Published in final edited form as:

Methods Mol Biol. 2019 ; 1920: 33–39. doi:10.1007/978-1-4939-9009-2_3.

Assaying NanoLuc Luciferase Activity from mRNA-Injected *Xenopus* Embryos

Michael D. Sheets

Abstract

The earliest steps of animal development depend upon posttranscriptional events that drive the embryonic cell cycle and guide cell fate decisions. The analysis of post-transcriptional regulatory events has relied upon the use of chimeric reporter mRNAs that encode firefly luciferase fused to potential regulatory sequences. A new and more sensitive luciferase developed recently called NanoLuc has the potential to improve reporter studies and provide new insights into the regulation of embryonic processes. Here I describe how to create and analyze reporter mRNAs encoding NanoLuc luciferase using extracts from microinjected *Xenopus* embryos.

Keywords

Xenopus embryos; mRNA microinjection; NanoLuc; Luciferase

1 Introduction

Developmental biologists have long embraced the use of reporter technologies for their studies. The myriad applications of these tools have provided significant new insights into the fundamental processes that occur in embryonic cells. For example, firefly luciferase-based reporters have been used extensively to quantitatively monitor spatial and temporal gene expression at both the transcriptional and post-transcriptional levels [1–4]. In addition, fluorescent reporters have been used extensively to follow the temporal and spatial expression and activities of specific proteins [5, 6].

The utility and adoption of the different reporters as experimental tools have largely paralleled technical advances. The wide-spread use of firefly luciferase was greatly facilitated by the cloning of the luciferase enzyme and development of sensitive instruments for measuring luciferase activity along with the establishment of assay conditions and substrates that produced prolonged and easily detectable light signals [7, 8]. The identification of fluorescent proteins coupled to the creation of tools to generate specific protein fusions has revolutionized the study of development and significantly advanced our knowledge of key embryonic processes [5, 6]. To further expand the utility of luciferase reporters a new luciferase, called NanoLuc, was developed using directed evolution of an enzyme from the deep-sea shrimp *Oplophorus gracilirostris* [9]. NanoLuc is a small monomeric protein that utilizes a novel substrate to generate stable luminescence as well as a higher specific activity compared to other luciferase enzymes. This reporter and its unique properties offer numerous advantages for developmental biologists. Particularly, the increased sensitivity of NanoLuc compared to firefly luciferase provides the potential to

analyze the activity of reporter mRNAs at concentrations approaching the physiological levels present in embryonic cells [10]. In the following sections I describe how to create and analyze reporter mRNAs encoding NanoLuc luciferase using extracts prepared from microinjected *Xenopus* embryos.

2 Materials

2.1 Plasmid for Creating Chimeric NanoLuc mRNAs

To generate mRNAs in vitro for analysis requires a plasmid containing the NanoLuc coding region downstream of a T7 promoter. The plasmids generated by Promega all lack a T7 promoter for generating mRNAs in vitro. To address this issue we created pT7-NanoLuc from pNL1.1 (Fig. 1). In addition to the T7 promoter the XbaI restriction site downstream of the NanoLuc coding region facilitates the cloning of 3'UTR fragments for analysis.

2.2 In Vitro Transcription to Generate NanoLuc mRNAs

1. Plasmid for transcription linearized with appropriate restriction enzyme.
2. T7 RNA polymerase.
3. 10×Transcription buffer: 400 mM Tris-HCl, 60 mM MgCl₂, 10 mM DTT, 20 mM spermidine, pH 7.9@25 °C.
4. Nucleotide solutions, ATP 100 mM, CTP 100 mM, UTP 100 mM, GTP 20 mM, CAP analog 40 mM.
5. Nuclease-free water.
6. G50 spin columns.
7. Agarose.
8. 5 ×Tris-borate-EDTA (TBE) buffer (450 mM Tris-borate, 10 mM EDTA).
9. RNA-loading dye (47.5% formamide, 0.01% SDS, 0.01% bromophenol blue, 0.005% xylene cyanol, 0.5 mM EDTA).

2.3 Microinjection into *Xenopus* Embryos

1. Microinjection apparatus.
2. Dissecting microscope.
3. Microinjection dishes with mesh grids to hold embryos in position.
4. Embryo culture media Marc's Modified Ringer's Solution (MMR)—10 stock: 1 M NaCl, 20 mM KCl, 10 mM MgSO₄, 20 mM CaCl₂, 50 mM HEPES (pH 7.8), 1 mM EDTA. Adjust pH to 7.4, sterilize by autoclaving, and store at room temperature.
5. MMR 0.25 (working solution).
6. MMR 0.25, 4% Ficoll 400.

2.4 Generating Embryo Extracts for NanoLuc Assays

1. Luciferase Cell Culture Lysis Reagent 5× (Promega).
2. Micro-pestles.

2.5 Assaying Embryo Extracts for NanoLuc Activity

1. Nano-Glo Luciferase Assay Substrate (Promega).
2. Nano-Glo Luciferase Assay Buffer (Promega).
3. Luminometer assay tubes.
4. Luminometer (for example, for single-tube analysis the Turner Designs 2020 or the Berthold Lumat LB9507 instruments work well). In addition, for the analysis of large number of samples there are a variety of luminometers capable of analyzing samples in microtiter plates.

3 Methods

3.1 Generate the Plasmid Template for Transcription

1. Plasmid DNA is linearized by restriction enzyme cutting to create a template for in vitro transcription.
2. The linearized DNA template is purified by extraction with an equal volume of 1:1 phenol/chloroform mixture.
3. The DNA is precipitated from the reaction by adding 1/10th volume of 3 M sodium acetate, pH 5.2, and three volumes of ethanol. Incubate at 20 °C for 60 min.
4. The DNA precipitate is spun out of solution using a microcentrifuge operating at 10,000 RPM (10,600 g), for 15 min at 4°.
5. The supernatant is removed and the DNA pellet is rinsed with 500 µL of cold 70% ethanol and centrifuged for 5 min.
6. Remove the wash, air-dry the pellet, and resuspend it in DEPC-treated water at a concentration of approximately 0.5–1.0 µg/µL.

3.2 In Vitro Transcription to Generate NanoLuc mRNAs [11] (See Note 1)

1. Assemble 20 µL reactions at room temperature in the following order (see Note 2):
Nuclease-free water X µL
10×Transcription buffer 2 µL
2 µL ATP 100 mM—10 mM final concentration
2 µL UTP 100 mM—10 mM final concentration
2 µL CTP 100 mM—10 mM final concentration

2 μ L GTP 20 mM—2 mM final concentration

4 μ L Cap Analog 40 mM—8 mM final concentration

Template DNA X μ L 1 μ g

T7 RNA polymerase 2 μ L

Total reaction volume 20 μ L

2. Mix by micropipetting and incubate at 37 °C for 2 h.
3. Add nuclease-free H₂O to the reaction until the volume reaches 100 μ L.
4. G50 spin columns are used to remove unincorporated nucleotides, proteins, and salts. Apply the reactions to the column and follow the manufacturer's instructions for sample purification.
5. Measure ultraviolet light absorbance at 260 nm (*see* Note 3).
6. Analyze an aliquot of the mRNA on a denaturing agarose gel to evaluate length, integrity, and quality [12, 13] (*see* Note 4).

3.3 Microinjection into *Xenopus* Embryos

1. Generate *Xenopus* embryos and microinject reporter mRNAs using standard protocols [14–17] (*see* Note 5).
2. Incubate injected embryos in 0.25MMR 4% Ficoll until the desired stage of development is reached (*see* Note 6).

3.4 Generating Embryo Extracts for NanoLuc Assays

1. Examine injected embryos and discard any damage during microinjection.
2. Place healthy embryos in 1.5 mL microcentrifuge tubes.
3. Remove excess culture media taking care not to lyse the embryos.
4. Add 100 μ L cell culture lysis reagent to each embryo sample (*see* Note 7).
5. Lyse the injected cells with a micro-pestle.
6. Spin the extracts at 4 °C for 10 min at 10,000 RPM (10,600 g) in a microfuge.
7. Transfer the supernatant extracts to a new microcentrifuge tube while leaving behind the particulate pellets and yolk.
8. Assay extracts for NanoLuc activity immediately or store extracts at 80 °C indefinitely (*see* Notes 8 and 9).

3.5 Assaying Embryo Extracts for NanoLuc Activity

1. Thaw extracts on ice or if generated fresh store on ice until use (*see* Notes 8 and 9).
2. Prepare a working solution of the NanoLuc substrate by diluting the concentrated substrate 1/50 in NanoLuc assay buffer (*see* Note 10).

3. Prepare samples by mixing 50 μ L of substrate with 50 μ L of extract and incubating at room temperature for 3 min protected from light (*see* Note 11).
4. Transfer samples to luciferase assay tubes and assay for NanoLuc activity using a luminometer (*see* Notes 12 and 13).

4 Notes

1. Follow precautions for working with mRNA to avoid RNase contamination that could degrade the mRNA. Wear gloves at all times and use only nuclease-free tubes and reagents.
2. Reactions are typically 20 μ L but can be scaled up if large amounts of mRNA are needed.
3. Calculate mRNA concentration, with one A260 unit being equivalent to an mRNA concentration of 40 μ L/mL.
4. Mix 0.2 μ g mRNA sample with RNA-loading dye. Denature the mRNA sample by heating at 65 °C for 5 min. Load sample onto a 1% agarose gel (TBE buffered) and electrophorese. Visualize RNA by staining the gel with ethidium bromide.
5. Concentrations of mRNAs in samples for injection will vary depending upon individual mRNA and specific application. Typically we use samples that contain 6 attomoles/nL (1 pg/nL) mRNA and inject 1–10 nL per embryo [14–16].
6. Embryos can be cultured at lower temperatures approaching 13 °C to slow development when collecting samples for time-course experiments.
7. The volume of cell culture lysis buffer is typically 100 μ L/embryo but the volume can be varied to accommodate different signal intensities.
8. Extracts can be assayed immediately after production or stored at 80 °C and assayed when convenient.
9. We have not observed a reduction of activity upon freezing and thawing extracts multiple cycles.
10. Each assay requires 50 μ L of substrate. The prepared substrate is kept at room temperature protected from light until use.
11. Assay each extract in triplicate to provide technical replicates. Analyze multiple dilutions of each extract to ensure that readings are in the linear range of the assay conditions and the luminometer. Prepare dilutions of extract with cell culture lysis buffer. Once prepared the cell culture lysis buffer can be stored at 20°until use.
12. Occasionally negative control extracts from uninjected embryos produce a background signal (>1000 RLUs). In such cases freezing and thawing extract often eliminate the background.

13. Although this chapter focuses on analyzing NanoLuc activities in *Xenopus* embryos similar approaches can be applied to *Xenopus* oocytes, eggs, and embryos of other model organisms used by developmental biologists.

Acknowledgments

Work in the Sheets lab was supported by NIH grants (R21HD076828, R01HD091921).

References

1. Sheets MD, Fox CA, Hunt T, Vande Woude G, Wickens M (1994) The 3'-untranslated regions of c-mos and cyclin mRNAs stimulate translation by regulating cytoplasmic polyadenylation. *Genes Dev* 8:926–938 [PubMed: 7926777]
2. Arnone MI, Dmochowski IJ, Gache C (2004) Using reporter genes to study cis-regulatory elements. *Methods Cell Biol* 74:621–652 [PubMed: 15575624]
3. Miraglia LJ, King FJ, Damoiseaux R (2011) Seeing the light: luminescent reporter gene assays. *Comb Chem High Throughput Screen* 14:648–657 [PubMed: 21564017]
4. Gallie DR (1991) The cap and poly(A) tail function synergistically to regulate mRNA translational efficiency. *Genes Dev* 5:2108–2116 [PubMed: 1682219]
5. Rizzo MA, Davidson MW, Piston DW (2009) Fluorescent protein tracking and detection: applications using fluorescent proteins in living cells. *Cold Spring Harb Protoc* 2009:pdb top64
6. Giepmans BN, Adams SR, Ellisman MH, Tsien RY (2006) The fluorescent toolbox for assessing protein location and function. *Science* 312:217–224 [PubMed: 16614209]
7. de Wet JR, Wood KV, Helinski DR, DeLuca M (1985) Cloning of firefly luciferase cDNA and the expression of active luciferase in *Escherichia coli*. *Proc Natl Acad Sci U S A* 82:7870–7873 [PubMed: 3906652]
8. Hooper CE, Ansorge RE, Rushbrooke JG (1994) Low-light imaging technology in the life sciences. *J Biolumin Chemilumin* 9:113–122 [PubMed: 7942114]
9. Hall MP, Unch J, Binkowski BF, Valley MP, Braeden LB, Wood MG et al. (2012) Engineered luciferase reporter from a deep sea shrimp utilizing a novel imidazopyrazinone substrate. *ACS Chem Biol* 7:1848–1857 [PubMed: 22894855]
10. England CG, Ehlerding EB, Cai W (2016) NanoLuc: a small luciferase is brightening up the field of bioluminescence. *Bioconjug Chem* 27:1175–1187 [PubMed: 27045664]
11. Nilsen TW, Rio DC (2012) In vitro transcription of labeled RNA: synthesis, capping, and substitution. *Cold Spring Harb Protoc* 2012:1181–1186 [PubMed: 23118360]
12. Rio DC (2015) Denaturation and electrophoresis of RNA with glyoxal. *Cold Spring Harb Protoc* 2015:223–226 [PubMed: 25646499]
13. Rio DC (2015) Denaturation and electrophoresis of RNA with formaldehyde. *Cold Spring Harb Protoc* 2015:219–222 [PubMed: 25646498]
14. Park S, Blaser S, Marchal MA, Houston DW, Sheets MD (2016) A gradient of maternal Bicaudal-C controls vertebrate embryogenesis via translational repression of mRNAs encoding cell fate regulators. *Development* 143:864–871 [PubMed: 26811381]
15. Zhang Y, Cooke A, Park s, Dewey CN, Wickens M, Sheets (2013) Bicaudal-C spatially controls translation of vertebrate maternal mRNAs. *RNA* 19:1575–1582 [PubMed: 24062572]
16. Zhang Y, Forinash KD, McGivern J, Fritz B, Dorey K, Sheets MD (2009) Spatially restricted translation of the xCR1 mRNA in *Xenopus* embryos. *Mol Cell Biol* 29:3791–3802 [PubMed: 19364820]
17. Sive HL, Grainger RM, Harland RM (2010) Microinjection of *Xenopus* embryos. *Cold Spring Harb Protoc* 2010:ip81

Fig. 1.

The pT7-NanoLuc plasmid for creating NanoLuc mRNAs in vitro. The pNL1.1 plasmid (Promega) was modified by cloning the promoter for T7 RNA polymerase upstream of the NanoLuc coding region. An XbaI restriction site is present 30 of NanoLuc and can be used for cloning DNA fragments that encode 3'UTRs for analysis