Automated Fluorescence Detection and Imaging of RNA Species in Live Cells

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Abstract

The determination of intracellular RNA levels is a critical component in elucidating the cellular responses of living cells to external stimuli. Many of the techniques traditionally used for RNA quantitation involve transfection, laborious sample preparation and RNA amplification, which can preclude large sample numbers. However, disease directed research, which often involves the screening of compound libraries, relies on the ability to rapidly make assay determinations on large numbers of samples. At the same time phenotypic information is also desired to assess the true cellular response. Towards that end, having multiple fluorescent probes capable of simultaneously detecting different cellular RNAs in live cells are of particular importance.

Here we describe the use of a combination microplate reader and imager to detect changes in RNA levels using a series of gold nanoparticle fluorescent probes (SmartFlare™ Probes from EMD Millipore). The multi-mode microplate reader is capable of digital microscopy and conventional microplate detection. Probes consist of a gold nanoparticle conjugated with multiple copies of double stranded oligonucleotides. The longer strand is complementary to the RNA target where as the shorter reporter strand contains a fluorescent molecule (CY3 or CY5) that is quenched when in proximity to the gold core. With exposure to the target RNA the reporter strand is displaced as the target strand binds to its complimentary strand located on the probe. Displacement removes the proximity associated quenching resulting in fluorescence. The degree of fluorescence is dependent on the amount of target RNA present.

Treatment of HeLa cells with positive and negative control probes, as well as probes specific to constitutively expressed housekeeping gene RNA demonstrates the utility of the technique. CY3 or CY5 detection probes can be specifically distinguished using either whole well PMT-based determinations or with image analysis in multiplex assays. Co-staining with a labeled antibody to the cell surface EGFR receptor in conjunction with a fluorescent probe for EGFR receptor in MCF-7 and SK-BR-3 cell lines which are positive and negative for the receptor respectively confirm the specificity of the technology. Cell stimulation with increasing serum concentrations results in a dose dependent increase in GAPDH RNA levels. Using this hardware reagent combination, several cell lines were screened for the presence or absence of cell line specific RNA.

Assay Process

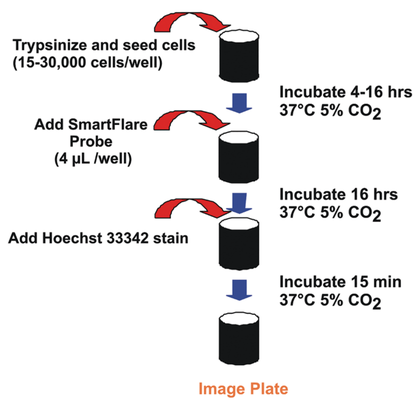

Figure 1 – *SmartFlare RNA Detection Probe Assay Process.*

BioTek Instrumentation

Figure 2 – *Cytation™ 3 Cell Imaging Microplate Reader with Injector Module.*

Molecular Mechanism

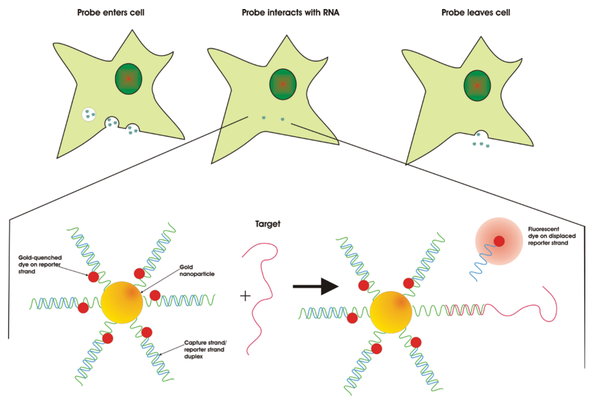

Figure 3 – Molecular Mechanism of SmartFlare RNA Detection Probe. *SmartFlare Probes are taken up by live cells where they interact with cellular RNA polymers. Over time, the probes are transported or diffuse out of the cell allowing the cells to be used for other experiments. SmartFlare probes consisting of gold nanoparticles conjugated to multiple copies of a double-stranded oligonucleotide, in which one strand (reporter) has a fluorophore that is quenched by its proximity to the gold core. Inside the cell, when the nanoparticle comes in contact with its target RNA, the target RNA binds to its complimentary capture strand and displaces the reporter strand. The unquenched reporter strand fluoresces and can be detected.*

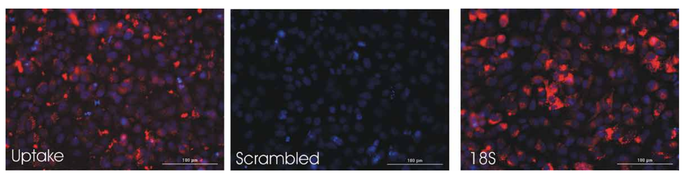

Figure 4 – CY3 Probe comparisons. *HeLa cells treated with CY3-Uptake, Cy3-Scramble, or CY3-18S probes were imaged using the Cytation 3 at the same exposure settings. The Uptake control has unquenched fluors tethered to the gold nanoparticle and subsequently is always "on" inside the cell, while the scramble probe does not recognize any sequence within the cell and is constitutively quenched or "off". The CY3-18S probe recognizes cellular 18s RNA.*

Probe Uptake and Signal Generation

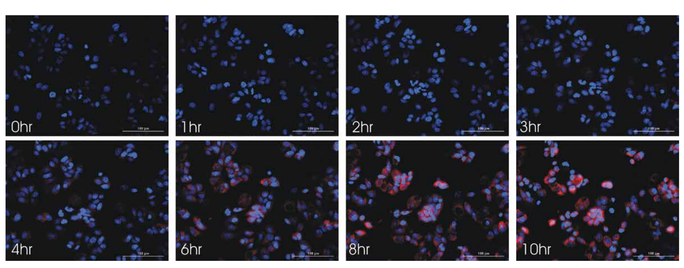

Figure 5 – Live Cell Imaging Time Course. *MCF-7 cells were seeded at 15,000 cells per well and grown overnight. The following day cells were stained with 0.5 µg/mL Hoechst 33342 dye for 15 min @ 37°C, treated with 4 µL CY3-GAPDH reagent and immediately imaged kinetically with the 20x objective using the DAPI and CY3 LED cube and the images overlaid. Scale bar indicates 100 µm.*

Probe Uptake and Signal Generation (*Cont.*)

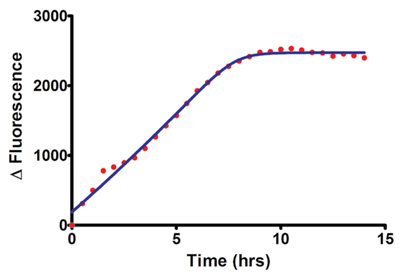

Figure 6 – Increase in CY3 Fluorescence in live MCF-7 cells. *MCF-7 cells were seeded at 15,000 cells per well and grown overnight. The following day cells were stained with 0.5 µg/mL Hoechst 33342 dye for 15 min @ 37°C, treated with 4 µL CY3-GAPDH reagent and immediately imaged every 30 minutes for 12 hours with the 20x objective using the DAPI and CY3 LED cube. The mean pixel intensity value of each image was recorded. Data point represents the average of three wells.*

Image Based Cellular Analysis

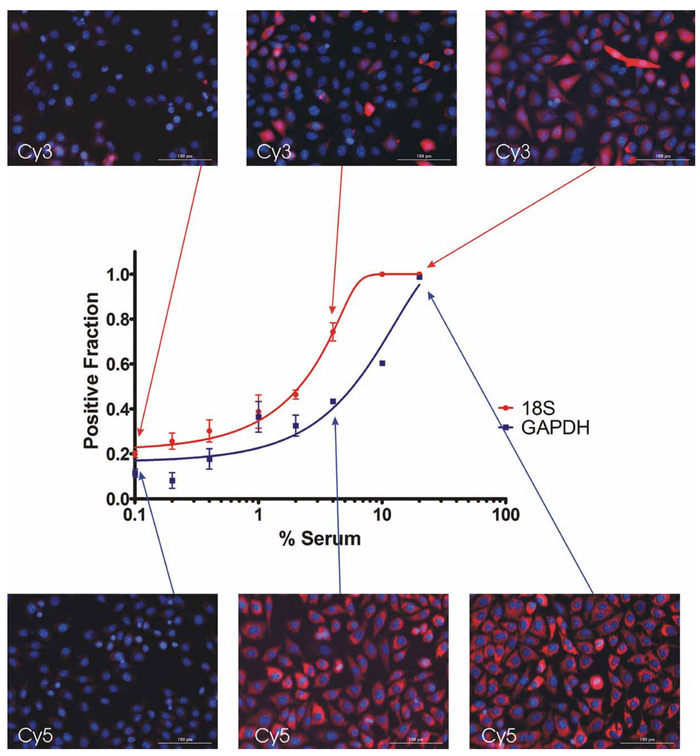

Figure 7 – Live Cell Image Analysis of cells treated with various concentrations of serum. *MCF-7 cells were seeded at 30,000 cells per well and treated with CY3-18S or CY5-GAPDH reagent. After 16 hour incubation, cells were stained with 5 µg/mL Hoechst 33342 for 15 min @ 37°C cells were imaged with the 4x and 20x objective using the DAPI and CY3 and CY5 LED cubes. Image object and subpopulation analysis were performed and the fraction of CY3 (18S) and CY5 (GAPDH) positive cells plotted as a function of serum concentration. 20x cell images of DAPI and either CY3 or CY5 images overlaid from indicated serum levels are shown. Scale bar indicates 100 µm.*

Cellular Localization

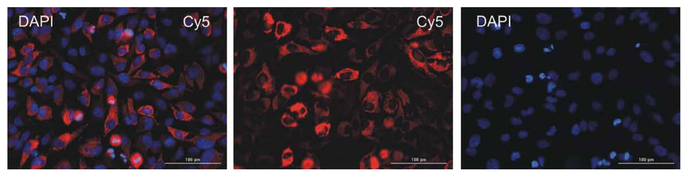

Figure 8 – Cytoplasmic localization of SmartFlare signal. *HeLa cells were seeded at 30,000 cells per well and treated with 4 µL CY5-Uptake reagent. After a 16 hour incubation, cells were stained with 5 µg/mL Hoechst 33342 for 15 min @ 37°C. Cells were imaged with the 20x objective using the DAPI and CY5 LED cube and the images overlaid. Images in the center and on the left have DAPI and CY5 data removed from the overlay respectively. Scale bar indicates 100 µm.*

Composite images with DAPI and CY5 signals demonstrate both fluorescent signals in HeLa cells. Hoechst 33342 stain binds DNA and indentifies cellular nuclei, while SmartFlare positive CY5-uptake control is seen primarily in the cytoplasm when composite images are separated.

Materials and Methods

Cell Culture

HeLa, MCF-7, NIH 3T3, and SK-BR-3 cells were cultured in Advanced Dulbecco Modified Eagle's medium (DMEM) supplemented with 10% fetal bovine serum and penicillin-streptomycin at 37°C in 5% CO_2. Cultures were routinely trypsinized (0.05% Trypsin-EDTA) at 80% confluency.

Cell Seeding

Cells were plated at a density of 15-30,000 cells per well in 100 µL growth medium into Corning black sided clear bottom 96-well plates (P/N 3904). Cells were allowed to attach and grow for 4-16 hours at 37°C in 5% CO_2.

Microplate Reading

Whole well PMT-based fluorescence intensity measurements were made using a Cytation 3 Cell Imaging Microplate Reader. All measurements were made from the bottom using twin dual grating monochromators. Cellular CY3 fluorescence was determined with an excitation of 545 nm and an emission 575 nm, CY5 was determined with at 635 nm and 670 nm excitation and emission wavelengths respectively. Likewise Hoechst 33342 staining was determined using an excitation wavelength of 360 nm and an emission wavelength of 460 nm. In order to reduce fluorescence variation as a result of intra-well cell density variation all wells were measured using the mean of a 3 x 3 area scan array.

Imaging

Cells were imaged using a Cytation 3 Cell Imaging Microplate Reader (BioTek Instruments) configured with DAPI, RFP and CY5 light cubes. The imaging microplate reader uses a combination of LED light sources in conjunction with band pass filters to provide appropriate wavelength light. The DAPI light cube is configured with a 357/50 excitation filter and a 447/60 emission filter; the RFP light cube uses a 531/40 excitation and 593/40 emission filters and the CY5 cube uses a 628/40 excitation and a 685/40 emission filters. Exposure settings were automatically determined for each color independently and fixed for all subsequent exposures, while focus was provided automatically on each well using the DAPI signal. The reader was controlled and data captured and analyzed using Gen5™ Data Analysis Software.

Signal Independence

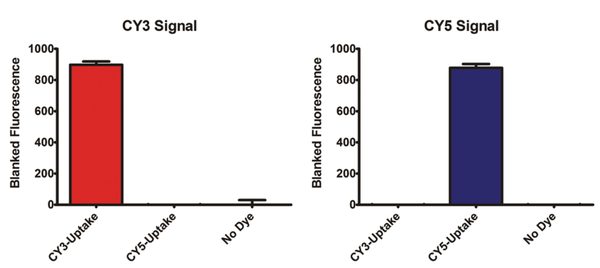

Figure 9 – CY3 and CY5 Probe Plate Read Signal Independence. *Uptake probes for both CY3 and CY5 were added to HeLa cells and the fluorescence measured using conventional top read whole well detection.*

Signal Independence (*Cont.*)

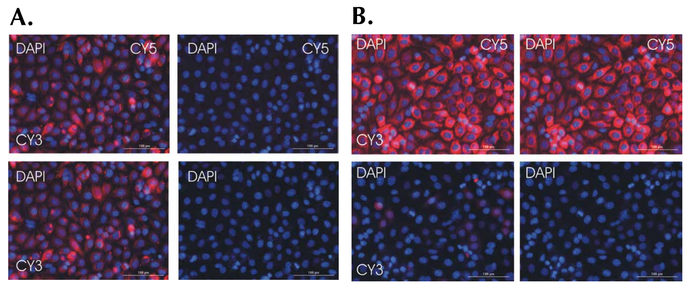

Figure 10 – Signal Independence of Live Cell imaging of HeLa cells treated with SmartFlare Reagents. *HeLa cells were seeded at 30,000 cells per well and treated with (A) CY3-18S or (B) CY5-GAPDH reagent. After 16 hour incubation cells were stained with 5 µg/mL Hoechst 33342 for 15 min @ 37°C cells were imaged with the 20x objective using the DAPI and CY3 and CY5 LED cubes and the images overlaid. Composite images consist of DAPI, CY3, and CY5 signals overlaid. Subsequent images represent the same image with one or more signals removed. In each image, the image signal present is indicated. Scale bar indicates 100 µm.*

The CY3 and CY5 probe signals show little bleed over when HeLa cells are treated with separate CY3 and CY5 probes for house keeping genes. This indicates that these two different probes can be multiplexed.

Cell Line Specific Expression

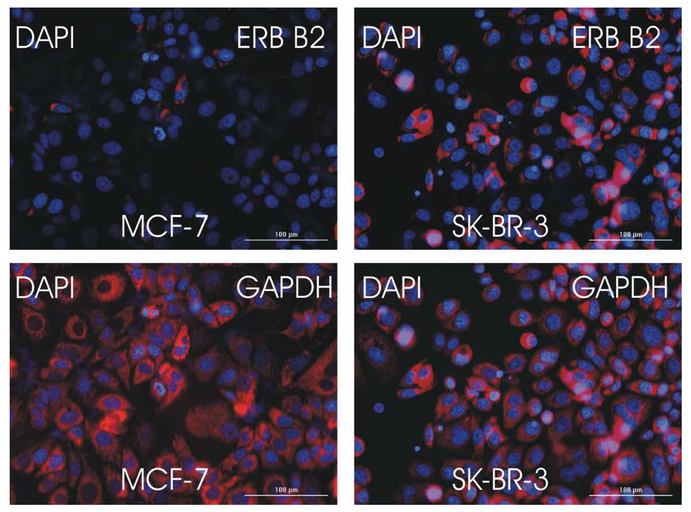

Figure 11 – Specificity of SmartFlare probes with Live Cell Imaging of MCF-7 and SK-BR-3. *MCF-7 or SK-BR-3 cells were seeded at 30,000 cells per well and treated with CY3-GAPDH and CY5-EGFR probes. After a 16 hour incubation, cells were stained with 5µg/mL Hoechst 33342 for 15 min @ 37°C. Cells were imaged with the 20x objective using the DAPI and CY3 and CY5 LED cubes.*

When MCF-7 and SK-BR-3 cells were treated with the SmartFlare probe for ERBB2, ERBB2 the gene encoding the EGF receptor, SK-BR-3 are shown to be a high expresser, while MCF-7 cells express only low amounts of the RNA (upper panels). The same cells show equivalent amounts of expression of the housekeeping gene GAPDH (lower panels).

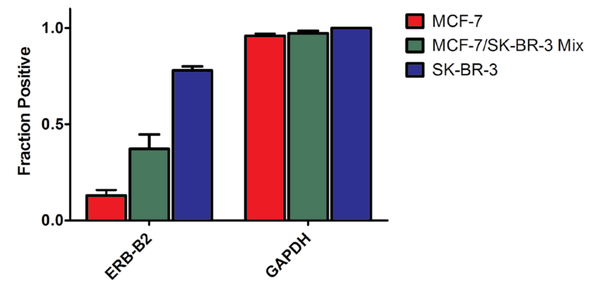

Figure 12 – Subpopulation analysis of Images of MCF-7 and SK-BR-3 cells treated with SmartFlare probes. *Mixtures of MCF-7 and SK-BR-3 cells were analyzed using Gen5 subpopulation analysis tools. MCF-7, SK-BR-3 or a 50/50 mixture of the two cell lines were seeded at 30,000 cells per well and treated with CY3-GAPDH and CY5-EGFR probes. After a 16 hour incubation, cells were stained with 5 µg/mL Hoechst 33342 for 15 min @ 37°C. Cells were imaged with the 20x objective using the DAPI and CY3 and CY5 LED cubes. Subpopulation analysis of DAPI stained objects was used to identify CY3 and CY5 positive cells. Data is expressed as the fraction of CY3 or CY5 objects and represents the mean of three wells.*

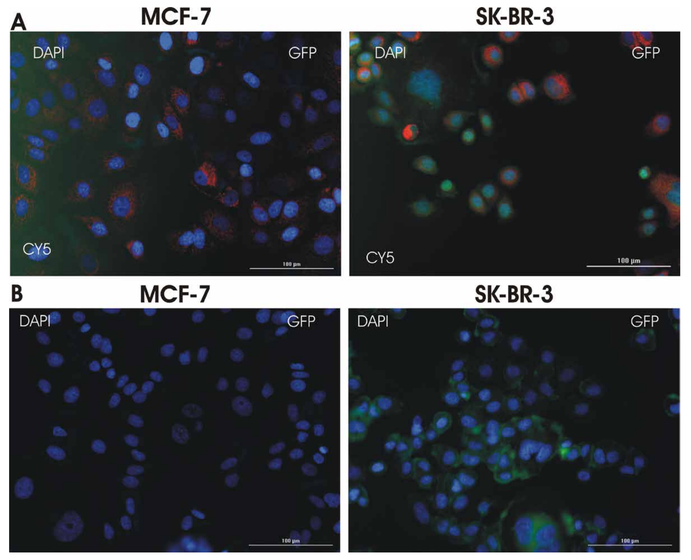

Figure 13 – Anti-EGFR antibody staining of Live MCF-7 and SK-BR-3 cells. *An Alexa Fluor 488 labeled anti EGFR antibody was incubated with live MCF-7 and SK-BR-3 cells and then imaged. In separate experiments cells were (A) exposed or (B) not exposed to a CY5-SmartFlare probe to ERB B2 for 16 hours prior to antibody staining.*

Conclusions

- Cytation 3 is capable of detecting mRNA in live cells using SmartFlare RNA Detection Probes
- Cytation 3 has a number of features that enable live cell imaging
  - Auto-focus and Auto-exposure
  - Multiple color Imaging capabilities
  - Gas Controller and Temperature Control allow long term studies and time lapse videos
- Quantitative Cellular Population Analysis Using Gen5 Software
  - Serum Titration Results in differential expression that can be quantified
  - Cell type specific expression can be identified
- SmartFlare Detection Probes (EMD Millipore) detects RNA expression in live cells in real time
  - Simultaneous Multiplex Determination of Multiple RNA Targets
  - Capable of working with multiple cell types
  - Compatible with downstream experimentation
  - Uptake and negative probe controls available