New Cell-Based High-Throughput Screening Platform for RNA-Mediated Neurodegenerative Diseases

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Introduction

Most neurodegenerative disorders are thought to be caused by misfolded proteins that form toxic aggregates and disrupt cellular homeostasis in neurons. However, recent advances have identified abnormal expansion of trinucleotide repeat sequences in disease genes as a new RNA-mediated mechanism that plays a causative role in neurodegeneration. Specifically, toxic expanded RNA sequences form large nuclear "sinks" to sequester RNA-binding proteins and splicing factors that prevent normal cellular functions and induce neuronal cell death. This RNA-mediated mechanism is now associated with at least 10 neurological disorders that include several forms of inherited ataxias, Huntington disease, and myotonic dystrophies. Currently, there is no drug approved to slow the progression or reverse the pathology for many if not most of the RNA-mediated diseases. The majority of the medically available treatments only serve to manage symptoms and disability. Therefore, there is a strong need to discover first-in-class drug treatments capable of preventing and/or reversing these disease progression.

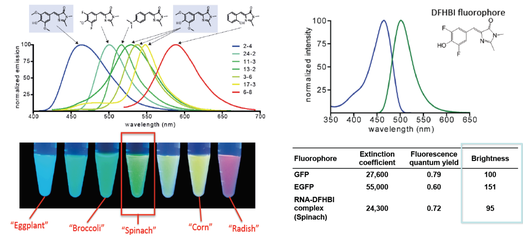
Our Solution

A new screening platform to accelerate the drug discovery of RNA-mediated neurodegenerative disease:

- A cell-based high-throughput screening system that will enable rapid identification of drugs that can specifically disrupt toxic RNA formations.
- A drug discovery platform that is both highly selective for the toxic RNA of interest and can be broadly applied across most RNA-mediated diseases.
- Compatible with standard fluorescence-based microtiter plate readers found in most HTS facilities

We believe that our technology offers an unique access to a new class of therapeutic targets and addresses a significant unmet medical market.

Results



Fluorophore	Extinction coefficient	Fluorescence quantum yield	Brightness
GFP	27,600	0.79	100
EGFP	55,000	0.60	151
RNA-DFHBI complex (Spinach)	24,300	0.72	95

Figure 1. Fluorescent RNA technology. We have successfully developed a palette of fluorescent RNA tags that can be used for live-cell imaging of biologically important RNA species. The "Spinach" tag is a RNA mimic of EGFP that turns on the fluorescence of the DFHBI fluorophore and exhibits spectral properties similar to EGFP. The Spinach RNA-fluorophore complex. We have recently generated a brighter and more thermostable version of Spinach that we termed "Spinach2."

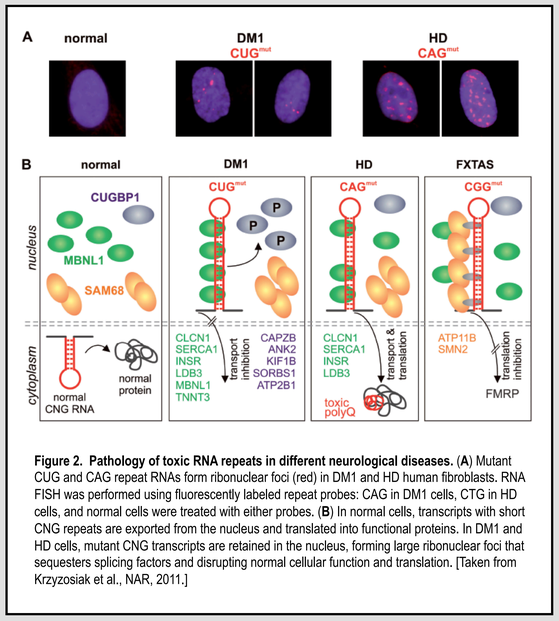


Figure 2. Pathology of toxic RNA repeats in different neurological diseases. (**A**) Mutant CUG and CAG repeat RNAs form ribonuclear foci (red) in DM1 and HD human fibroblasts. RNA FISH was performed using fluorescently labeled repeat probes: CAG in DM1 cells, CTG in HD cells, and normal cells were treated with either probes. (**B**) In normal cells, transcripts with short CNG repeats are exported from the nucleus and translated into functional proteins. In DM1 and HD cells, mutant CNG transcripts are retained in the nucleus, forming large ribonuclear foci that sequesters splicing factors and disrupting normal cellular function and translation. [Taken from Krzyzosiak et al., NAR, 2011.]

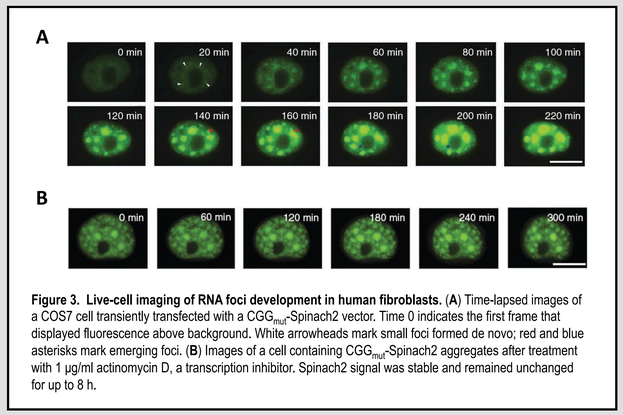


Figure 3. Live-cell imaging of RNA foci development in human fibroblasts. (**A**) Time-lapsed images of a COS7 cell transiently transfected with a CGG_{mut}-Spinach2 vector. Time 0 indicates the first frame that displayed fluorescence above background. White arrowheads mark small foci formed de novo; red and blue asterisks mark emerging foci. (**B**) Images of a cell containing CGG_{mut}-Spinach2 aggregates after treatment with 1 μg/ml actinomycin D, a transcription inhibitor. Spinach2 signal was stable and remained unchanged for up to 8 h.

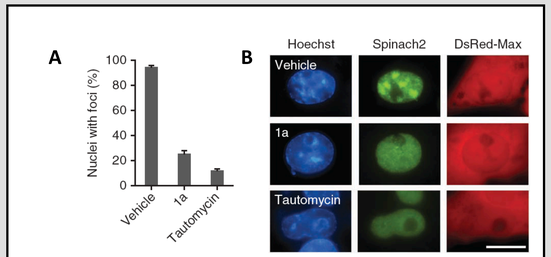


Figure 4. CGG_{mut}-Spinach2 can be used to dynamically assess the effects of small molecule inhibitors on toxic CGG RNA foci in human fibroblasts. (**A**) Presence of nuclear foci in COS7 cells co-expressing CGG_{mut}-Spinach2 and DsRed, treated with vehicle, 20 μM 1a or 5 μM tautomycin (two drugs that have been shown to prevent the formation of CGG foci in transfected cells). After 24 h, 100 DsRed-positive cells were analyzed for the presence of nuclear foci for each condition. 94 ± 1.4%, 25 ± 4.9%, and 12 ± 4.2% of nuclei contained foci with vehicle, 1a, and tautomycin, respectively. (**B**) Images representative of 100 COS7 cells (per treatment) expressing CGG_{mut}-Spinach2 after 24 h of treatment with vehicle, 1a, and tautomycin. Images show nuclei labeled using Hoechst, fluorescence signal from Spinach2, and fluorescence signal from DsRed-Max, a transfection control.

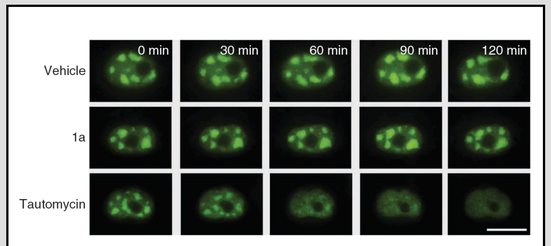


Figure 5. Tautomycin, but not 1a, can disrupt existing toxic CGG_{mut} RNA foci. To determine if small molecule inhibitors can also induce disaggregation of toxic RNA foci, we treated COS7 cells expressing CGG_{mut}-Spinach2 with vehicle, 20 μM 1a, or 5 μM tautomycin and imaged the cells every 5 min for 2 h after drug treatment. (**Top and Middle**) We observed no changes in foci in cells treated with vehicle or 1a for 2 h. After 48 h treatment, the number of cells with foci only changed slightly from 94 ± 2.8% to 86 ± 3.5%. Furthermore, no dissociation of SAM68 from CGG_{mut}-Spinach2 foci, indicating that 1a does not disrupt protein binding from foci. (**Bottom**) In contrast, tautomycin induced disaggregation of toxic RNA foci within 1 h. The disaggregated CGG_{mut}-Spinach2 RNA remained as diffused nucleoplasmic staining in cells. Removal of tautomycin after a 2 h treatment was not sufficient to restore foci, suggesting that tautomycin induce cellular changes that prevent reaggregation.

Disease	Repeat Unit	Normal Repeat	Expanded Repeat	Clinical Symptoms
Diseases caused by RNA toxicity:				
DM1	CUG	5-37	50-10,000	Muscle weakness, mental retardation
DM2	CCUG	10-26	75-11,000	Muscle weaknss
FXTAS	CGG	6-60	60-200	Tremor/ataxia, parkinsonism, cognitive deficits
ALS/FTD	GGGGCC	3	>30	Muscle weakness, cognitive impairments
Diseases with RNA toxicity as a component:				
SCA3	CAG	13-36	61-84	Ataxia and parkinsonism
SCA8	CAG	16-34	>74	Ataxia and slurred speech
SCA10	CUG	10-20	500-4,500	Ataxia, tremor, and dementia
SCA12	CAG	7-45	55-78	Ataxia and seizures
HDL2	CUG	7-28	66-78	Chorea, cognitive deficits

Table 1. A list of neurological diseases where expanded-repeat RNA toxicity plays an important role in disease pathology.

Conclusions

Demonstration of a new approach to identity drugs that target RNA-mediated neurodegenerative diseases:

- We have developed a novel technology that enables real-time imaging RNA movements in living cells.
- We demonstrate that we can monitor the formation and dissolution of toxic ribonuclear foci that contain expanded CGG transcripts and RNA-binding proteins.
- Our platform can report the effects of small molecule inhibitors on toxic RNA foci aggregation
- Our platform can be easily adapted for other RNA-mediated neurodegenerative diseases including ones that are listed in Table 1.

Publications

- Paige JS, Wu KY, Jaffrey SR. (2011) RNA mimic of green fluorescent protein. *Science*.
- Strack RL, Disney MD, Jaffrey SR. (2013) A superfolding Spinach2 reveals the dynamic nature of trinucleotide repeat RNA. *Nature Methods*.
- Strack RL, Jaffrey SR. (2014) Using RNA mimics of GFP to image RNA dynamics in mammalian cells. *Advanced Fluorescence Microscopy*, 1st ed. Academic Press, Elsevier, Inc.

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