invitrogen

PrimeFlow RNA Assay

Simultaneous detection of RNA and protein by flow cytometry

Conjugated Antibodies

Flow RNA

Flow cytometry instrument

ThermoFisher SCIENTIFIC

Detect RNA and protein simultaneously by flow cytometry

With the novel Invitrogen™ PrimeFlow™ RNA Assay, scientists can now reveal the dynamics of RNA and protein expression simultaneously within millions of single cells. This assay employs a proprietary FISH and bDNA amplification technique for simultaneous detection of up to four RNA transcripts labeled with Invitrogen™ Alexa Fluor™ 488, 568, 647, and 750 dyes in a single cell, using a standard flow cytometer. RNA detection may be combined with intracellular and cell-surface antibody staining to elevate the understanding of single-cell dynamics to a new dimension.

Key features:

- See gene expression heterogeneity at the single-cell level
- Compare RNA and protein kinetics in the same cell
- Detect noncoding RNA in cellular subsets
- Evaluate viral RNA in infected cells
- Analyze mRNA expression levels when antibody selection is limited
- Analyze up to four RNA transcripts simultaneously
- Detect microRNA (miRNA) by flow cytometry

Assay technology

Fluorescence *in situ* hybridization (FISH) is a powerful technique that allows specific localization of RNA targets in fixed cells. The basic premise of the application relies on detecting nucleic acids through sequential hybridization of nucleic acid probes that provide gene expression information at the single-cell level. Traditional FISH techniques are generally limited by high background and low sensitivity due to nonspecific binding and inefficient signal amplification.

Principle of the PrimeFlow RNA Assay

The PrimeFlow RNA Assay incorporates a proprietary oligonucleotide probe set design and branched DNA (bDNA) signal amplification technology to analyze RNA transcripts by flow cytometry. bDNA technology provides a unique approach to RNA detection and signal amplification by amplifying the reporter signal rather than the target sequence (e.g., PCR) to help enable more consistent results than those obtained using PCR-based assays. In the PrimeFlow RNA Assay, target-specific probe sets contain 20–40 oligonucleotide pairs that hybridize to the target RNA transcript (Figure 1). Signal amplification is achieved through specific hybridization of adjacent oligonucleotide pairs to bDNA structures, formed by preamplifiers, amplifiers, and fluorescently labeled probes (Figure 2), resulting in excellent specificity, low background, and high signal-to-noise ratios.

Development of the PrimeFlow RNA Assay is based upon proven and well-published Invitrogen™ ViewRNA™ technology designed for microscopic analysis of RNA in cells and tissues. The assay combines paired oligonucleotide probe design with bDNA signal amplification to robustly detect up to four RNA transcripts at the single-cell level using a standard flow cytometer.

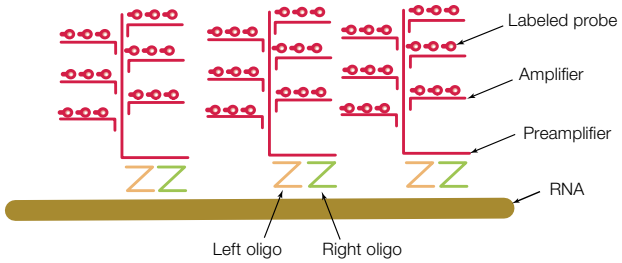


Figure 1. PrimeFlow RNA Assay using bDNA technology.

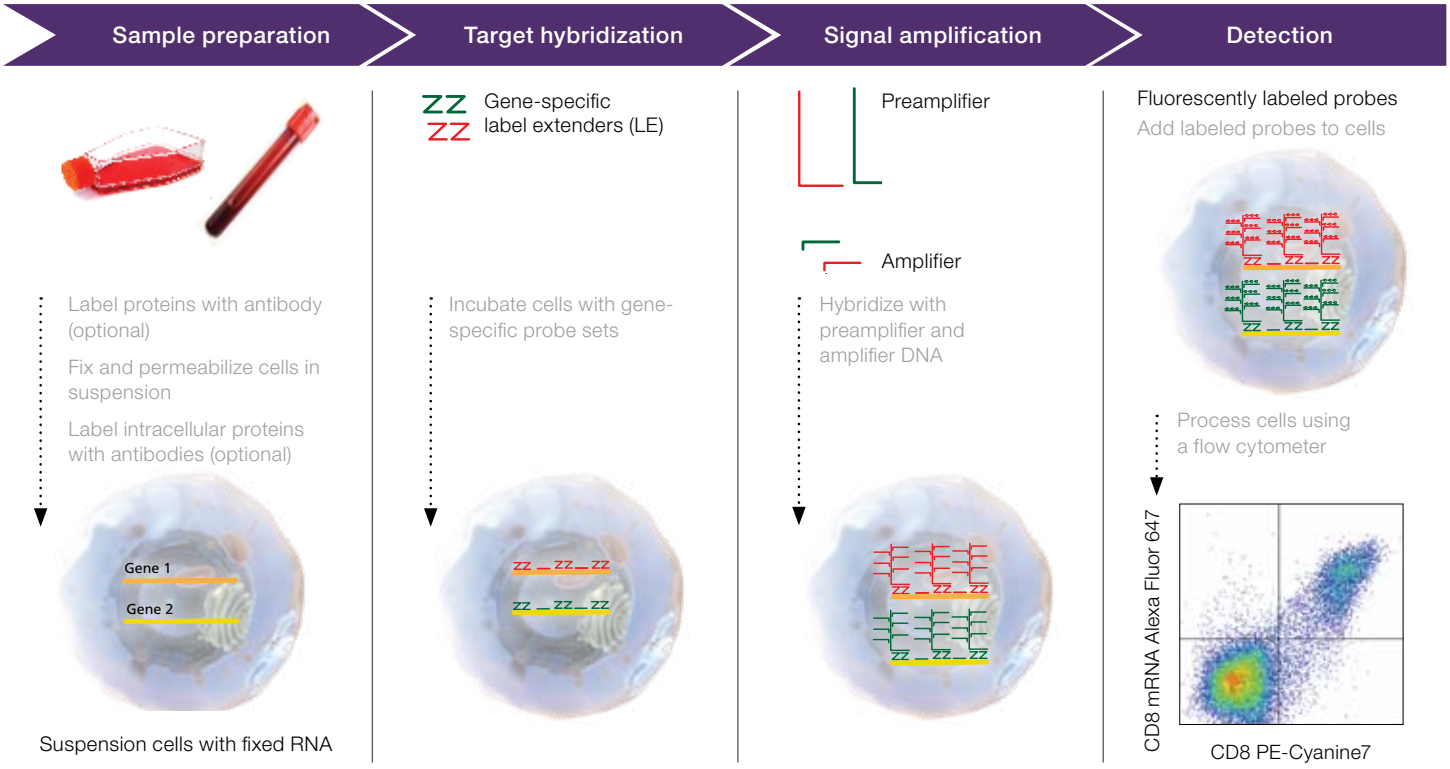


Figure 2. The PrimeFlow RNA Assay workflow. The assay workflow contains several steps: antibody staining; fixation and permeabilization, including intracellular staining, if desired; followed by target hybridization with a target-specific probe set containing 20–40 oligonucleotide pairs.

Compare RNA and protein kinetics in the same cell

In response to external stimuli, differential regulation of RNA transcription leads to changes in protein level. However, the levels of RNA and protein products of specific genes may vary at any given point in time. Using current methods such as qPCR or microarrays, researchers must choose between measuring mRNA or protein because they cannot be measured simultaneously due to the limitations of prevailing techniques. PrimeFlow technology can reveal the unique kinetics of mRNA and protein in different cell subsets, and identify expression differences as they change over time in response to the stimulus.

Correlation and kinetics of IFNγ and TNFα transcription and translation

Aims

Intracellular staining and flow cytometric analysis of lymphocytes is commonly used to assess cytokine production at the single-cell level in heterogeneous samples. Here, the PrimeFlow RNA Assay is used in combination with intracellular antibody staining to study the kinetics of the transcription and translation of IFNγ and TNFα in lymphocyte subsets.

Results

IFNγ mRNA was upregulated in $CD8^+$ and $CD8^-$ lymphocytes within 1 hour after stimulation, while protein levels were not detected until 2 hours. Both IFNγ mRNA and protein were maintained for the next 3–4 hours (Figures 3A and 3C). In contrast, TNFα mRNA and protein were both upregulated within 1 hour after stimulation and expression was maintained in $CD8^+$ cells, while expression in $CD8^-$ cells peaked between 1–2 hours and then decreased over the next 4 hours, with the decrease in mRNA preceding the decrease in protein (Figures 3B and 3D).

Conclusions

Using the PrimeFlow RNA Assay, we find that induction of IFNγ and TNFα mRNA and protein exhibit unique kinetics, and that TNFα protein and mRNA are differentially regulated in $CD8^+$ and $CD8^-$ lymphocytes. This assay enables the study of gene expression at the single-cell level in heterogeneous samples without the need for sorting specific subsets, as well as the ability to compare and contrast the kinetics of mRNA and protein induction.

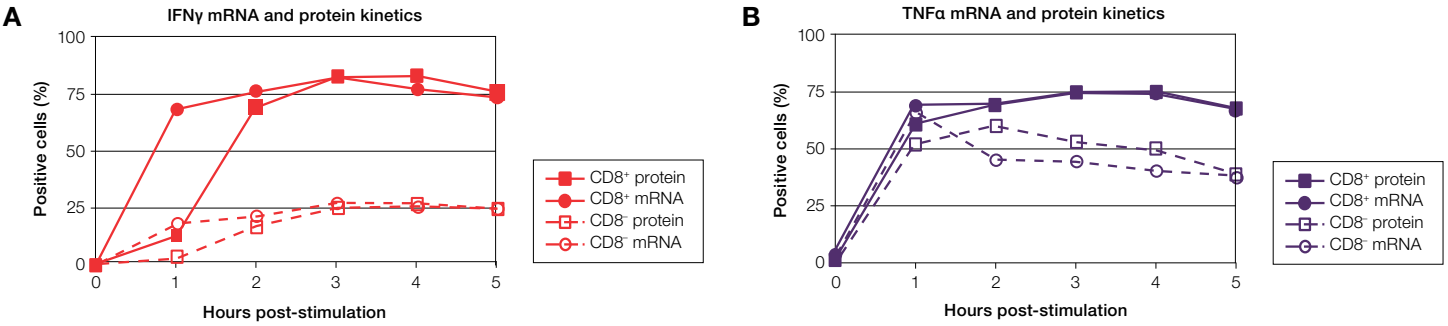
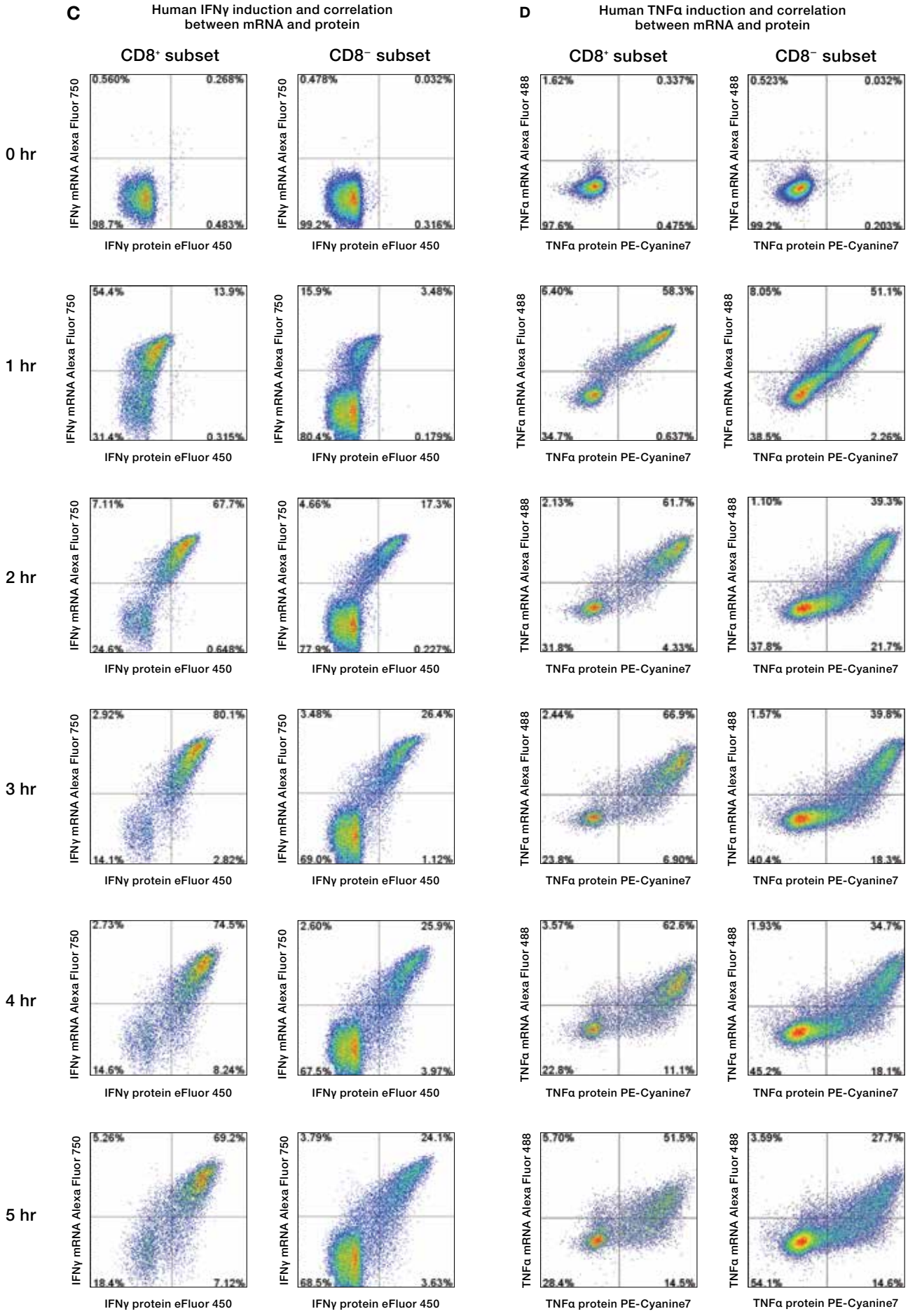


Figure 3. Kinetics of IFNγ and TNFα transcription and translation measured by the PrimeFlow RNA Assay. Normal human peripheral blood mononuclear cells were stimulated with the Invitrogen™ eBioscience™ Cell Stimulation Cocktail (plus protein transport inhibitors) (Cat. No. 00-4975) for 0–5 hours. Using the PrimeFlow RNA Assay, cells were fixed, permeabilized, and intracellularly stained with antibodies for CD8, IFNγ, and TNFα. Next, cells underwent a series of hybridization steps to label mRNA for IFNγ and TNFα. Viable $CD8^+$ and $CD8^-$ cells in the lymphocyte gate were used for analysis. **(A)** and **(C)** illustrate the kinetics of IFNγ, and **(B)** and **(D)** illustrate the kinetics of TNFα.


C
Human IFNγ induction and correlation between mRNA and protein
CD8+ subset
CD8− subset
0 hr
1 hr
2 hr
3 hr
4 hr
5 hr
IFNγ mRNA Alexa Fluor 750
IFNγ protein eFluor 450
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0.483%
0.478%
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CD8− subset
TNFα mRNA Alexa Fluor 488
TNFα protein PE-Cyanine7
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54.1%
14.6%

Observe gene expression heterogeneity at the single-cell level

Cellular heterogeneity is present in any biological sample, from low- to high-protein levels in cell subsets or gene expression differences from cell to cell. Paradoxically, most of our understanding of gene expression is based upon bulk population averages. This analysis, although informative, often leads to conclusions that assume ensemble averages reflect the dominant biological mechanism operating within an entire population. Using such measurements and assumptions can mask the presence of rare or small subpopulations of cells or bimodal cellular behaviors, and ignores essential cell-to-cell differences. To fully understand whether cellular heterogeneity contributes to biological function or contains relevant information, a single-cell approach must be applied. The PrimeFlow RNA Assay reveals differential responses following stimulation and, thus, the hidden story that is otherwise masked by using quantitative RT-PCR (RT-qPCR) to analyze the entire population.

Aims

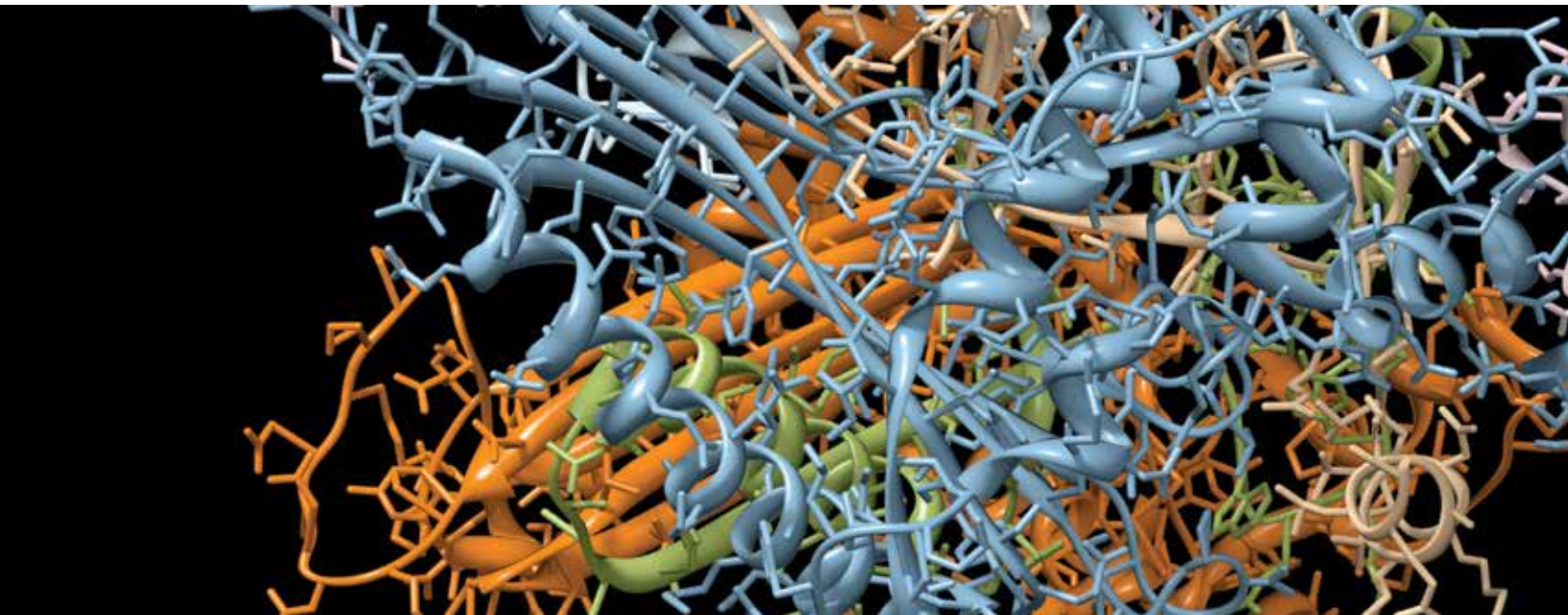
mRNA is commonly assessed by RT-qPCR where cells are isolated after stimulation so that total RNA can be extracted and then undergo RT-qPCR reactions. This results in amplified bulk measurements that mask the differences in gene expression that occur at the single-cell level. In this example, PrimeFlow RNA data are compared to RT-qPCR.

Results

The same cell populations in Figure 3 (pages 4 and 5) were analyzed by RT-qPCR. Using RT-qPCR, it appears that after IFNγ and TNFα are initially induced at similar levels, IFNγ level plateaus, while TNFα level declines slowly (Figure 4). However, with the PrimeFlow RNA Assay, it is observed that TNFα mRNA is rapidly and highly induced at 1 hour, dramatically declines between 1–2 hours, and continues to decline more slowly 3–5 hours after stimulation. In contrast, IFNγ is maintained throughout the time course. As shown in Figure 3, PrimeFlow RNA data can be further analyzed into $CD8^+$ or $CD8^-$ cell subsets, which reveal additional sample heterogeneity that RT-qPCR cannot achieve from a single sample. The PrimeFlow RNA Assay shows that the percentage of cells expressing IFNγ is less than the percentage of cells expressing TNFα.

Conclusions

Examination of stimulated cells over time using current RT-qPCR technology shows similar kinetics as with PrimeFlow RNA technology. However, the PrimeFlow RNA Assay uncovers the finer details of the kinetics at a single-cell level and allows users to study multiple parameters within the same sample, thereby eliminating the bulk averages previously masking cellular heterogeneity.



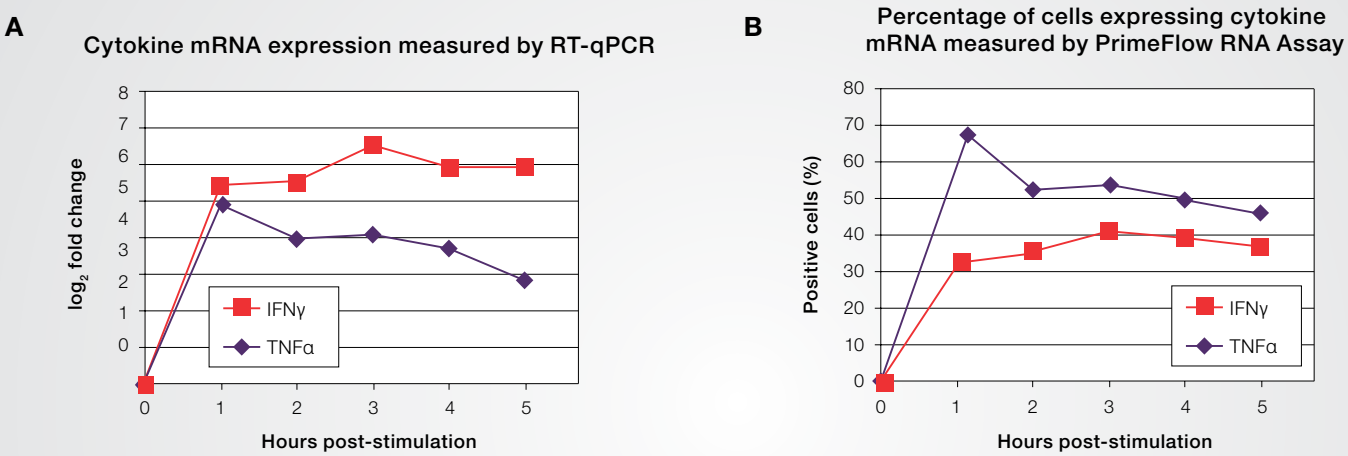
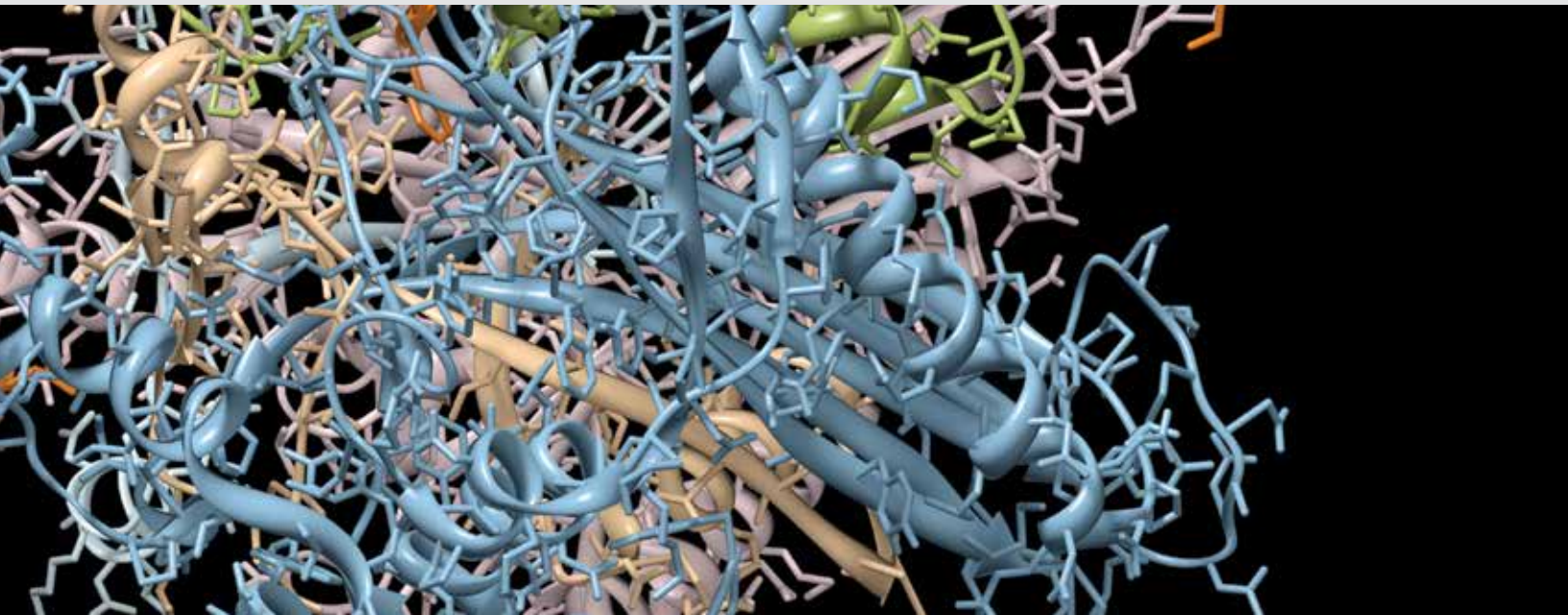
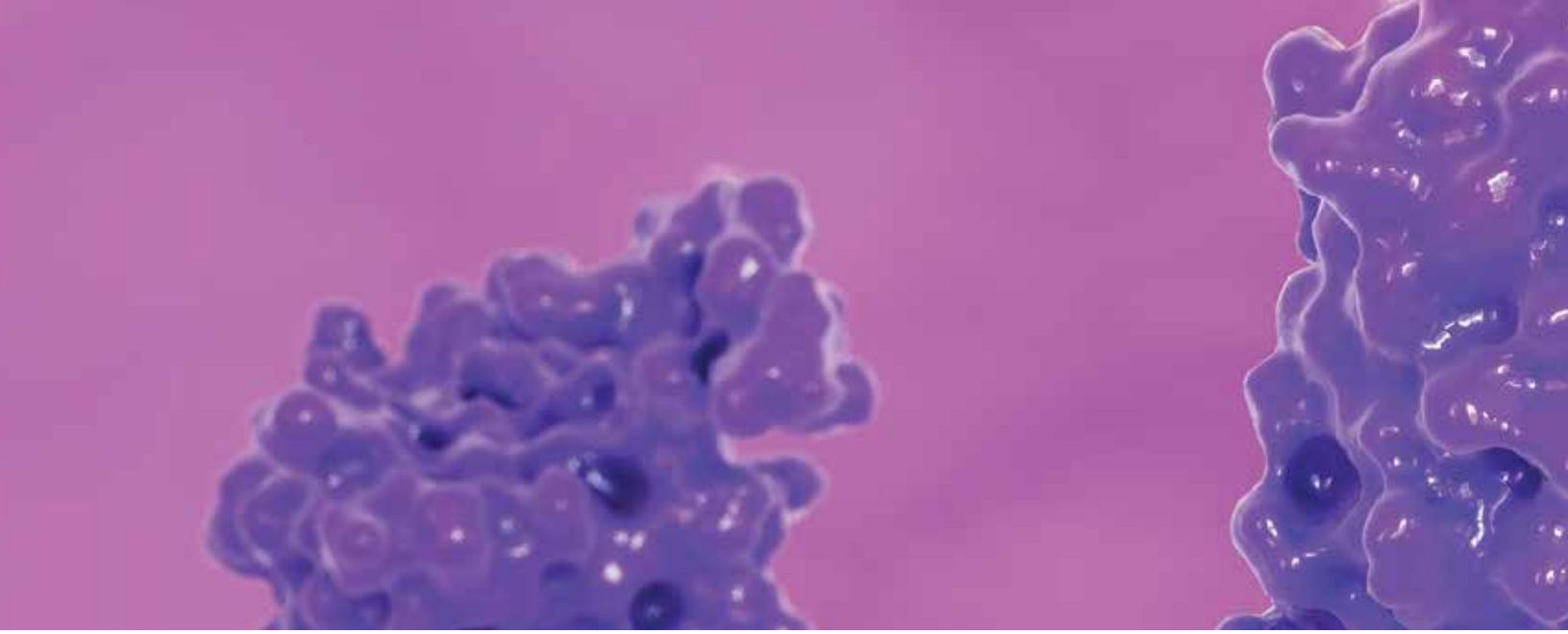


Figure 4. IFNγ and TNFα mRNA measured by RT-qPCR and PrimeFlow RNA Assays. (A) Normal human peripheral blood mononuclear cells were stimulated with the Invitrogen™ eBioscience™ Cell Stimulation Cocktail (plus protein transport inhibitors) (Cat. No. 00-4975) for 0–5 hours. **(B)** RNA was isolated and was analyzed by RT-qPCR. Using the PrimeFlow RNA Assay, the cells were fixed, permeabilized, and intracellularly stained with antibodies for CD8, IFNγ, and TNFα. Next, cells underwent a series of hybridization steps to label mRNA for IFNγ and TNFα. Viable cells in the lymphocyte gate were analyzed for percentage of cytokine-producing cells.





Analyze mRNA expression levels when antibody is limited

Flow cytometry has the ability to look at millions of cells with multiplexing capabilities. The straightforward workflow to detect both cell-surface and intracellular proteins with single-cell resolution makes flow cytometry the gold-standard technique for the study of heterogeneous cell populations. However, flow cytometry historically has been constrained by lack of availability and inadequacy of antibodies. Noncoding RNA, viral transcripts, unique model organisms and/or targets, or markers for which antibody development is troublesome have not benefited from the power of flow cytometry and have required numerous disconnected experiments to analyze their impact on cell subsets. The PrimeFlow RNA Assay can detect target-specific RNA for which available flow cytometry antibodies are nonexistent.

IL-23R mRNA expression by flow cytometry

Aims

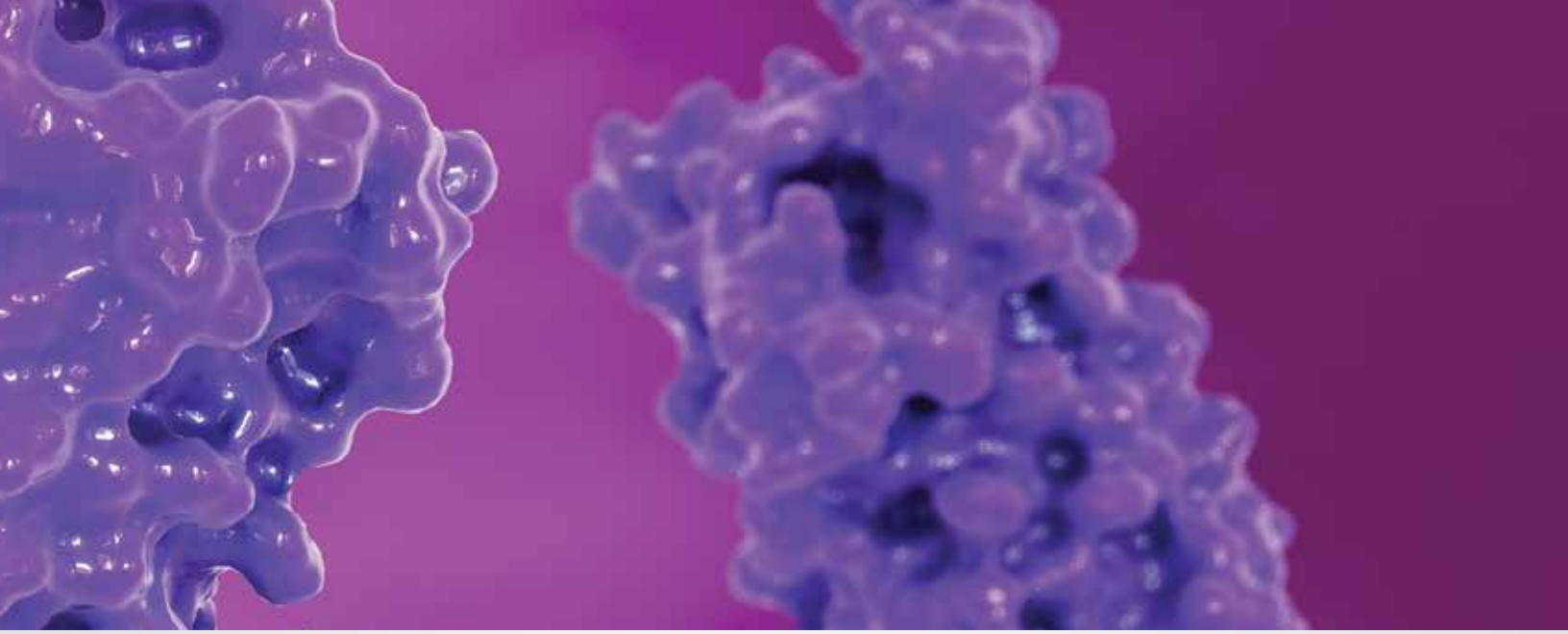
IL-23R is expressed by Th17 cells, a subset of activated $CD4^+$ T cells that play a key role in defense of mucosal barriers against extracellular bacteria and fungi. While Th17 cell differentiation is controlled by TGFβ, IL-6, and IL-1, IL-23 is crucial for their survival and function and has been implicated in many autoimmune diseases. The study of IL-23R is impaired by the lack of antibodies with appropriate sensitivity; most studies involving IL-23R in Th17 cells examine gene expression at the population level. The data obtained are unquestionably informative; however, they mask the actual expression heterogeneity. Here, the PrimeFlow RNA Assay is used in combination with antibody staining to interrogate the heterogeneity of IL-23R mRNA expression in polarized Th17 cell subsets.

Results

Normal human peripheral blood cells were cultured under Th17-polarizing conditions for 3 days and then restimulated. Under these conditions, only a subset of $IL\text{-}17A^+$ or $IL\text{-}17AF^+$ cells expressed low levels of IL-23R mRNA (Figure 5).

Conclusions

A strength of the PrimeFlow RNA Assay is its ability to detect most mRNAs in individual cells without being limited by antibody availability. This applies to targets for which antibody development is difficult (e.g., IL-23R, GPCRs), unique model organisms (e.g., canine, fish), or esoteric markers for which no commercial antibodies are available. The PrimeFlow RNA Assay can also be used to detect non–protein-coding RNA targets (lncRNA, viral transcripts) against which antibodies cannot be made.



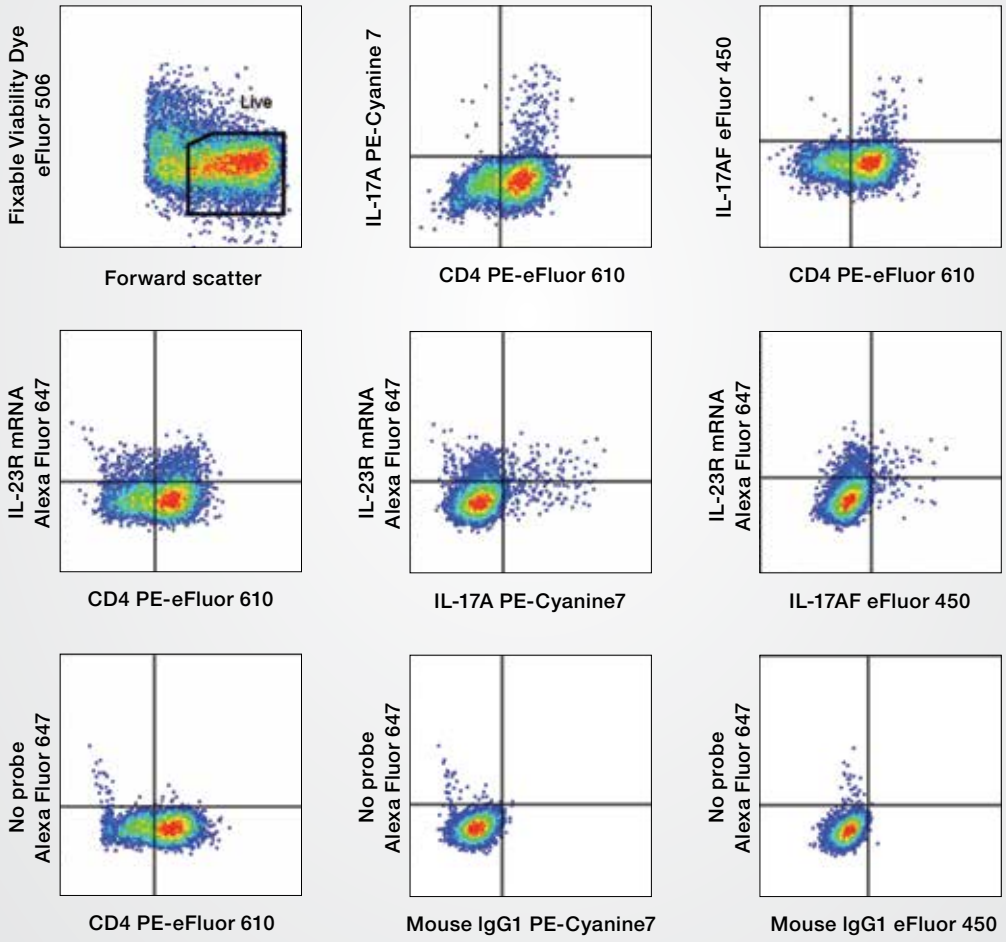


Figure 5. IL-23R mRNA expression in Th17 cell subsets by the PrimeFlow RNA Assay. Normal human peripheral blood cells were cultured under Th17-polarizing conditions for 3 days, then restimulated with eBioscience Cell Stimulation Cocktail (plus protein transport inhibitors) (Cat. No. 00-4975) for 5 hours. Cells were labeled with Invitrogen™ eBioscience™ Fixable Viablity Dye eFluor™ 506 (Cat. No. 65-0866), fixed and permeabilized using the PrimeFlow RNA Assay buffers, then intracellularly stained with Anti-CD4 PE-eFluor™ 610, Anti-IL-17A PE-Cyanine7, and Anti-IL-17AF eFluor™ 450. Next, cells underwent a series of hybridization steps to label mRNA for IL-23R.

Evaluate viral RNA in infected cells

Coinfection by multiple virus particles can lead to increased pathology and morbidity, as is the case of human immunodeficiency virus (HIV) and hepatitis C virus (HCV) coinfections seen in older AIDS patients, or super-infection with hepatitis B and HCV. Understanding the lack of virus interference in these cases, on a per-cell basis, may have implications in the future design and delivery of vaccines. To date, there are no robust methods to track and study individual coinfected cells. The PrimeFlow RNA Assay can be used for the direct detection of multiple viral transcripts within a single cell by flow cytometry, thus facilitating the detailed study of coinfected populations of cells.

Detection of hepatitis C viral RNA in coinfected human hepatocytes

Method

Hepatocytes were coinfected with two strains of HCV, JFH1-wild type (WT) and JFH1-codon altered (CA). Probes were designed to differentiate the two strains of HCV to study dominant relationships in coinfected cells. These probes were then applied using the PrimeFlow RNA Assay and Invitrogen™ ViewRNA™ Assay.

Results

The PrimeFlow RNA Assay clearly defined JFH1-WT single-positive, JFH1-CA single-positive, and double-positive populations (Figure 6). The orthogonal ViewRNA Assay was used to verify that the probe design was highly specific for the indicated strains of HCV (Figure 7).

Conclusions

The PrimeFlow RNA Assay is the first commercial assay that can study individual cells that have been coinfected.

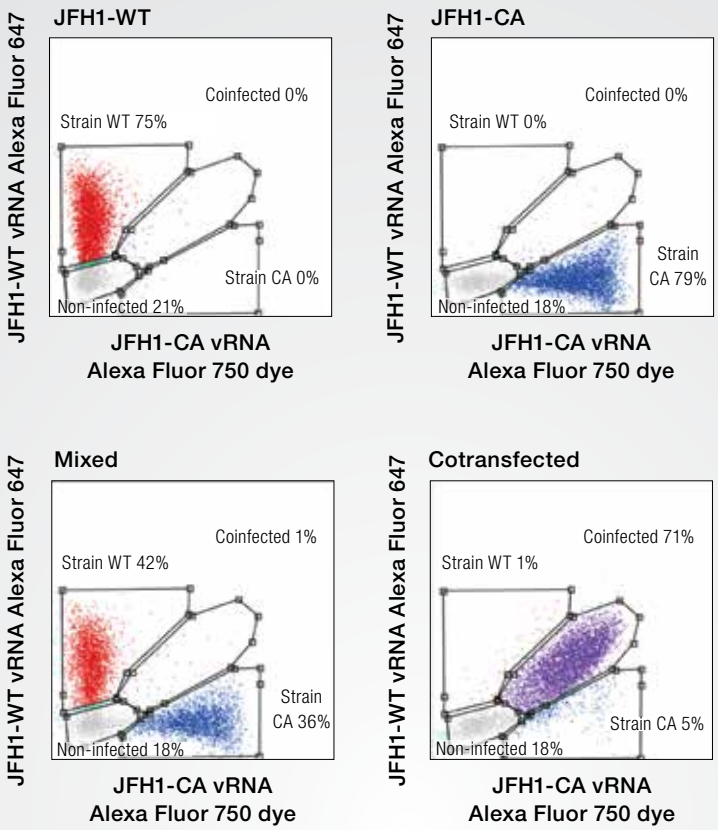


Figure 6. Coinfected viral RNA proof of principle.* Hepatocytes were infected with JFH1-WT strain (top left) or JFH1-CA (top right). Hepatocytes from these two single infections were mixed post-infection (bottom left), or hepatocytes were coinfected with JFH1-WT and JFH1-CA (bottom right). Using the Invitrogen™ QuantiGene™ FlowRNA Assay, hepatocytes were analyzed for strain-specific viral RNA.

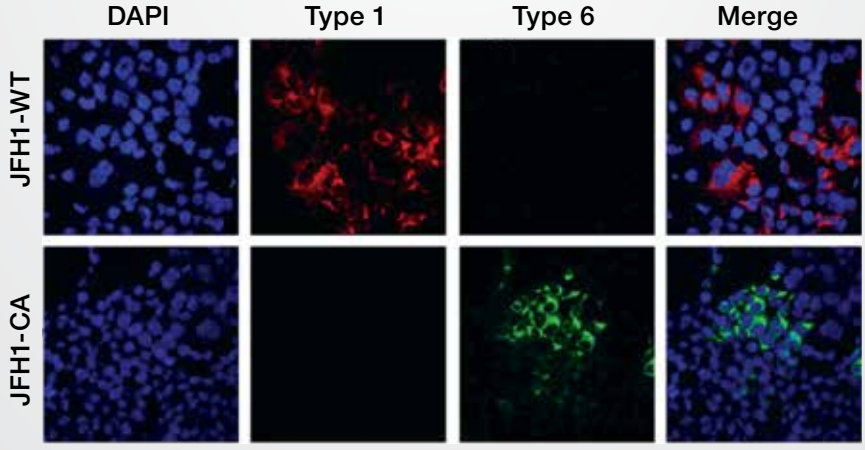


Figure 7. Visualization of specificity.* Hepatocytes infected with JFH1-WT (top row) or JFH1-CA (bottom row) were analyzed using the ViewRNA Assay and the same target probe sets used in the PrimeFlow RNA Assay.

* Data courtesy of Nicholas J. van Buuren and Karla Kirkegaard (PI), Stanford University School of Medicine. Data obtained using version 1 of the QuantiGene FlowRNA Assay.
The QuantiGene FlowRNA Assay and PrimeFlow RNA Assay have the same RNA hybridization and bDNA amplification protocol.

Make new discoveries and challenge current conventions

Most diseases, such as cancer, are complex and highly heterogeneous. With its ability to look at millions of single cells in a high-throughput manner, flow cytometry is one of the most prevalent technologies used to study complex diseases. However, there is still a great need to improve therapeutic targeting and efficacy through improved understanding of disease states. RNA detection, teamed together with intracellular and cell-surface antibody staining, elevates the understanding of single-cell dynamics to a new dimension, allowing researchers to further unmask these complex diseases with a unique tool.

Cell cycle regulation

Aims

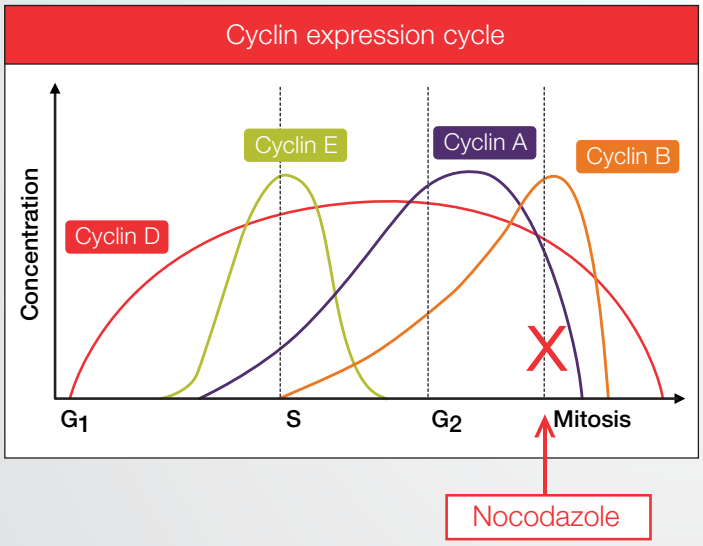
Proliferation and cell cycle progression are tightly controlled biological processes mediated by cyclins and cyclin inhibitors. Each phase of the cell cycle can be characterized by the expression of specific cyclins; however, cells must be synchronized *in vitro* in order to assay the kinetics of cyclin expression, using bulk sample methods currently available. Here, the PrimeFlow RNA Assay is applied to the detection of cyclins in unsynchronized U937 cells, untreated or treated with nocodazole, allowing for analysis of cells in different stages of the cell cycle.

Results

U937 cells showed upregulation of cyclin A and cyclin B mRNA, but not of cyclin E mRNA, in treated cells when compared to untreated cells. Expression of the positive control gene (GAPDH) was minimally affected by nocodazole treatment. Therefore, the mechanics of nocodazole were confirmed with the PrimeFlow RNA Assay (Figure 8).

Conclusions

The PrimeFlow RNA Assay answers previously unanswerable questions in numerous fields, from immunology to cancer research to cell biology. Life happens at a single-cell level but also as a collection of millions of cells working together in complex environments. The assay is the first and only tool that combines single-cell resolution with the acquisition of millions of cells to study both RNA and protein expression, allowing researchers to create new discoveries and challenge current conventions.



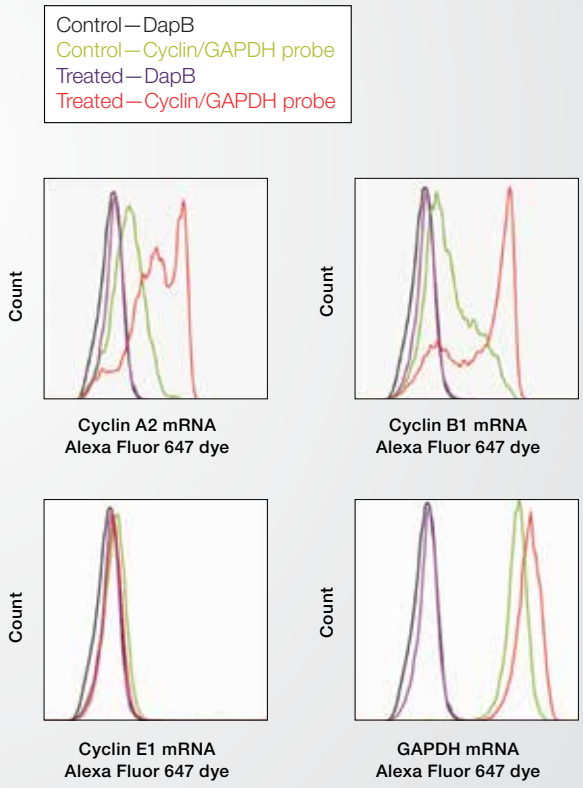


Figure 8. Cyclin detection in unsynchronized U937 cells. U937 human monocytic cells were untreated (black and green histograms) or treated with nocodazole for 16 hours (purple and red histograms). Nocodazole disrupts microtubules, leading to cell cycle arrest at the G_2/M checkpoint. After stimulation, cells were analyzed using the PrimeFlow RNA target probe sets for cyclin A2, cyclin B1, or cyclin E1. The target probe set for GAPDH was used as a positive control.

Ordering information

Required products for the PrimeFlow RNA Assay

mRNA/lncRNA detection

- **PrimeFlow RNA Assay Kit**—contains all the reagent needed to conduct the assay
- **Invitrogen™ PrimeFlow™ Probe Sets**—target-specific probes (labeled with Alexa Fluor 488, Alexa Fluor 568, Alexa Fluor 647, and Alexa Fluor 750 dyes); see the PrimeFlow RNA Probe Set catalog

miRNA detection

- **PrimeFlow RNA Assay Kit**—contains all the reagent needed to conduct the assay
- **PrimeFlow Probe Sets**—target-specific probes; due to the short length of miRNA, the signal generated per molecule of miRNA is expected to be significantly lower than mRNA. Therefore, it is highly recommended to use Alexa Fluor 647 or Alexa Fluor 568 dye, for detection of miRNA for maximal sensitivity.
- **Invitrogen™ PrimeFlow™ microRNA Pretreatment Buffer**—improves the retention of some small RNA targets, resulting in higher signal and better sensitivity

Optional kits, each sold separately

- **Positive control**—use positive-control probe sets in every experiment to ensure proper assay performance: RPL13a for human leukocytes and β-actin (ACTB) for mouse tissues; for specific cell types and other recommended genes, refer to Appendix 5 in the product manual of the PrimeFlow RNA Assay Kit
- **Negative control**—such as samples with the target-specific probe omitted, or samples labeled with a probe against a target not expressed in the cells of interest (e.g., dapB, a bacterial gene); highly recommended
- **Temperature validation kit**—the Invitrogen™ ViewRNA™ Temperature Validation Kit uses a calibrated thermometer to assess the accuracy of the temperature of the incubator used in the assay
- **Incubator**—capable of maintaining temperature at 40 ± 1°C
- **Metal heat block for 1.5 mL microcentrifuge tube**—placed inside the validated incubator
- **Antibodies for protein detection**—see **thermofisher.com/antibody**

Product	Quantity	Cat. No.
PrimeFlow RNA Assay Kit	40 tests	88-18005-204
	100 tests	88-18005-210
PrimeFlow microRNA Pretreatment Buffer	100 tests	88-18006

Product	Quantity	Cat. No.
ViewRNA Temperature Validation Kit	1	QV0523

PrimeFlow Probe Sets

PrimeFlow Probe Sets consist of target-specific oligonucleotide pairs, preamplifier oligonucleotide, and amplifier oligonucleotide. Four types of Alexa Fluor dye—labeled probe sets are currently available to enable detection of RNA: Alexa Fluor 647 (type 1 probe sets), Alexa Fluor 488 (type 4 probe sets), Alexa Fluor 750 (type 6 probe sets), and Alexa Fluor 568 (type 10 probe sets) dyes. Go to **thermofisher.com/primeflow** to view a complete listing of over 8,200 synthesized probe sets.

Probe sets

Product	Size	Cat. No.
PrimeFlow Probe Sets, mRNA/lncRNA	40 tests	PF-204
	100 tests	PF-210
PrimeFlow Probe Sets, miRNA	40 tests	PM-204
	100 tests	PM-210

Custom probes

PrimeFlow Probe Sets can be designed and synthesized. Please go to **thermofisher.com/custom-bdna** and provide the following information when ordering: accession number (including version or GI number) or RNA sequence for the target of interest, species, gene name or symbol, PrimeFlow Probe Set type, and any special design requirements.

Assay details

Specification	Description
Sample type	Single-cell suspensions, including human whole blood and peripheral blood mononuclear cells (PBMC), mouse dissociated tissues, and cell lines. See Appendix 5 in the product manual for a complete list of validated cell types.
Species	Mammalian
Multiplex level	Up to four RNA targets simultaneously
Assay format	1.5 mL microcentrifuge tube or 96-well v-bottom plate. See Appendix 7 in the product manual for the protocol for using 96-well plates.
Instrumentation	Flow cytometer equiped with: • Blue (488 nm), yellow-green (561 nm), and red (633–640 nm) lasers • Filter sets for FITC (bandpass 530/30), PE–eFluor 610 (PE–Texas Red) (bandpass 610/20), APC (bandpass 660/20), and APC–eFluor 780 (APC-Cyanine7) (bandpass 780/60)

invitrogen

Find out more at **thermofisher.com/primeflow**

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