ATTO™ 550 labeled Alt-R™ Cas9 tracrRNA allows for FACS sorting and intracellular RNP-complex visualization

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Introduction

Cas9 protein is increasingly being used for genome editing by direct transfection of an active guide RNA:Cas9 ribonucleoprotein (RNP) complex into cells. The RNP complex can be delivered via lipofection, electroporation, or microinjection into cells of interest, but the efficiency of delivery can vary widely between cell types. We investigated the use of dye-labeled synthetic crRNAs and tracrRNAs as an aid to assess RNP complex delivery efficiency and the ability to enrich for transfected cells via FACS (fluorescence-activated cell sorting).

Of the variety of dyes tested, some suffered from non-specific binding to the cells, making evaluation of transfection efficiency and for use in FACS to enrich for transfected cells. In addition, intracellular location of the dye-labeled complexes can be visualized using fluorescence microscopy.

Functional testing of dye-labeled RNAs

T7EI editing efficiencies of labeled guide RNA complexes. Labeled crRNAs were complexed to unlabeled tracrRNAs, whereas labeled tracrRNAs were complexed to unlabeled crRNAs. crRNA oligos were fluorescently labeled on the 3' end. tracrRNA oligos were fluorescently labeled on the 5' end. These guide RNA complexes were bound to Cas9 protein as RNP, and the RNP (10 nM) were reverse transfected into HEK293 cells using RNAiMAX™ (Thermo Fisher). Genomic DNA was isolated using QuickExtract™ (Epicentre) after cells were incubated for 48 hr. Total editing efficiency was measured using a T7 Endonuclease I assay.

Flow cytometric analysis of dye-labeled RNAs

Flow cytometric resolution of positively transfected cells. Jurkat cells were electroporated using the Neon™ Transfection System (Thermo Fisher) in the presence of Alt-R™ Cas9 Electroporation Enhancer (single-stranded, non-targeting DNA) with 1.5 µM RNP consisting of either labeled crRNA or labeled tracrRNA. Jurkat cells subjected to the same RNP but without electroporation were used as background controls and were used to set the gates. The frequency of cells within each gate is reported on each histogram. Plots shown were previously gated on FSC-A x SSC-A and FSC-A x FSC-W. Dye-labeled tracrRNAs gave better results than dye-labeled crRNAs, with ATTO™ 550 labeled tracrRNA yielding the best results.

Optimal flow cytometric resolution occurs at 24 hr post-transfection

Effect of washing cells prior to flow cytometric resolution. Jurkat and HEK293 cells were electroporated using the Neon™ Transfection System in the presence of Alt-R™ Cas9 Electroporation Enhancer with 1.5 or 0.15 µM RNP consisting of unlabeled crRNA and ATTO™ 550 labeled tracrRNA. Cells were sorted 24 hr post-transfection. Prior to sorting, cells were washed 1 time with PBS + 1% FBS. Histogram plots were generated as previously described to show fluorescence intensities and percent positive cells. Cells were sorted 24 hr post-transfection. Prior to sorting, cells were washed 1, 0, or 1 times with PBS + 1% FBS. Histogram plots show fluorescence intensities

Enrichment of editing in positive cells by FACS sorting

Enrichment of sorted cells leads to higher editing efficiencies. Jurkat cells or HEK293 cells were electroporated using the Neon™ Transfection System in the presence of Alt-R™ Cas9 Electroporation Enhancer with 1.5 or 0.5 µM RNP consisting of labeled tracrRNA (ATTO™ 550). Cells subjected to RNP but without electroporation were used as background controls and were used to set the gates. The frequency of cells within each gate was reported on each histogram. Positive cells were sorted at 72 hr. Total editing efficiency was measured using a T7 Endonuclease I assay. FACS sorting enriched the detected editing at a sub-optimal dose of 0.5 µM.

Conclusions

- Dye-labeled tracrRNAs gave better results than dye-labeled crRNAs, with ATTO™ 550 labeled tracrRNA giving the best results
- It is possible to visualize cellular localization and transfection efficiency by fluorescence microscopy using ATTO 550 labeled tracrRNA
- A wash step before FACS analysis is recommended to reduce non-specific binding to the cells
- Optimal FACS sorting occurred at 24 hr post-transfection
- FACS sorting allowed for enrichment of editing by selecting positive cell fraction

Effect of washing cells prior to flow cytometric resolution. Jurkat and HEK293 cells were electroporated using the Neon™ Transfection System in the presence of Alt-R™ Cas9 Electroporation Enhancer with 1.5 or 0.15 µM RNP consisting of unlabeled crRNA and ATTO™ 550 labeled tracrRNA. Histogram plots were generated as previously described to show fluorescence intensities and percent positive cells.

Washing cells increases positive fraction by flow cytometry

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