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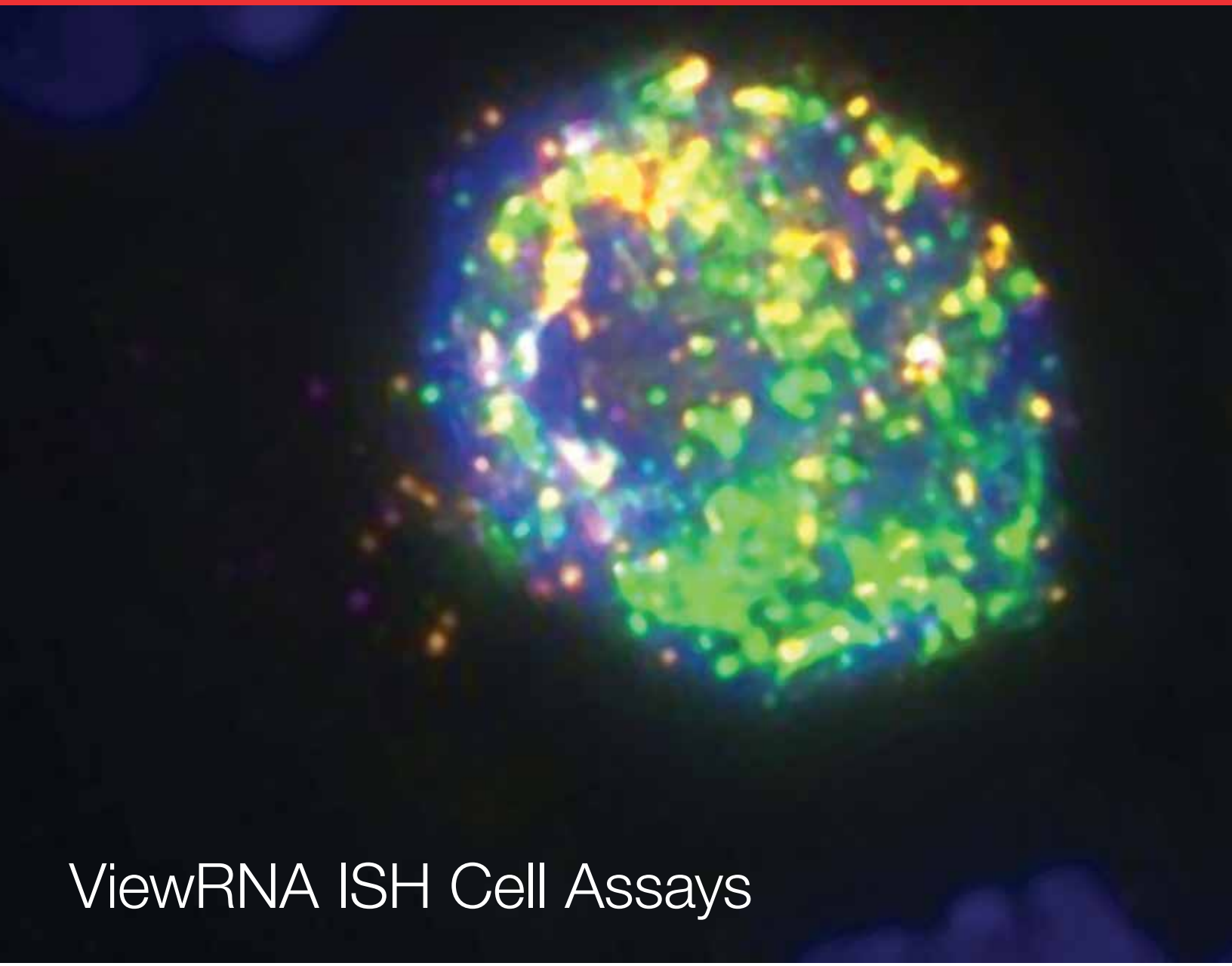

ViewRNA ISH Cell Assays

Visualize RNA with single-molecule sensitivity and single-cell resolution

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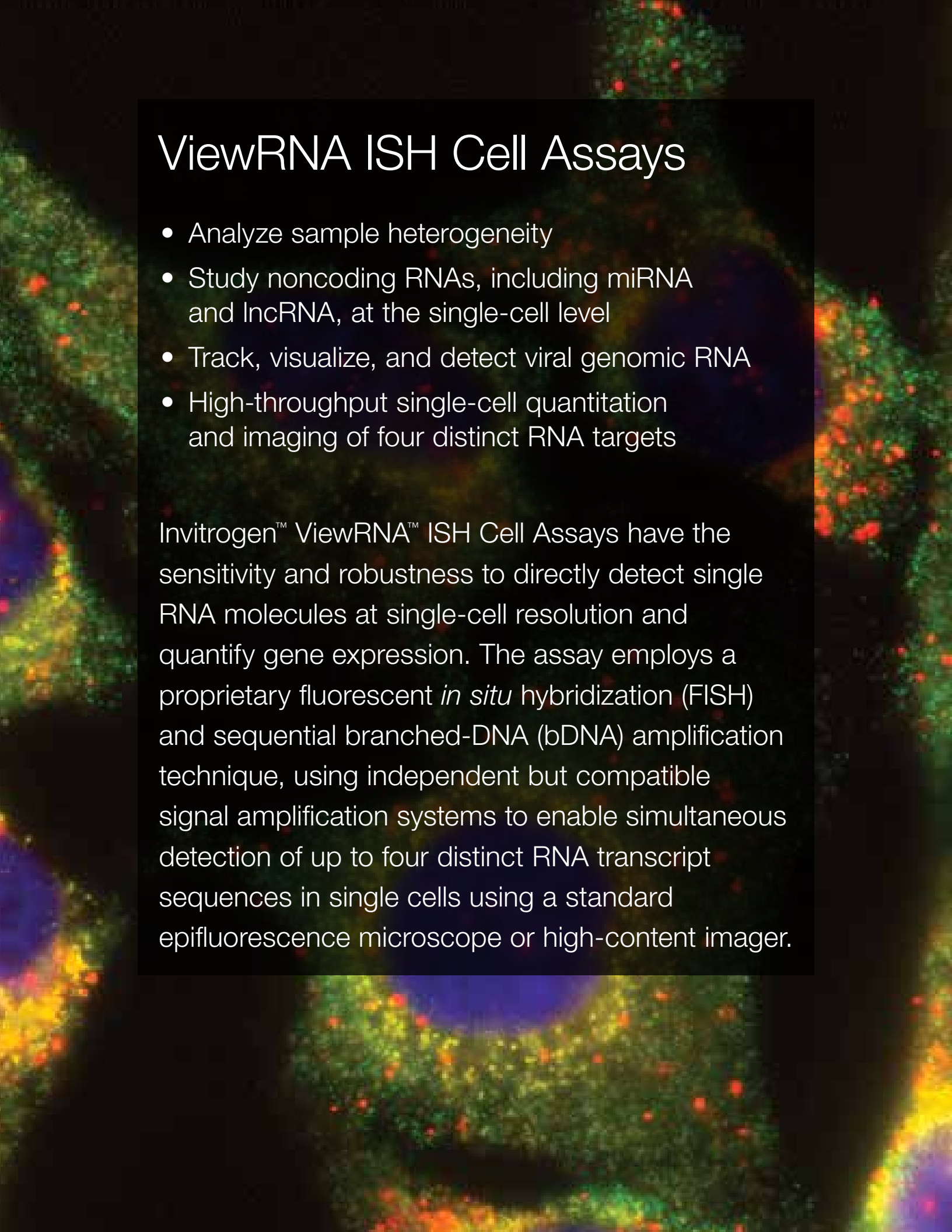


ViewRNA ISH Cell Assays

- Analyze sample heterogeneity
- Study noncoding RNAs, including miRNA and lncRNA, at the single-cell level
- Track, visualize, and detect viral genomic RNA
- High-throughput single-cell quantitation and imaging of four distinct RNA targets

Invitrogen™ ViewRNA™ ISH Cell Assays have the sensitivity and robustness to directly detect single RNA molecules at single-cell resolution and quantify gene expression. The assay employs a proprietary fluorescent *in situ* hybridization (FISH) and sequential branched-DNA (bDNA) amplification technique, using independent but compatible signal amplification systems to enable simultaneous detection of up to four distinct RNA transcript sequences in single cells using a standard epifluorescence microscope or high-content imager.

Assay principle

Fluorescent *in situ* hybridization (FISH) is a powerful technique that allows specific localization of ribonucleic acid (RNA) targets in fixed cells. Traditional FISH techniques using large oligonucleotide sequences labeled with 1–5 fluorophores are generally limited by high background and low sensitivity due to nonspecific binding and insufficient signal amplification.

ViewRNA ISH Cell Assays incorporate a proprietary probe set design and bDNA signal amplification technology. A target-specific probe set of approximately 20 oligonucleotide pairs hybridizes to the target RNA. Signal amplification is achieved through specific hybridization of adjacent oligonucleotide pairs to bDNA structures, formed by preamplifiers, amplifiers, and fluorophore-conjugated label probes, resulting in excellent specificity, low background, and high signal-to-noise ratios (Figures 1 and 2). ViewRNA ISH Cell Assays represent the most sensitive and specific RNA ISH method on the market.

In comparison, to create images with similarly discernible spots, traditional FISH can require a 600-times longer exposure and a 100-times greater camera gain than ViewRNA ISH Cell Assays. Furthermore, under equivalent imaging conditions, ViewRNA ISH Cell Assays are 100 times brighter, creating a minimum 2–3 times higher signal-to-noise ratio [1].

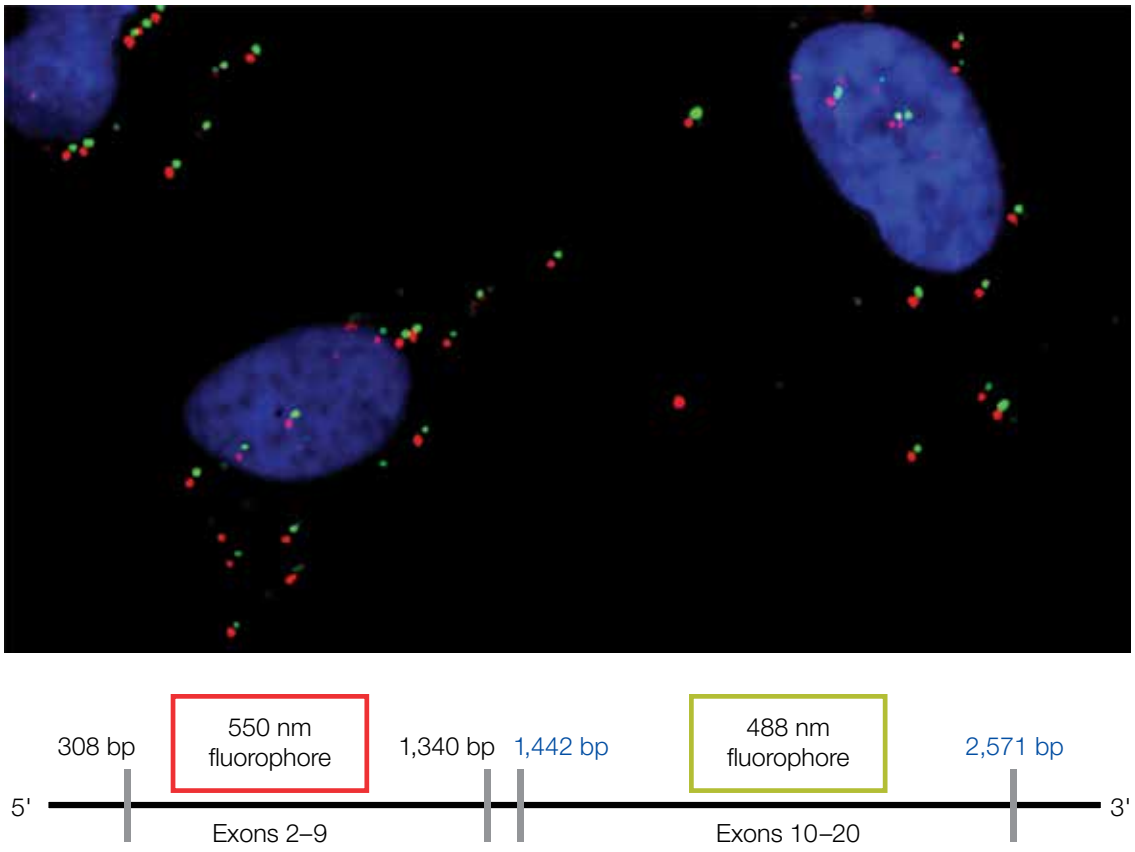


Figure 1. Validation of single-molecule detection. To demonstrate single-molecule sensitivity, where one spot corresponds to one target molecule, two probe sets were designed to target different regions of ERBB2 (Her2) mRNA. One probe set targeted the region from exons 2 through 9 (Invitrogen™ Alexa Fluor™ 546 dye, red spots) and the other probe set targeted the region from exons 10 through 20 (Invitrogen™ Alexa Fluor™ 488 dye, green spots). Although the two signals cannot be resolved on a single target, images were captured slightly offset to enable visualization of both signals. If one spot represents detection of one target, one would expect to see pairs of red and green spots as seen in the image. Nuclei were stained with DAPI (blue).

Key benefits

- **Efficient**—simultaneous analysis of up to four RNA targets per cell
- **Sensitive**—single-copy sensitivity at single-cell resolution
- **Customizable**—ability to design probe sets for any RNA target
- **Flexible**—robust results in adherent or suspension cells (see Table 1 for formats and specifications)
- **Fast**—1-day assay

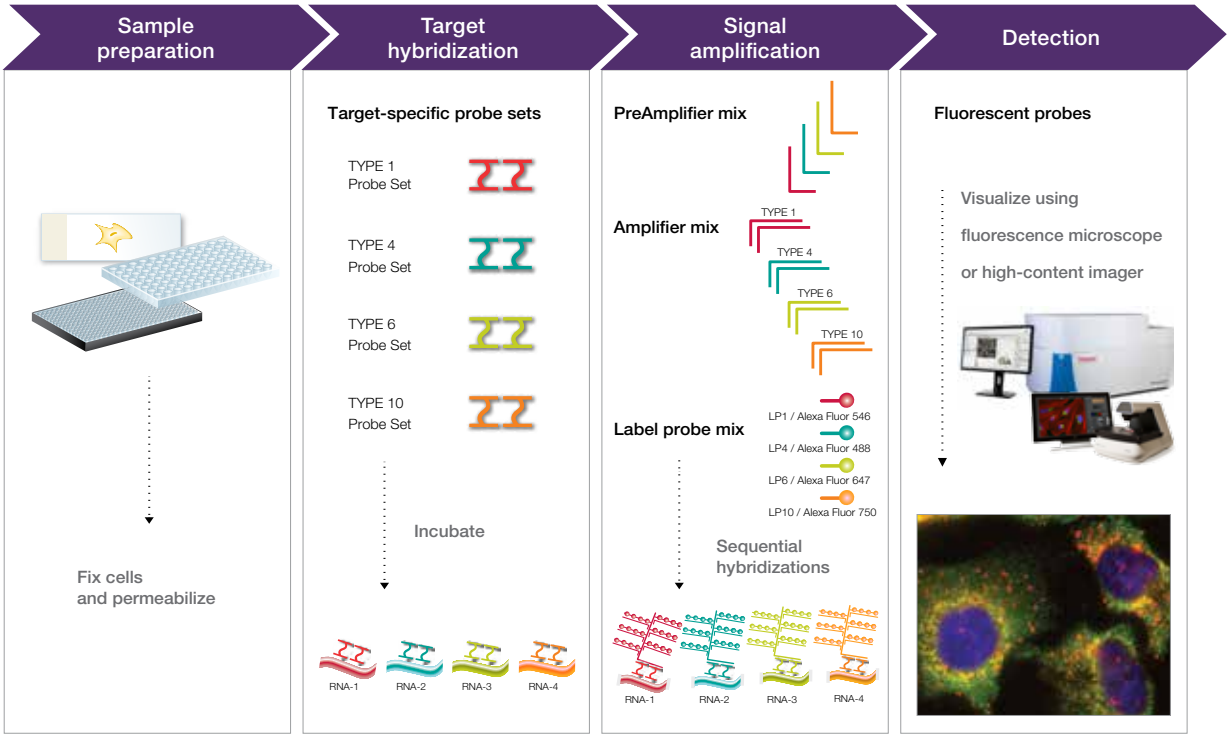


Figure 2. ViewRNA ISH Cell Assay workflow.

Table 1. ViewRNA formats and specifications.

	mRNA ISH	miRNA ISH	High-content ISH
Cell samples	Cultured cells (adherent or suspension), circulating tumor cells (CTCs)	Cultured cells (adherent or suspension)	Cultured cells (adherent or suspension)
Multiplexing	Up to four RNA targets	One miRNA and up to two mRNA targets	Up to four RNA targets
Detection signal	Fluorescence: Alexa Fluor™ 488, 546, 647, and 750 dyes	Fluorescence: Fast Red substrate for miRNA; Alexa Fluor 488 and 750 dyes for mRNA	Fluorescence: Alexa Fluor 488, 546, 647, and 750 dyes
Automation compatible	No	Yes	Yes
Instrumentation	Fluorescence microscopy or high-content imaging system	Fluorescence microscopy or high-content imaging system	High-content imaging system
Assay format	Coverslips mounted on slides or 96-well plates	Coverslips mounted on slides or 96-well plates	96- or 384-well plates

Analyze sample heterogeneity

Cellular heterogeneity is present in any biological sample. However, most of our understanding of gene expression is based upon bulk population averages from qPCR, microarrays, or sequencing methodologies. These methods can mask the presence of rare cells, ignore essential cell-to-cell differences, and obscure the importance of subcellular localization. To fully understand how gene expression levels among individual cells (such as those in cocultures or homogeneous cell lines that are cycling but not synchronized) contribute to biological function, a single-cell approach must be applied. ViewRNA ISH Cell Assays reveal the hidden story at the single-cell level.

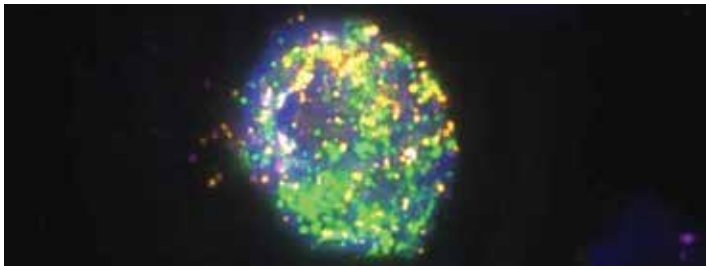


Figure 3. Identification of breast cancer CTCs from human bone marrow. A putative breast cancer tumor cell from a bone marrow sample was fixed and analyzed using the ViewRNA ISH Cell Assay for ERBB2 (green), pan-keratin panel (cytokeratin 8, 18, and 19) (yellow), and BACT (purple). The technology was used to identify the only two such CTCs on the slide. Data courtesy of Radium Hospital, Oslo, Norway.

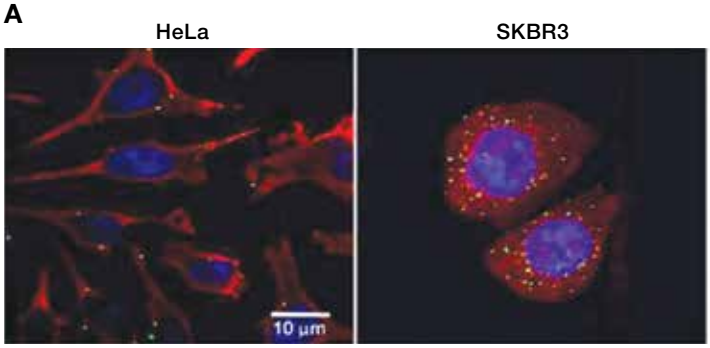
Unmasking rare cells

Circulating tumor cells (CTCs) are cells that detach from a primary tumor and circulate in the bloodstream. With only 1–10 CTCs per milliliter of whole blood, isolation becomes challenging because hundreds of thousands of white blood cells may contaminate the sample. Single-cell approaches with the highest specificity, rather than bulk preparations, must be applied. Because ViewRNA ISH Cell Assays can be utilized downstream of enrichment processes, they provide a robust four-parameter analysis of the rare and highly specific cell population (Figure 3).

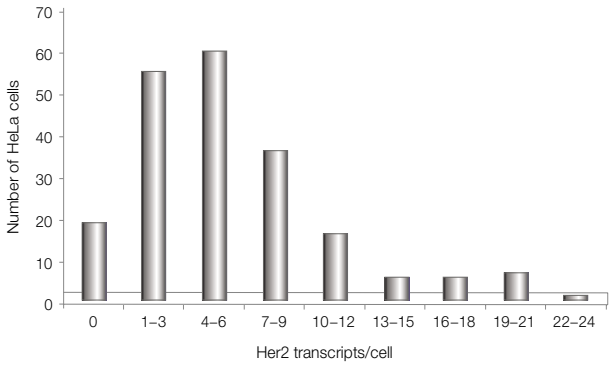
Population heterogeneity

mRNA is commonly assayed by reverse transcription quantitative PCR (RT-qPCR) or lysate-based bDNA technology, such as the Invitrogen™ QuantiGene™ Plex Assay. Although informative, these techniques result in bulk measurements that mask the differences in gene expression that occur at the single-cell level. In Figure 4, quantitation of Her2 transcripts in HeLa cells and SKBR3 cells is analyzed using both the ViewRNA ISH Cell Assay and the QuantiGene Plex Assay. The ViewRNA ISH Cell Assay accurately detected Her2 transcripts in HeLa cells known to express an average of 4–6 copies per cell, and in SKBR3 breast cancer cells known to have higher expression levels of Her2 (Figure 4A). Her2 spots were counted in 200 HeLa cells and plotted in a histogram (Figure 4B). The histogram of Her2 expression derived from 200 cells clearly indicates a high degree of expression variation from cell to cell, with a range of expression from 0 to 21 transcripts/cell and an average of 5.4 transcripts/cell (Figure 4B). The QuantiGene Plex Assay was used to confirm these measurements, and the result (average of 6.4 transcripts/cell) correlated well with the results of the ViewRNA ISH Cell Assay (Figure 4C).

A



B



C

Assay	Average number of transcripts/cell
ViewRNA ISH Cell Assay	5.4
QuantiGene Plex Assay	6.4

Figure 4. Visualization and quantitation of transcript heterogeneity in cell lines. (A) Multiplex analysis of Her2 mRNA (green) and control 18S rRNA (red) in HeLa cells and SKBR3 cells. Nuclei were stained with DAPI (blue) and visualized by fluorescence microscopy. **(B)** Histogram of Her2 expression per HeLa cell, based on counting spots per cell for 200 cells. **(C)** Comparison of Her2 expression results from the ViewRNA ISH Cell Assay vs. the QuantiGene Plex Assay.

Cells within a population are expected to respond differentially to external stimuli. While bulk averaging methods will identify the set of genes regulated by the stimuli, the ViewRNA ISH Cell Assay reveals the unique response in each cell and discerns the population heterogeneity (Figure 5).

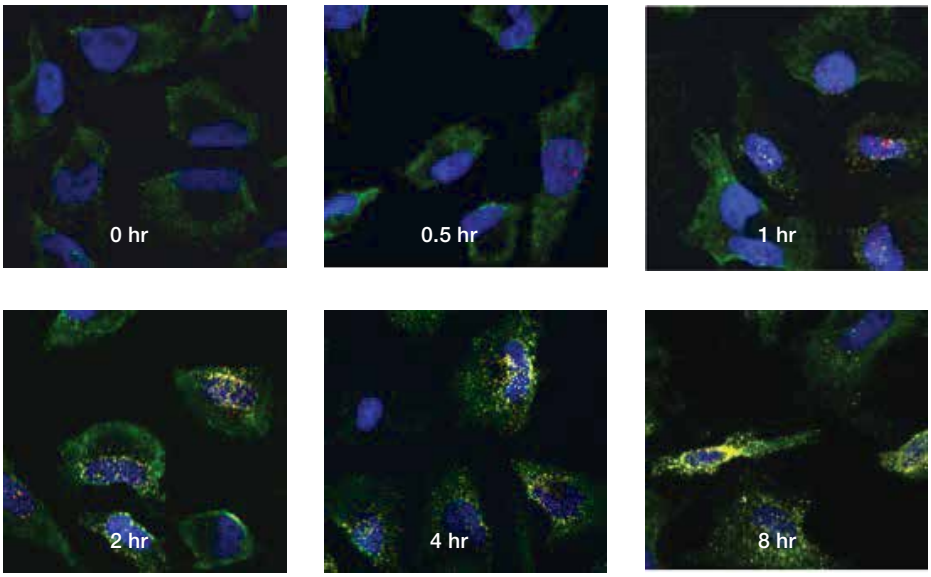


Figure 5. Time course of gene expression after cytokine induction, at the single-cell level. HeLa cells were treated with PMA for 0–8 hours. Then the cells were fixed and analyzed using the ViewRNA ISH Cell Assay for induction of IL-6 (red) and IL-8 expression (green). Nuclei were stained with DAPI (blue). The results show post-induction peaks at 1–2 hours for IL-6 and 4 hours for IL-8. Furthermore, the assay reveals the heterogeneity of individual cell responses.

Track, visualize, and detect viral genomic RNA

Numerous methods exist to detect and study viruses, each with benefits and limitations. Historically, single-cell approaches (e.g., immunofluorescence and flow cytometry) have used antibodies against viral proteins. Antibodies, however, can lack viral strain specificity, and viral proteins are only present after the virus has commandeered host cell function. Genomic approaches such as RT-qPCR, multiplex Luminex® assays, microarrays, and sequencing are highly sensitive assays at the bulk population level.

Other assays use viral-driven expression of a recombinant fluorescent reporter protein (e.g., GFP). This approach provides a very useful tool for single, live-cell imaging, but requires time postinfection to allow for reporter protein translation. ViewRNA ISH Cell Assays can detect and localize viral genomic RNA within individual cells, facilitating a precise fixed–time point analysis of the viral life cycle at the level of transcriptional control (Figures 6 and 7).

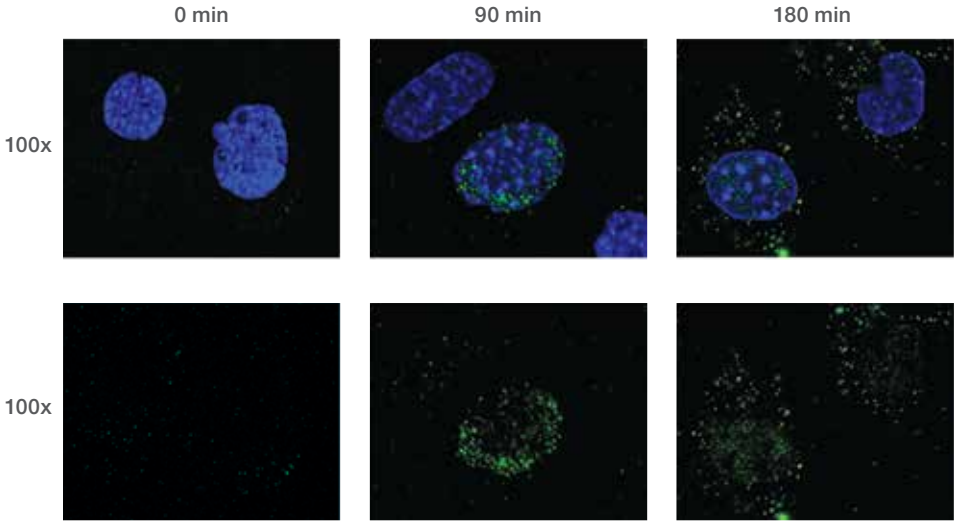


Figure 6. Detection of H1N1 influenza A RNA migration in cells. Nuclear translocation of viral RNA is necessary for replication of the influenza virus. Murine embryonic fibroblasts (MEFs) were incubated on ice with influenza A virus (H1N1, PR8 strain). At time 0, virus was removed and medium (warmed to 37°C) was added. At the indicated time points after warming, the cells were fixed and processed using the ViewRNA ISH Cell Assay Kit with a probe set against the nucleoprotein (NP) viral genomic segment (green). Nuclei are stained blue with DAPI. Time-dependent nuclear translocation of the NP genome is seen at 90 min post-infection, and by 180 min the export of newly synthesized viral genomes from the nuclei to the cytosol is also observed. Data courtesy of Dr. Abraham L Brass, Ragon Institute of MGH, MIT, and Harvard.

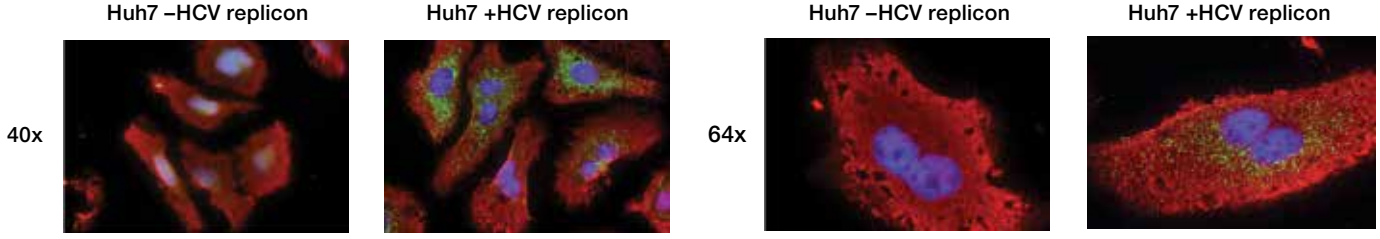


Figure 7. Multiplexing of viral RNA and mRNA in Huh7 cells. Multiplex fluorescence *in situ* detection of HCV genomic viral RNA (green) and 18S rRNA (red) in Huh7 cells lacking (–HCV) or containing (+HCV) a hepatitis C virus (HCV) replicon. Nuclei are stained with DAPI (blue).

Single-cell analysis of noncoding RNAs, including miRNA and lncRNA

Approximately 75% of the human genome can be transcribed into RNA; however, only 1.5% of the human genome codes for mRNA, which typically gets translated into protein. The majority of nontranslated RNA or noncoding RNA is functionally involved in many cellular processes. Two critical epigenetic regulators are long noncoding RNA (lncRNA), which often binds to chromatin-modifying proteins to help regulate gene expression, and microRNA (miRNA), short RNAs that have emerged as key translational regulators for 30% of all protein-coding genes in diverse biological processes.

With advances in transcriptomic techniques, researchers have been able to identify, profile, validate, and functionally analyze relevant noncoding RNAs in different models and diseases. However, analysis at the single-cell level, especially for miRNA, has been limited by low sensitivity and poor resolution. Invitrogen™ ViewRNA™ ISH Cell and ViewRNA™ miRNA ISH Cell Assays enable the simultaneous visualization and quantitation of noncoding RNA and miRNA, with single-molecule sensitivity and single-cell resolution (Figure 8). For the ViewRNA miRNA ISH Cell Assay, an miRNA target probe set covering the entire mature miRNA sequence is used in combination with bDNA and Fast Red substrate signal amplification for increased sensitivity.

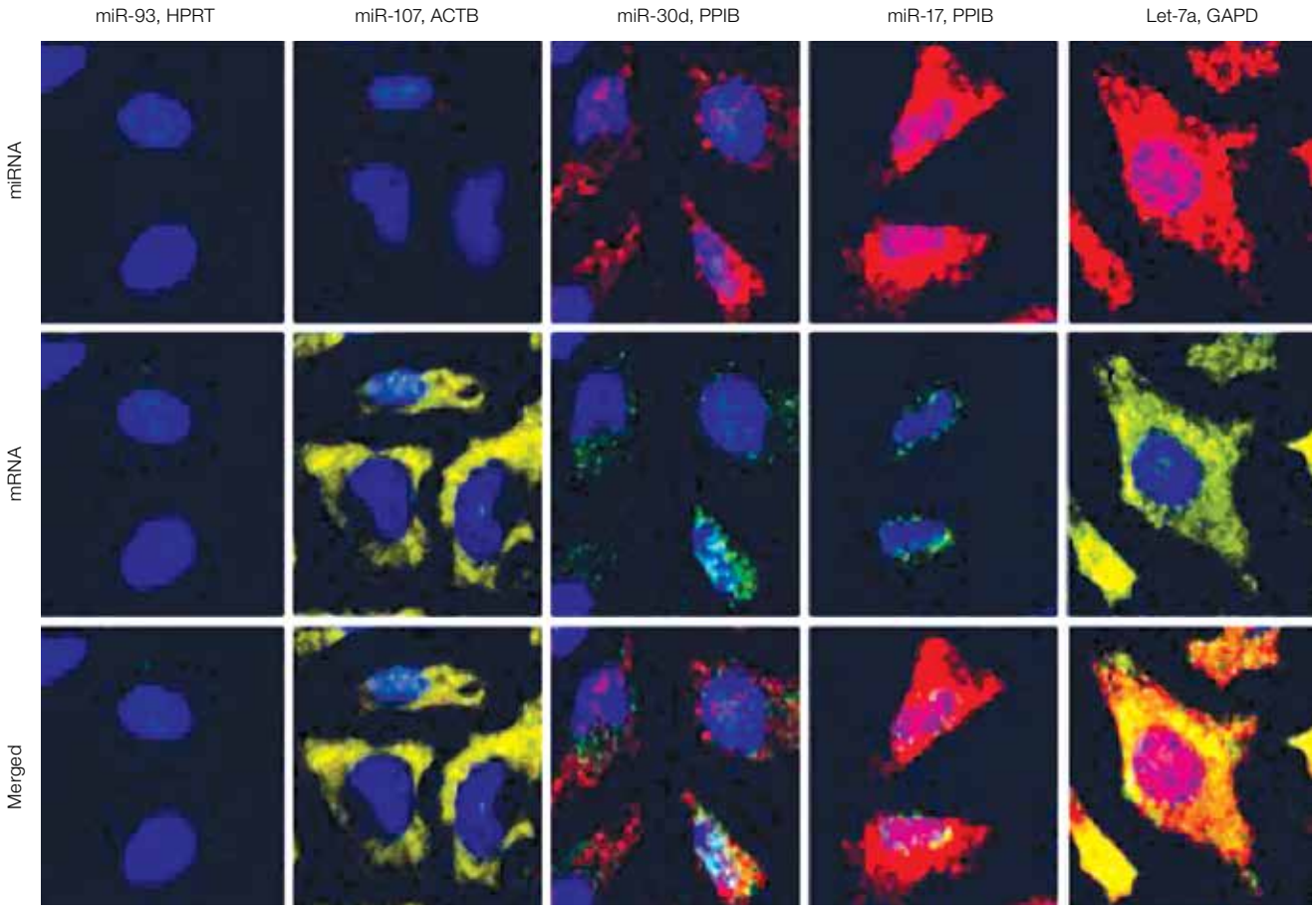


Figure 8. Multiplex analysis of miRNA and mRNA of various expression levels. Simultaneous detection of miRNA (Fast Red substrate, Cy®3/550 channel) and mRNA (in FITC channel or in Cy®7 channel) expression in any given cell. Shown are duplex assays showing expression of different miRNA and mRNA targets in HeLa cells. HPRT, ACTB, and PPIB were detected in the FITC channel, and GAPD was detected in the Cy7 channel. Based on known expression levels, the data confirm accurate and precise study and visualization of miRNA and mRNA of various expression levels, where one distinct spot corresponds to one molecule of an miRNA or mRNA target.

Circular RNAs (circRNAs) are a class of low-abundance cellular RNAs with no 5′ or 3′ end. Neuronal circRNAs are composed of protein-coding exons, but do not appear to be translated. With no poly(A) tail and no protein expression, circRNAs are difficult to detect. A team at the Berlin Institute for Medical Systems Biology, Max Delbrueck Center, and Max Planck Institute believe they may be the first ever to have visualized individual circRNA species directly in mouse hippocampus using ViewRNA technology (Figure 9) [2].

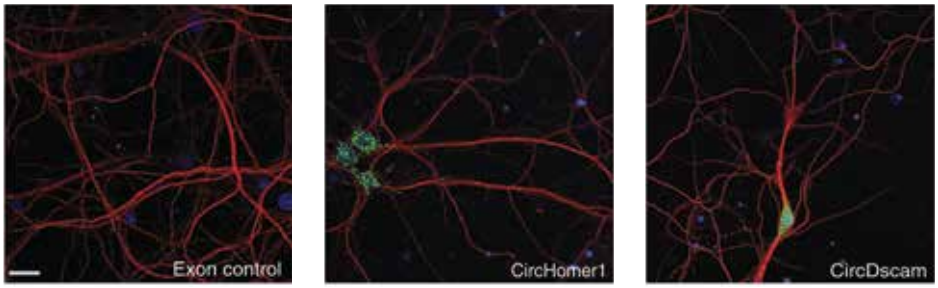
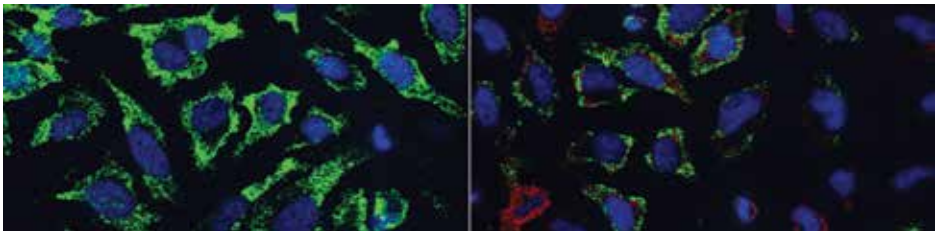


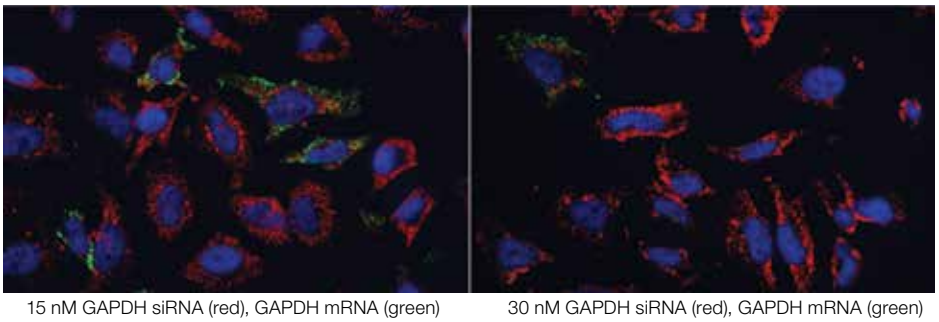
Figure 9. Visualization of circular RNA. A ViewRNA miRNA ISH Cell Assay was adapted to detect the indicated circRNA (green) in cultured hippocampal neurons. CircRNA is present both in the cell bodies surrounding nuclei (DAPI, blue) and in dendritic processes (visualized with anti-MAP2 antibody, red). A negative control exon probe that was designed to detect noncontiguous regions was used to assess background. Adapted by permission from Macmillan Publishers Ltd: *Nature Neuroscience*, advance online publication, 25 February 2015 (doi:10.1038/nn.3975).

ViewRNA miRNA ISH Cell Assays can also detect other small noncoding RNA molecules such as small interfering RNA (siRNA) and circRNA (Figure 10). Direct visualization of siRNA in single cells after transfection and target gene knockdown enables fast and easy monitoring of siRNA knockdown efficiency.



0 nM GAPDH siRNA (red), GAPDH mRNA (green)

7.5 nM GAPDH siRNA (red), GAPDH mRNA (green)



15 nM GAPDH siRNA (red), GAPDH mRNA (green)

30 nM GAPDH siRNA (red), GAPDH mRNA (green)

Figure 10. Visualize and quantitate siRNA knockdown efficiency *in situ*. HeLa cells were transfected with various concentrations of GAPDH siRNA. Following transfection, cells were analyzed for GAPDH siRNA and GAPDH mRNA. The images clearly show that the level of GAPDH knockdown is directly proportional to the level of siRNA present in the cells as detected by the ViewRNA miRNA ISH Cell Assay. Nuclei were stained with DAPI (blue).

High-throughput single-cell quantitation and imaging of four RNA targets

The Invitrogen™ ViewRNA™ High-Content Screening Assay is a 96- or 384-well plate-based assay, compatible with standard liquid-handling automation systems and high-content imaging systems, that can simultaneously detect four RNA targets in single cells (Figure 11). Unlike high-throughput PCR screening, image-based screening enables the study and subcellular localization of gene expression at the single-cell level in heterogeneous samples without the need to isolate specific cell types or the extraction, purification, and amplification of a transcript (Figures 12 and 13). This powerful platform can be used across the drug discovery pipeline, from primary screens of compound libraries to downstream companion assays in clinical trials.

- **Cost-effective**—high-content gene expression assay for screening
- **Single-copy sensitivity**—single-cell resolution for up to four RNA targets in 96- or 384-well plate formats
- **Flexible**—works with primary or cultured cells
- **Accurate**—direct detection of viral life cycle
- **Versatile**—alternative for reporter gene assays
- **Powerful**—ability to perform RNAi library screens
- **Fast**—1-day assay, specifically designed for automation
- **Compatible**—works with most automated high-content imaging systems

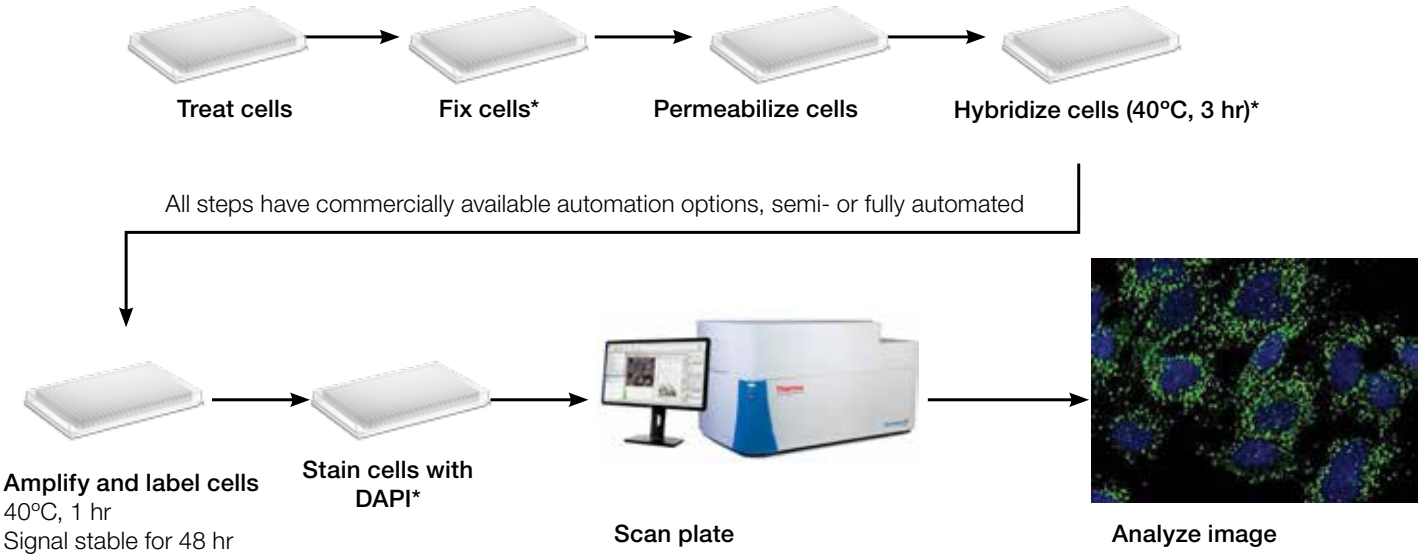


Figure 11. Workflow for using the ViewRNA High-Content Screening Assay with multiwell plates and high-content imaging systems.

Compound dose-response profiling

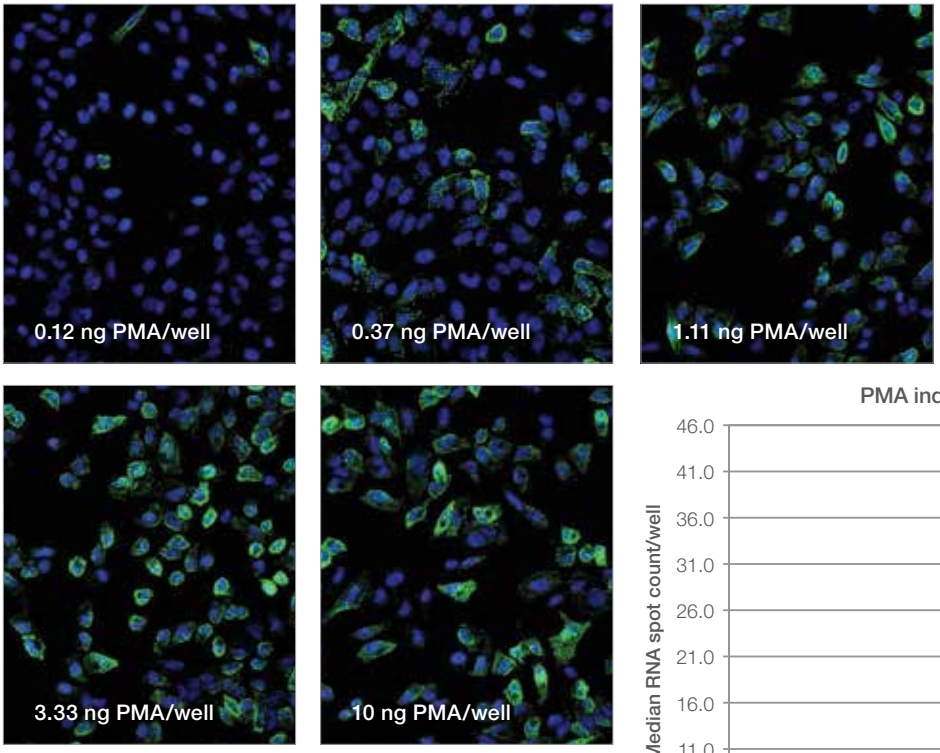


Figure 12. Visualization and quantitation of induction in cultured cells. HeLa cells were cultured in a 384-well clear-bottom plate and treated with various concentrations of PMA. Following a 4-hour PMA treatment, cells were fixed and analyzed for IL-8 expression (green). Nuclei were stained with DAPI (blue).

Compound screening with multiple markers

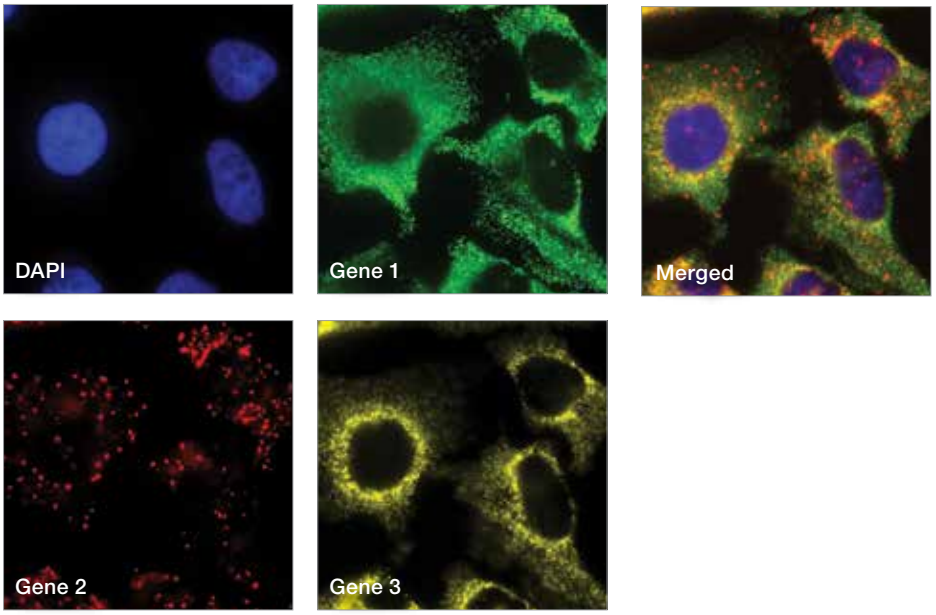


Figure 13. Multiplex analysis of compound screens. Individual images show nuclei and RNA targets: gene 1, gene 2, and gene 3. The merged image shows simultaneous detection of the three RNA targets. Nuclei were stained with DAPI (blue). Data courtesy of Dr. Dan Garza, Proteostasis, Inc.

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Ordering information

Required products for the ViewRNA ISH Cell Assay

- **Invitrogen™ ViewRNA™ ISH Cell Assay Kit**—contains all the assay reagents for signal amplification and detection required for visualization of 1–3 target RNAs (mRNA and lncRNA) using adherent or suspension cells
- **Invitrogen™ ViewRNA™ Probe Sets**—target-specific probes (TYPE 1, 4, 6, or 10); see the ViewRNA Probe Set catalog

Optional kits, each sold separately

- **Invitrogen™ ViewRNA™ ISH Cell 740 Module**—designed to be used in conjunction with ViewRNA ISH Cell Assays and allows analysis of an additional RNA target in the 740 channel
- **Invitrogen™ ViewRNA™ ISH Cell Accessory Kit**—intended to provide many of the components not supplied in the reagent kit but needed to perform the assay; see the package insert for a complete list of materials provided in the kit

ViewRNA ISH Cell Assay Kits

Product	Quantity	Cat. No.
ViewRNA ISH Cell Assay Kit	1*	QVC001
ViewRNA miRNA ISH Cell Assay Kit	1*	QVCM0001
ViewRNA ISH Cell 740 Module	1*	QVC0200
ViewRNA High Content Screening Assay Kit	Contact local account manager	

* 24 assays when using coverslips in a 24-well plate, 96 assays when using coverslips mounted on glass slides (additional 10X PBS, detergent solution, and wash buffer components are required), 16 assays when using the four-chambered dish format (four-chambered dish is required), or 144 assays when using a 96-well plate (96-well optical bottom plate is required).

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.

Validation kit

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

ViewRNA ISH Cell Assay accessories

Product	Quantity	Cat. No.
ViewRNA ISH Cell Accessory Kit	1	QVC0700
Hydrophobic Barrier Pen*	1	QVC0500
Forceps*	1	QVC0501
Tissue-Tek Staining Dish (Clear)*	1	QVC0502
Tissue-Tek Slide Rack*	1	QVC0503
Tissue Culture 24-Well Plate*	1	QVC0504
Microscope Slides*	12	QVC0505
Rectangular Cover Glass*	15	QVC0506
Cover Slips*	50	QVC0507
Four-Chambered Dish	10	QVC0510

* Included in the ViewRNA ISH Cell Accessory Kit.

ViewRNA Probe Sets

Invitrogen™ ViewRNA™ Probe Sets are designed for use with ViewRNA™ ISH Cell Assays, ViewRNA™ ISH Tissue Assays, and PrimeFlow™ RNA Assays. Visit our website to view a complete listing of over 6,500 synthesized probe sets. By request, new probe sets can be designed and synthesized in less than 2 weeks at no additional cost.

Probe sets

Product	Quantity	Cat. No.
ViewRNA Probe Sets, catalog*	30 assays**	VX#-#####-06
	110 assays**	VX#-#####-01
	360 assays**	VX#-#####-02
	1,800 assays**	VX#-#####-03
ViewRNA Probe Sets, by request†	110 assays**	VX1-99999-01
	110 assays**	VX4-99999-01
	110 assays**	VX6-99999-01
	110 assays**	VX10-99999-01

* Catalog: Available ViewRNA Probe Sets, where "X" identifies the species (A = human, B = mouse, C = rat, X = others); "#" corresponds to the ViewRNA Probe Set TYPE (1 for TYPE 1: 550 nm Ex; 4 for TYPE 4: 488 nm Ex; 6 for TYPE 6: 650 nm Ex; and 10 for TYPE 10: 740 nm Ex), and "#####" is a unique number associated with each target.
** Number of assays based on 24-well assay format.
† By request, ViewRNA Probe Sets can be designed and synthesized at no additional cost. Please 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, ViewRNA Probe Set TYPE, and any special design requirements. Please allow 5–7 days for delivery.

References

1. Battich N et al. (2013) Image-based transcriptomics in thousands of single human cells at single-molecule resolution. *Nat Methods* 10(11):1127–1133.
2. You X et al. (2015) Neural circular RNAs are derived from synaptic genes and regulated by development and plasticity. *Nat Neurosci* 18(4):603–610.

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