. All other reagents are commercially available.

Data and code availability

This study did not generate/analyze datasets/code

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AUTHOR CONTRIBUTIONS

The protocol was developed by D.N.H. with assistance from M.K.S. and K.L.M. The manuscript was written by D.N.H. with contributions from M.K.S. and K.L.M.

DECLARATION OF INTERESTS

The authors declare no competing interest.

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