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 phenotyping 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 is 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-modal 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 complimentary to the RNA target where it is quenched in proximity to the gold core. With exposure to the target RNA the reporter strand is displaced as the target strand binds to its complementary 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.

Treatments 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 was successful 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.

Molecular Mechanism (Continued)

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 fluorescence until 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.

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 Inconclusively with the DAPI and CY3 LED cube and the images overlaid. Scale bar indicates 100 µm.

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 the 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 Inconclusively with the DAPI and CY3 LED cube and the images overlaid. Scale bar indicates 100 µm.

Cellular Localization

Figure 8 – Flow Cytometry localization of SmartFlare signal. HeLa cells were seeded at 30,000 cells per well and treated with 4 µL CY3-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 LED cube and the images overlaid. Cells were then imaged with a low power objective (DAPI and CY3 cube) after the overlay was removed from the overlay respectively. Scale bar indicates 100 µm.

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 at 5% CO₂. Cultures were routinely transfected (0.50% Trypan Blue) at 80% confluency.

Cell Seeding

Cells were plated at a density of 15,000 cells per well at 100 µl growth medium into 96-well black aseptically clear bottom 96-well plates (F:N:R:40A). Cells were allowed to attach and grow for 4-16 hours at 37°C at 5% CO₂.

Microplate Reading

Whole well PMT-based fluorescence intensity measurements were made using a Cytation 3 Cell Imaging Microplate Reader. All measurements were made with 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 635 nm and 670 nm excitation and emission wavelengths respectively. Likewise Hoechst 33342 staining was determined using an excitation wavelength of 340 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 405 nm excitation length (520 nm emission filter) then the RFP light cube is configured with a 561 nm excitation length (580 nm emission filter) and at last the CY5 light cube uses a 628 nm excitation and a 685 nm emission filter. Exposure settings were automatically determined for each strand independently and fixed for all subsequent exposures, while focus was provided automatically on each well using the DAPI light. 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.

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 different SmartFlare agents. After 16 hour incubation all 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 LED cube and the images overlaid. Cells were then imaged with the DAPI and CY5 LED cube after the overlay was removed from the overlay respectively. Scale bar indicates 100 µm.

Cell Line Specific Expression

Figure 11 – Specificity of SmartFlare probes with Live Cell Imaging of MCF-9 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-EF1A 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.

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 panel). The same cells show equivalent amounts of expression of the housekeeping gene GAPDH (lower panel).

Conclusions

• Cy3 signal is capable of detecting mRNA in live cells using SmartFlare RNA Detection Probes
• Cy5 signal has a number of features that enable live cell imaging
  - Auto-focus and Auto-exposure
  - Multiple color imaging capabilities
  - One 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 Multiples Determination of Multiple RNA Targets
  - Capable of working with multiple cell types
• Compatible with downstream experimentation
• Uptake and negative probe controls available

References

- Automated fluorescence detection and imaging of RNA species in live cells
- Molecular mechanism of SmartFlare RNA detection probe
- Cellular localization of SmartFlare signal
- Materials and methods
- Signal independence of SmartFlare probes
- Cell line specific expression
- Conclusions