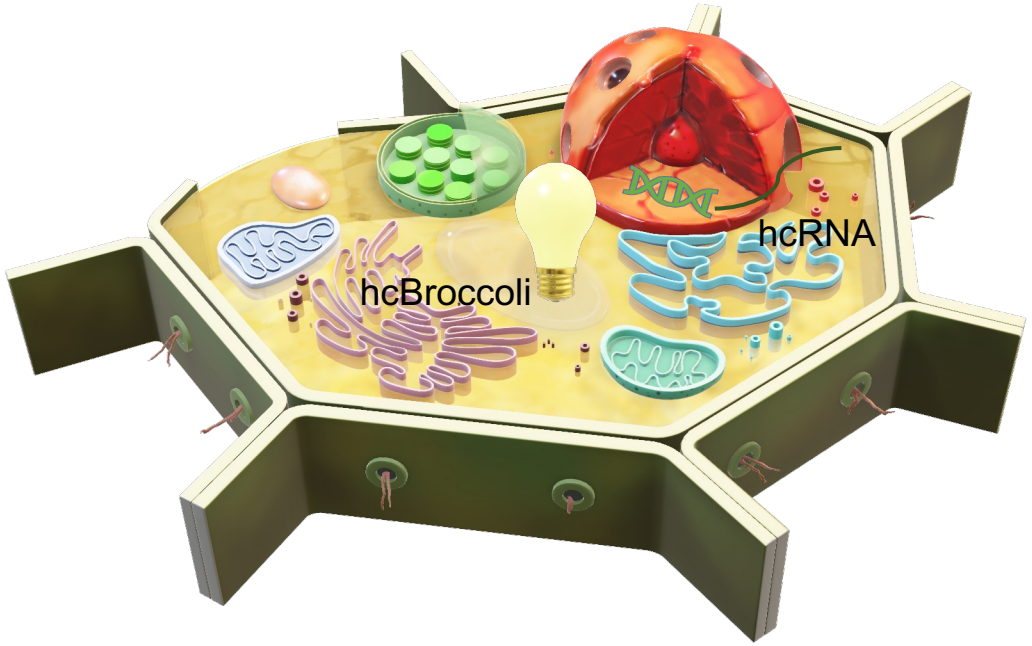
University of Massachusetts Amherst
College of Natural Sciences
Department of Chemistry

Stabilizing RNA Aptamers in Mammalian Cells via Protein-Mediated Circularization



An Original Research Proposal Presented
By

Yumeng Zhang

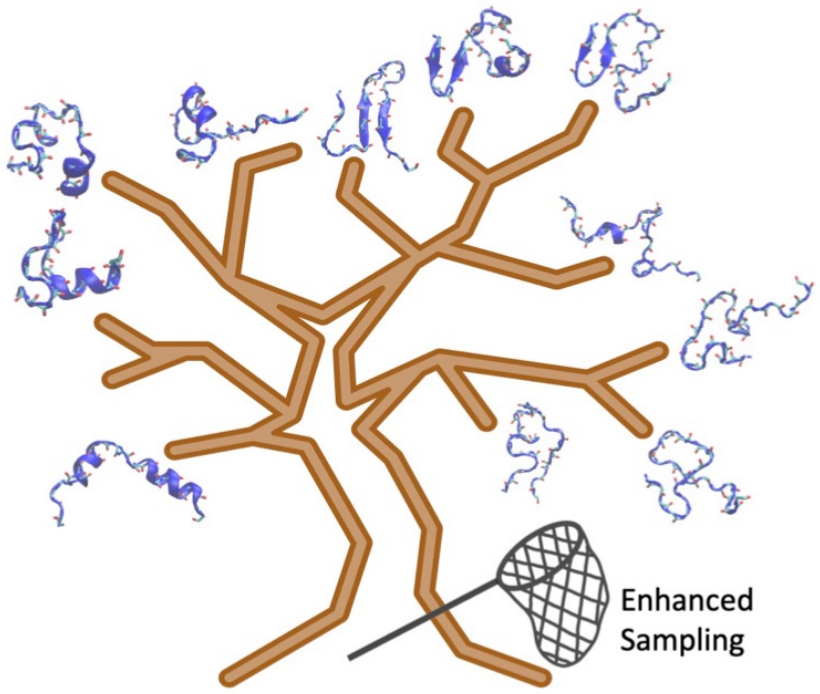
Jianhan Chen Research Group

Dec 13, 2021

[Research Update]$ Multi-scale simulations for IDP studies

Objective:

- Characterize the conformation and dynamics of intrinsically disordered proteins with MD simulations.



IDP Conformational Landscape

- ❖ IDP high structural heterogeneity.
- ❖ IDP high dynamics.
- ❖ Timescales of IDP conformational fluctuations.

Method:

1. Speed up the atomistic simulations with enhanced sampling method: **REST** (Replica Exchange with Solute Tempering)
2. Realize long-time scale simulations using coarse-grained model: **HyRes** (Hybrid Resolution)

Specific Aims:

1. Resolve the high temperature collapse in REST2.
2. Solve the over-compactness in HyRes model.

Approaches:

1. Develop REST3 method:
   Re-balance the protein-water interactions, which is underestimated in REST2 high temperatures.
2. Develop HyRes2 force field
   a. Downscale the protein-protein intra-interactions.
   b. Introduce the implicit solvent SASA model.
   c. Re-adjust the hydrogen bonding strength.

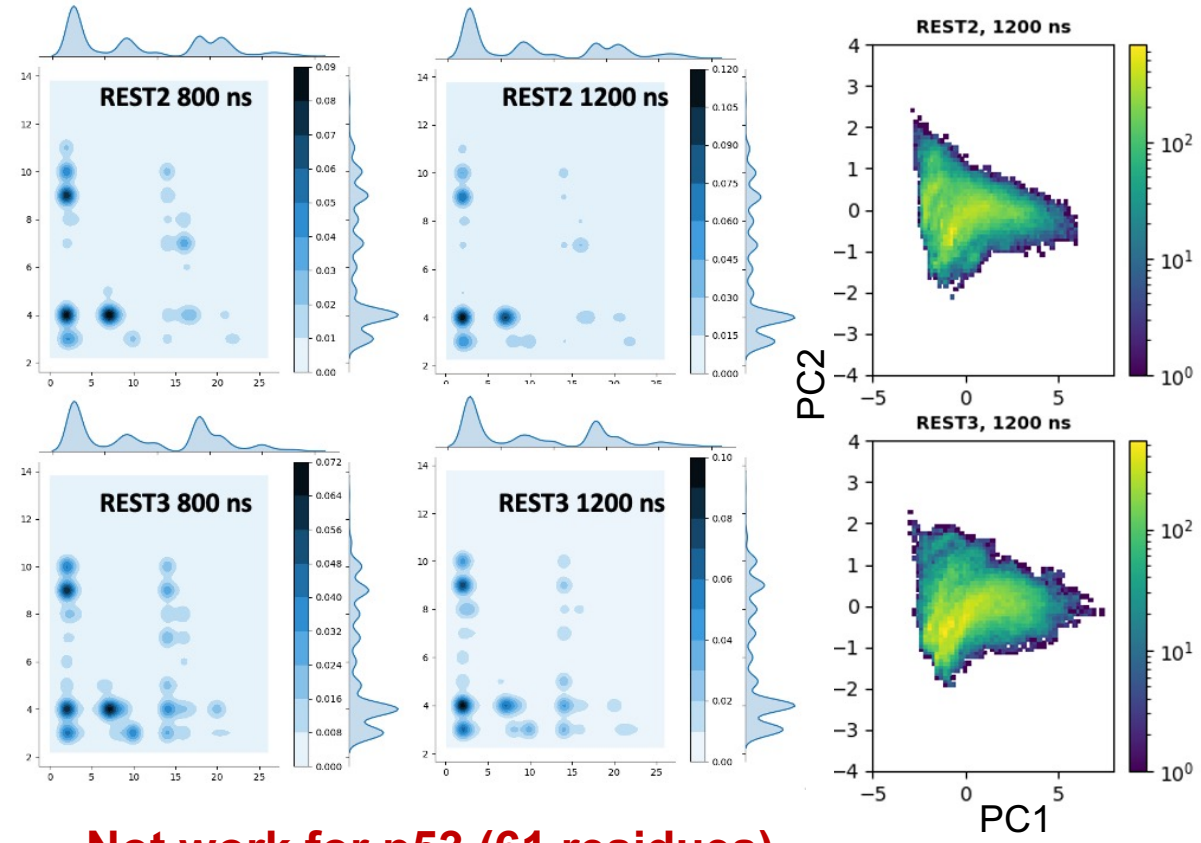
Biomolecules **2021**, *11*(10), 1416

[Research Update]$ Multi-scale simulations for IDP studies

1. REST3

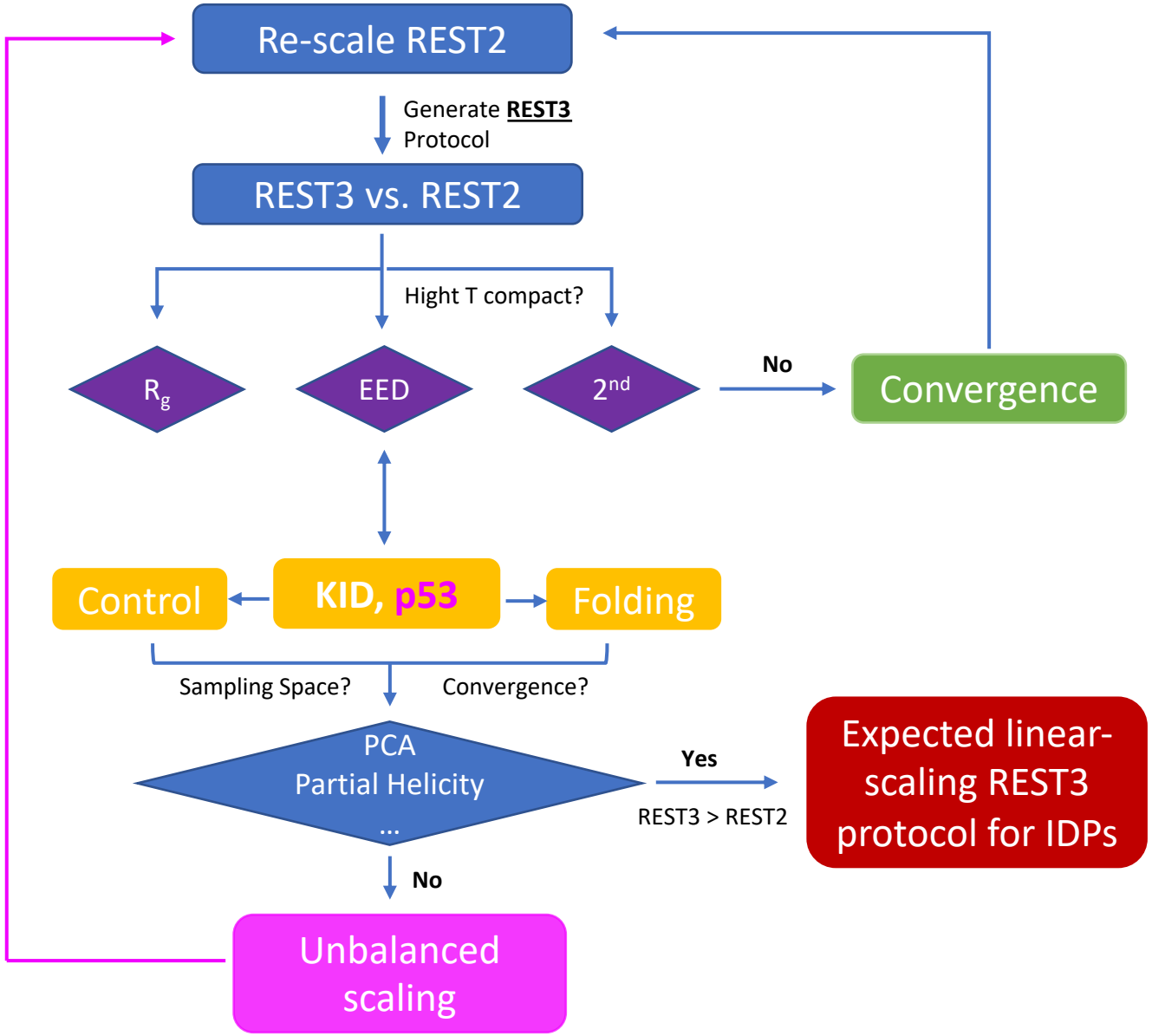
Work for KID (28 residues)

1. Faster convergence rate than REST2.
2. More conformational space sampled.



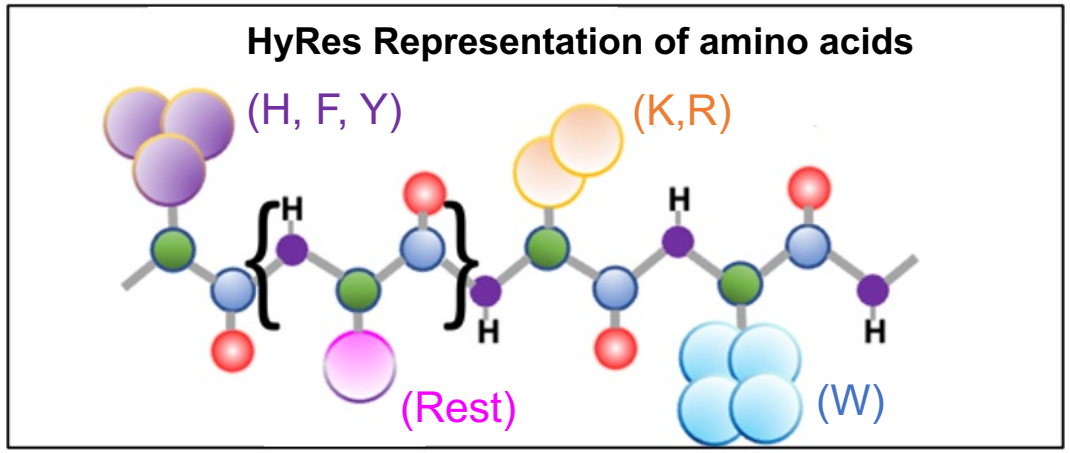
Not work for p53 (61 residues)

- Need to re-calibrate the p-w/p-p interactions.



[Research Update]$ Multi-scale simulations for IDP studies

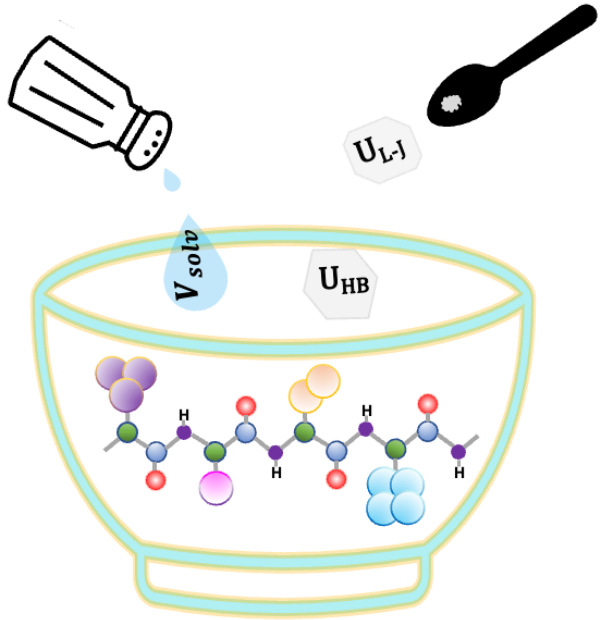
2. HyRes2



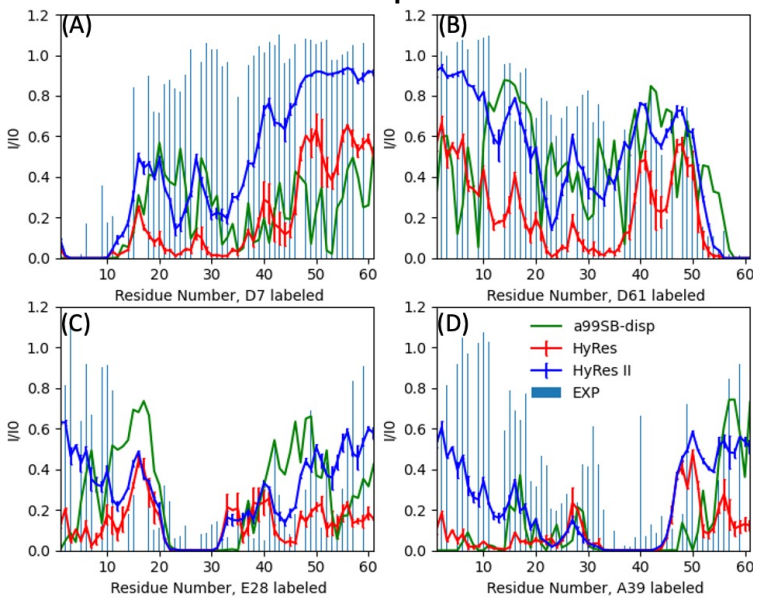
Preliminary data

1. More extended conformation for large IDPs (61 res).
2. More accurate protein-protein inter/intra interactions.
3. More conformational space sampled for large system (~ 400 residues).

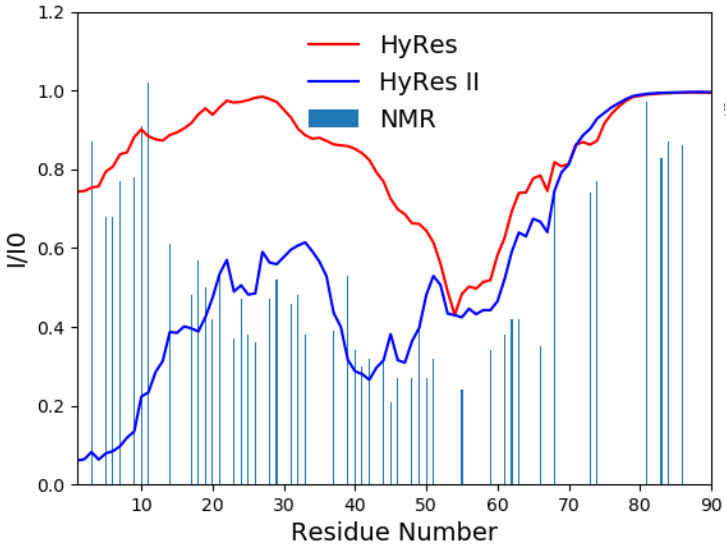
PDBID	Chain Length	Ref (EED, Å)	HyRes (EED, Å)	HyRes II (EED, Å)	Ref (R_g, Å)	HyRes (R_g, Å)	HyRes II (R_g, Å)
1VII	36	25.97	13.74 ± 0.04	18.07 ± 0.2	10.80	9.49 ± 0.02	11.04 ± 0.04
1BDC	46	20.69	19.87 ± 0.27	26.87 ± 0.09	9.95	10.42 ± 0.02	12.77 ± 0.07
3GB1	56	9.24	17.82 ± 0.54	25.86 ± 0.05	9.80	11.04 ± 0.02	13.31 ± 0.03
KID	28	~ 29	17.81 ± 0.12	22.87 ± 0.2	~ 11	9.18 ± 0.1	10.95 ± 0.12
NTAD	70	~ 70	19.71 ± 0.15	45.78 ± 2.49	~23.8	16.39	23.57 ± 0.33

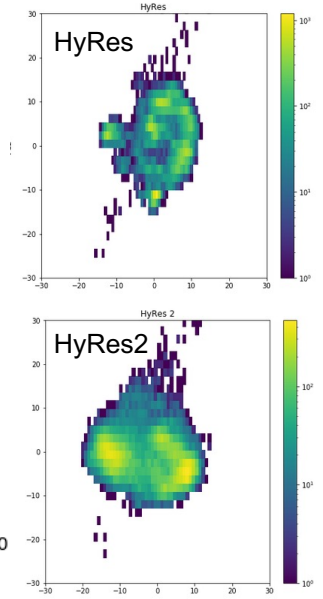


PRE of p53-NTD



PRE of full-length p53

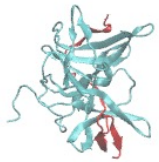


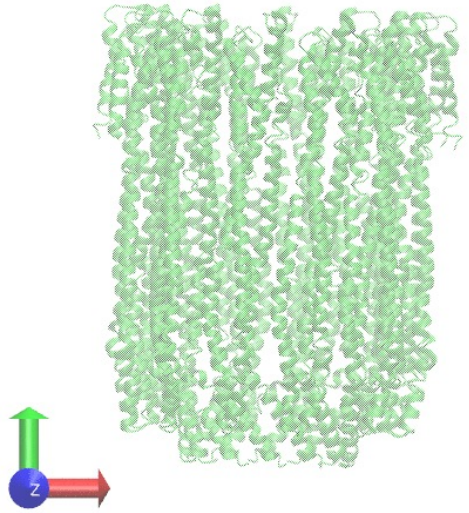


[Research Update]$ ClyA/NS2B-NS3 simulations

Objective:

- Explore the dynamics of NS2B-NS3 in ClyA nanopore.





- ❖ Large and complicated system.
- ❖ Conformational dynamics of proteases.

Method:

1. HyRes model.
2. Steered MD.

Specific Aims:

1. Resolve pore-protease interactions at atomistic level.
2. Gain insight on the dynamic of proteases in ClyA.

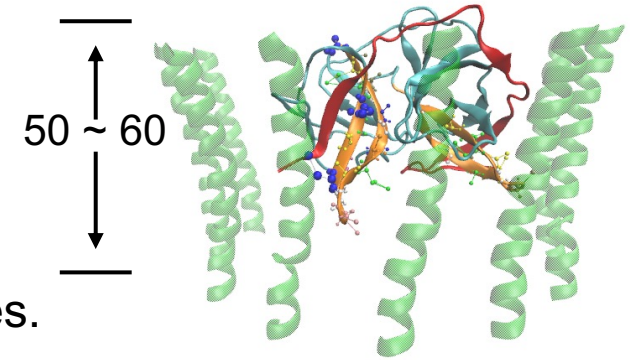
Future Work:

1. Resolve the dynamics of proteases at atomistic level.
2. Trap protease at mid-trap state then explore the ligand binding interactions.

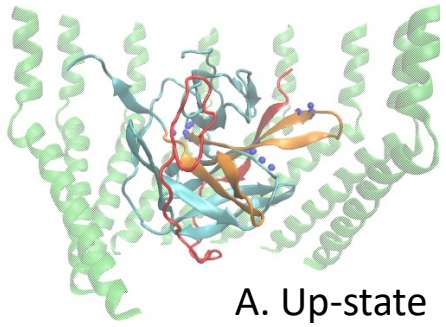
Preliminary data:

1. One potential trapping site on the mid-region of ClyA.
2. Apo state is easier to have translocation events.
3. Two potential conformations of proteases when being trapped at the constriction region.

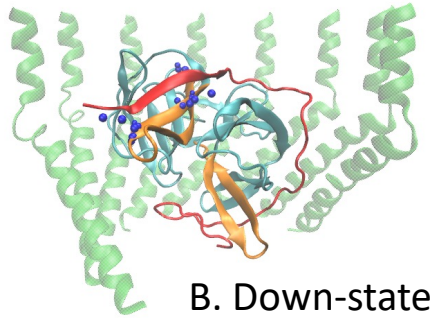
Mid-trap state



Constriction-trap states



A. Up-state



B. Down-state

ORP

Introduction & Significance

Approaches

Aim 1: **Encoding circular RNA in mammalian cells.**

Aim 1a: Encode Broccoli with 5’ cap and 3’ poly(A) tail.

Aim 1b: Characterize hcBroccoli in cells.

Aim 2: **Functional evaluation of encoded RNAs.**

Aim 2a: Evaluate hcBroccoli functionalities.

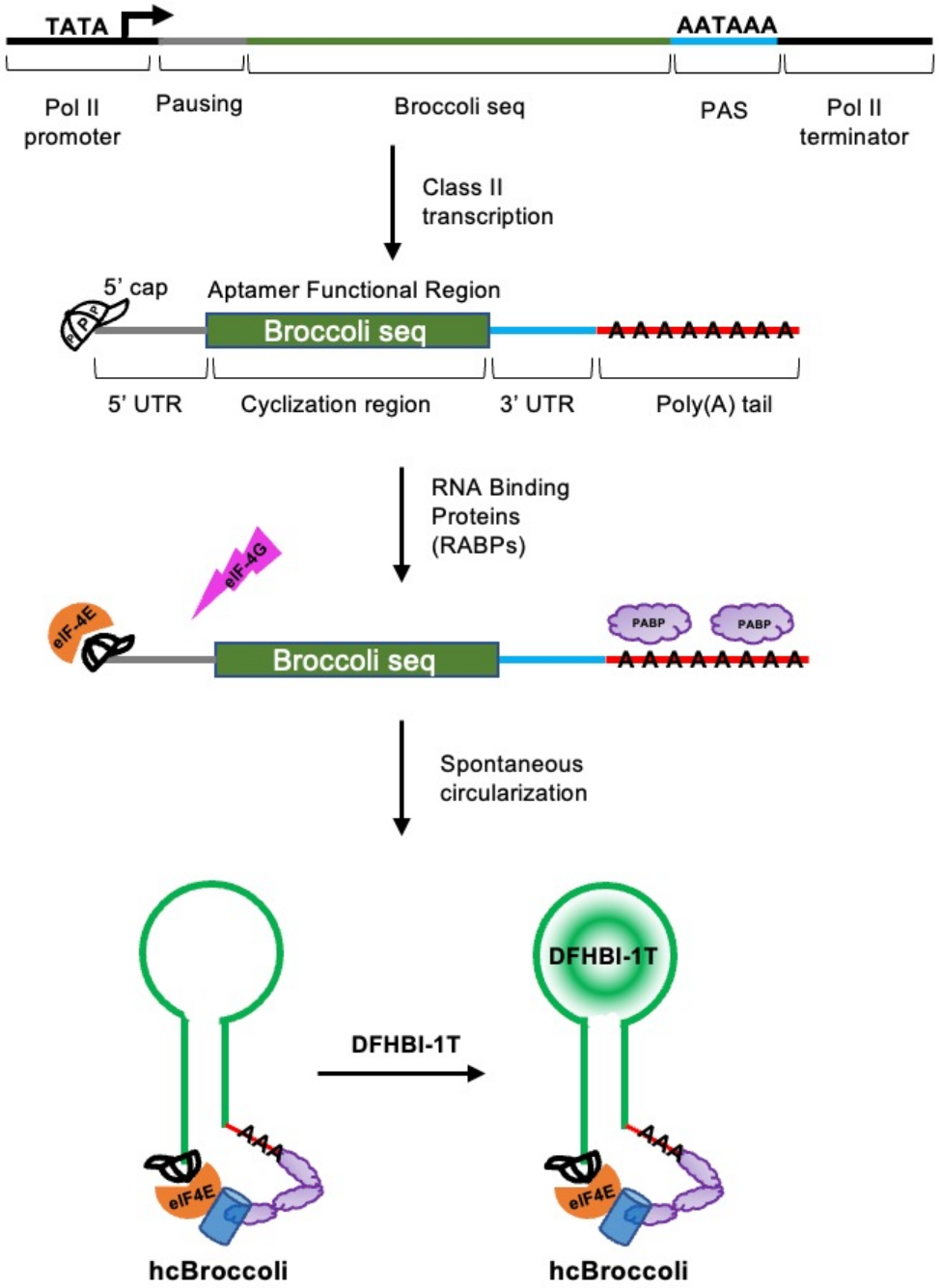
Aim 2b: Examine cytotoxicity of hcBroccoli.

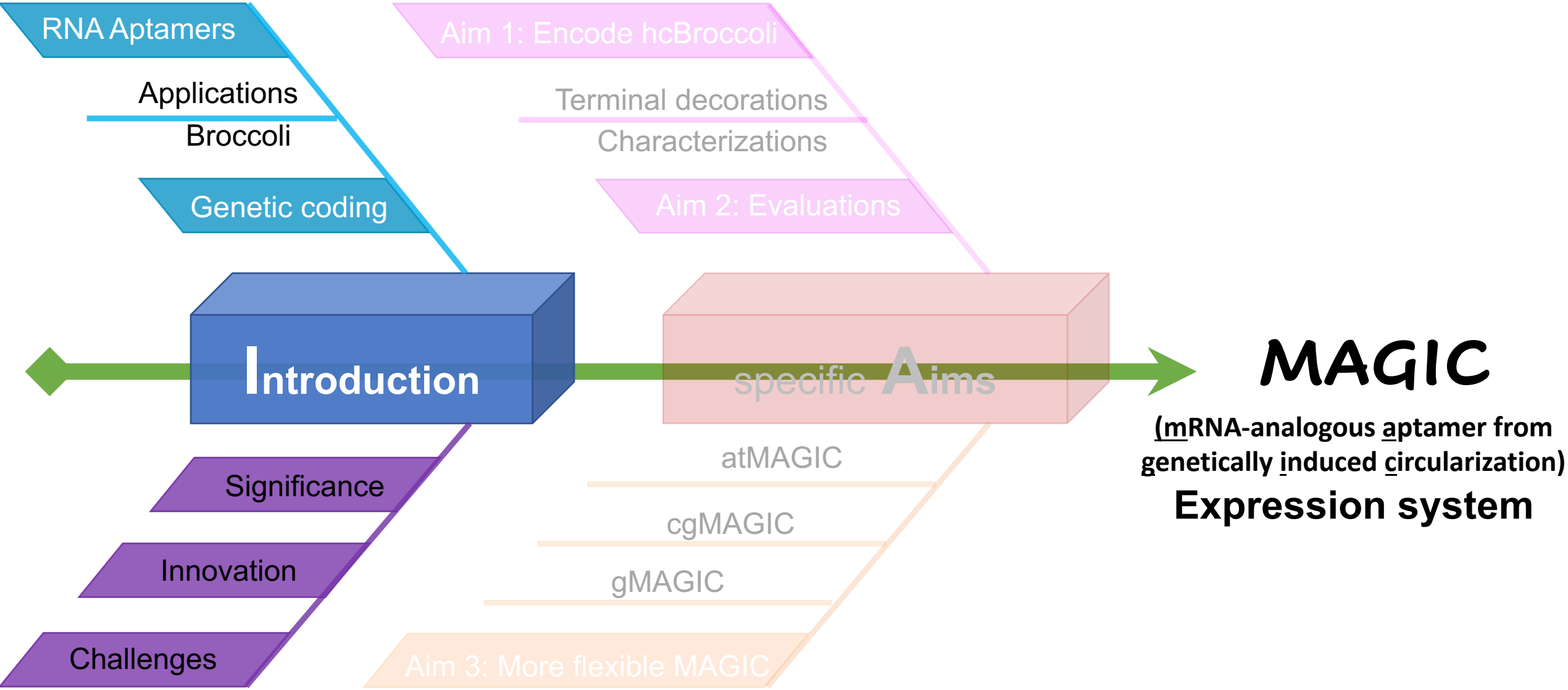
Aim 3: **More generalized expression systems.**

Aim 3a: Poly(A) independent cgMAGIC system.

Aim 3b: Cap independent atMAGIC system.

Aim 3c: Protein independent gMAGIC system.

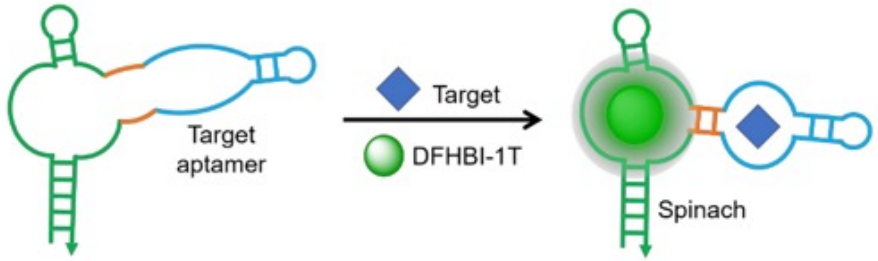



RNA Aptamers
Applications
Broccoli
Genetic coding
Aim 1: Encode hcBroccoli
Terminal decorations
Characterizations
Aim 2: Evaluations
Introduction
specific Aims
Significance
Innovation
Challenges
atMAGIC
cgMAGIC
gMAGIC
Aim 3: More flexible MAGIC
MAGIC
(mRNA-analogous aptamer from genetically induced circularization)
Expression system

Introduction

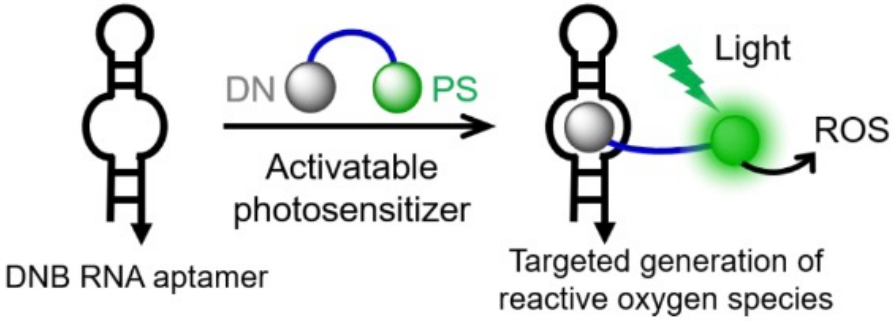
• RNA Biodevices

a. Biosensing



b. Gene regulation

c. Cell regulation



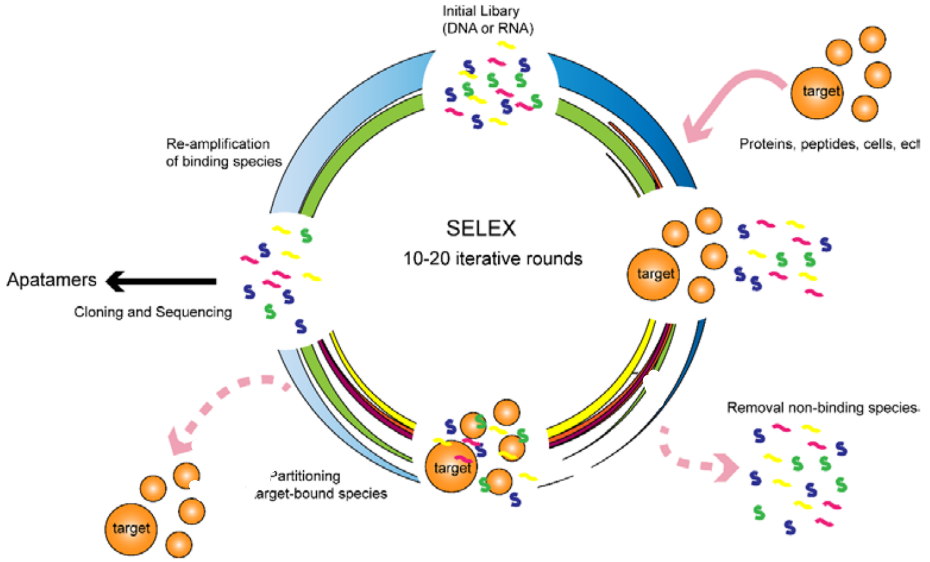
• RNA aptamers

a. Can selectively bind to target with high affinity

RNA aptamer	Fluorophore	K_D (nM)	E_x/E_m (nm)	ε (M^{-1} cm^{-1})	Φ	Length (nt)
Spinach	DFHBI	540	469/501	24 300	0.72	98
Spinach2	DFHBI-1T	560	482/505	31 000	0.94	95
Spinach2	DFHBI	530	447/501	22 000	0.72	95
Spinach2	DFHBI-2T	1300	500/523	29 000	0.12	95
Spinach2	DFHBI-CM	N/A	447/502	N/A	N/A	95
Broccoli	DFHBI-1T	360	472/507	29 600	0.94	49
Broccoli	BI	51	470/505	33 600	0.67	49
Red Broccoli	DFHO	206	518/582	35 000	0.34	49
Orange Broccoli	DFHO	230	513/562	34 000	0.28	49
Red Broccoli	OBI	23	541/590	47 300	0.67	54

b. Can be identified by SELEX

(Systematic evolution of ligands by exponential enrichment)

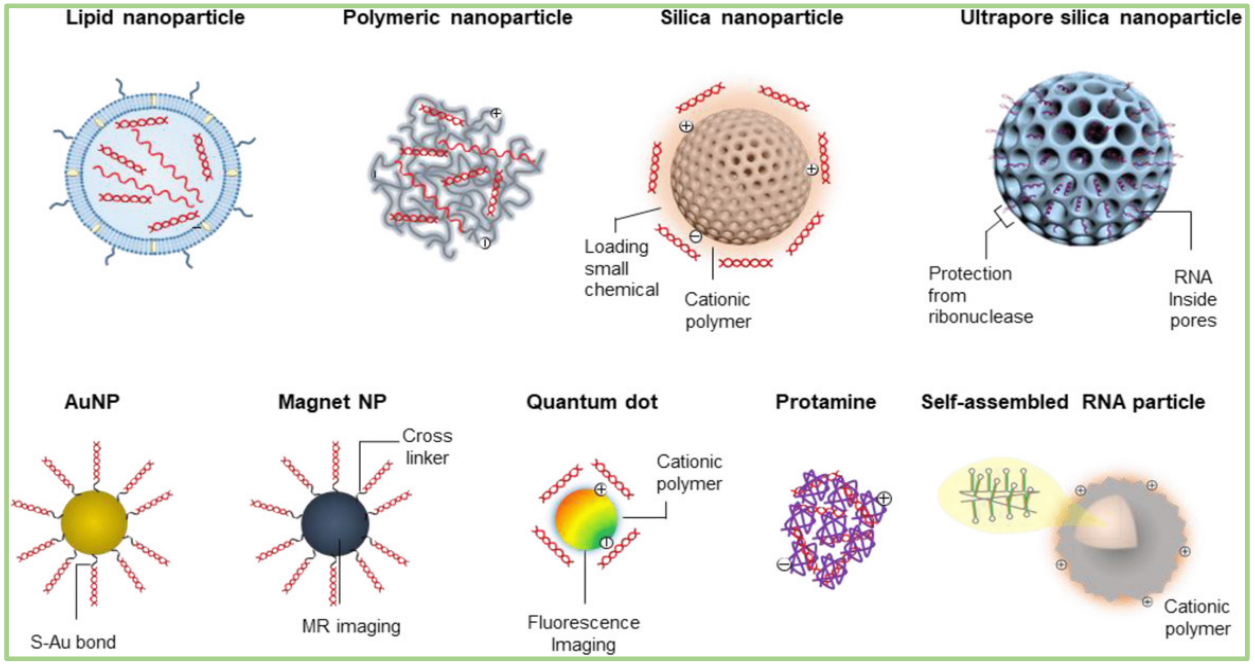


- Versatile applications.
- Generalizable for various targets.
- **Genetically encodability.**

Nanoscale, **2021**, 13, 7988
Int. J. Mol. Sci. **2017**, *18*(10), 2142

Significance & Innovation

- **In vitro synthetic RNA aptamers**
- Challenges
  - Delivery.
    a. Susceptibility to degradations *in vivo*.
    b. Poor intracellular uptake.
    c. Immunogenicity and cytotoxicity.
    d. Molecular weight confined.
  - Intracellular concentration.



- **Genetically encoded RNA aptamers**
- Advantages
  - Easy to implement.
  - No delivery system required.
  - Intracellular concentration maintainable.
  - Functionalities unaffected.
- **Challenges**

  High susceptibility to degradations in mammalian cells. Only be applied to bacteria.

- **Feasible approaches**

  Engineer the encoded RNA via capping, protein binding, or circularization.

  Rational: Mimic the **natural RNA** self-protection approaches to resist enzymatic degradations triggered by RNases or exonucleases.

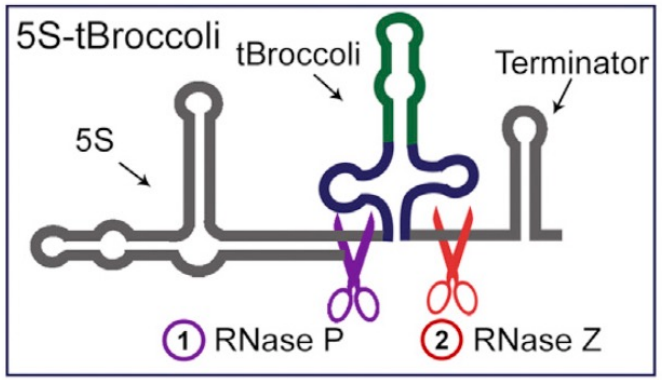
  One successful case: a high efficient circular RNA expression system: **Tornado system**.

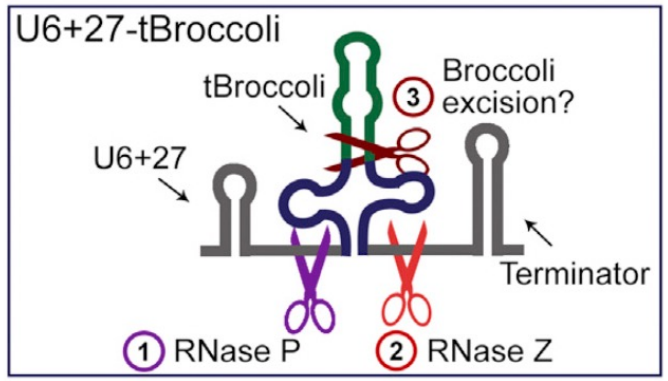
  Our goal: design **MAGIC** expression system that can protect RNAs via protein bindings and protein-mediated circularization.

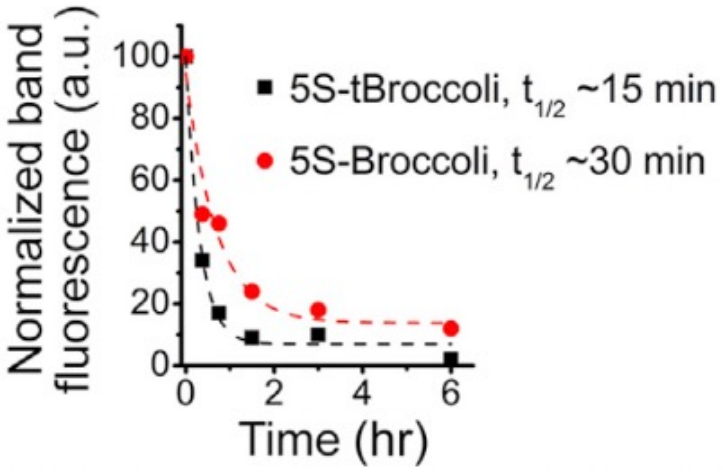
Advanced Therapeutics 1, no. 7 (**2018**): 1800065.

Challenges

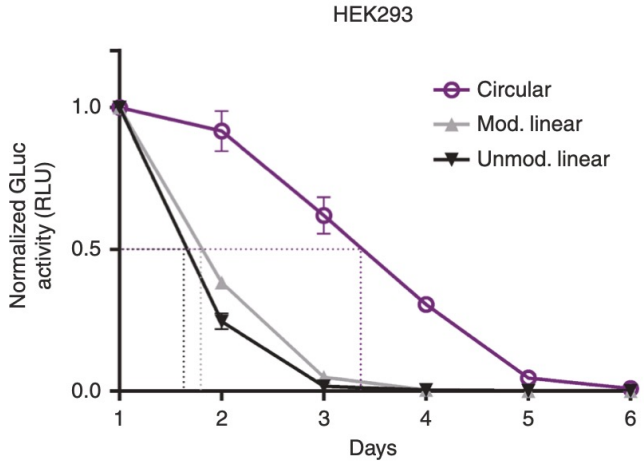
Instable aptamers in mammalian cells.







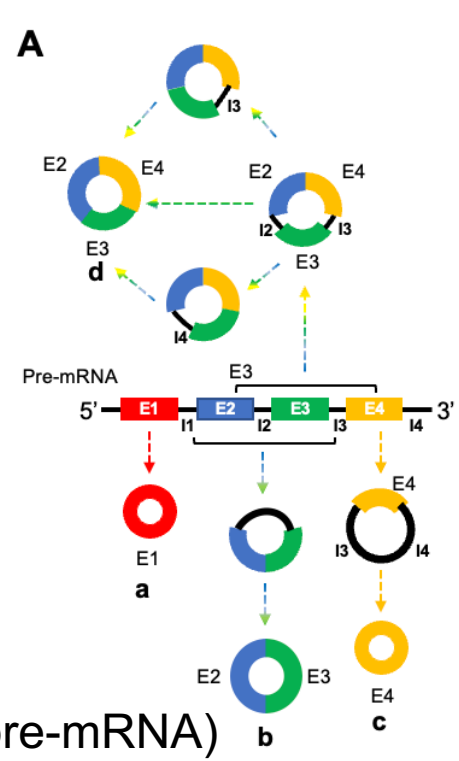
Chem Biol. **2015** May 21;22(5):649-60

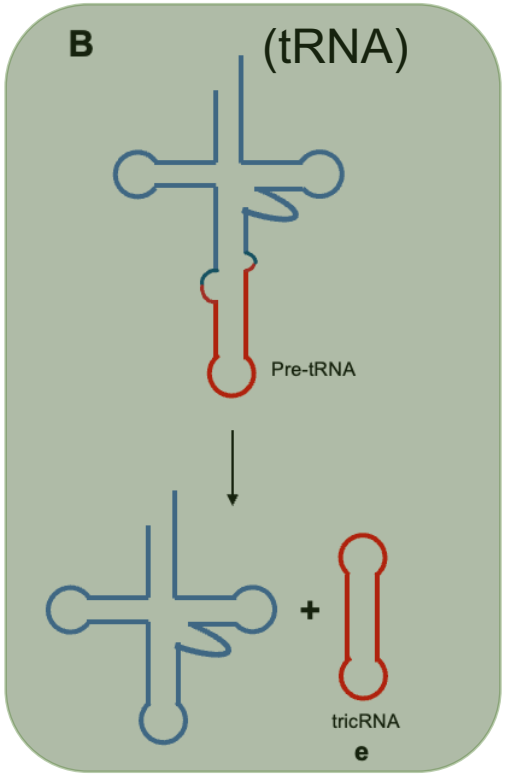


Nat Commun **9,** 2629 (**2018**)

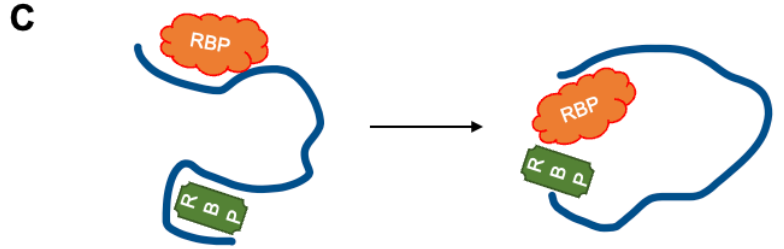
Circularization stabilizes RNA.

❖ Biogenesis of circular RNAs (A,B)

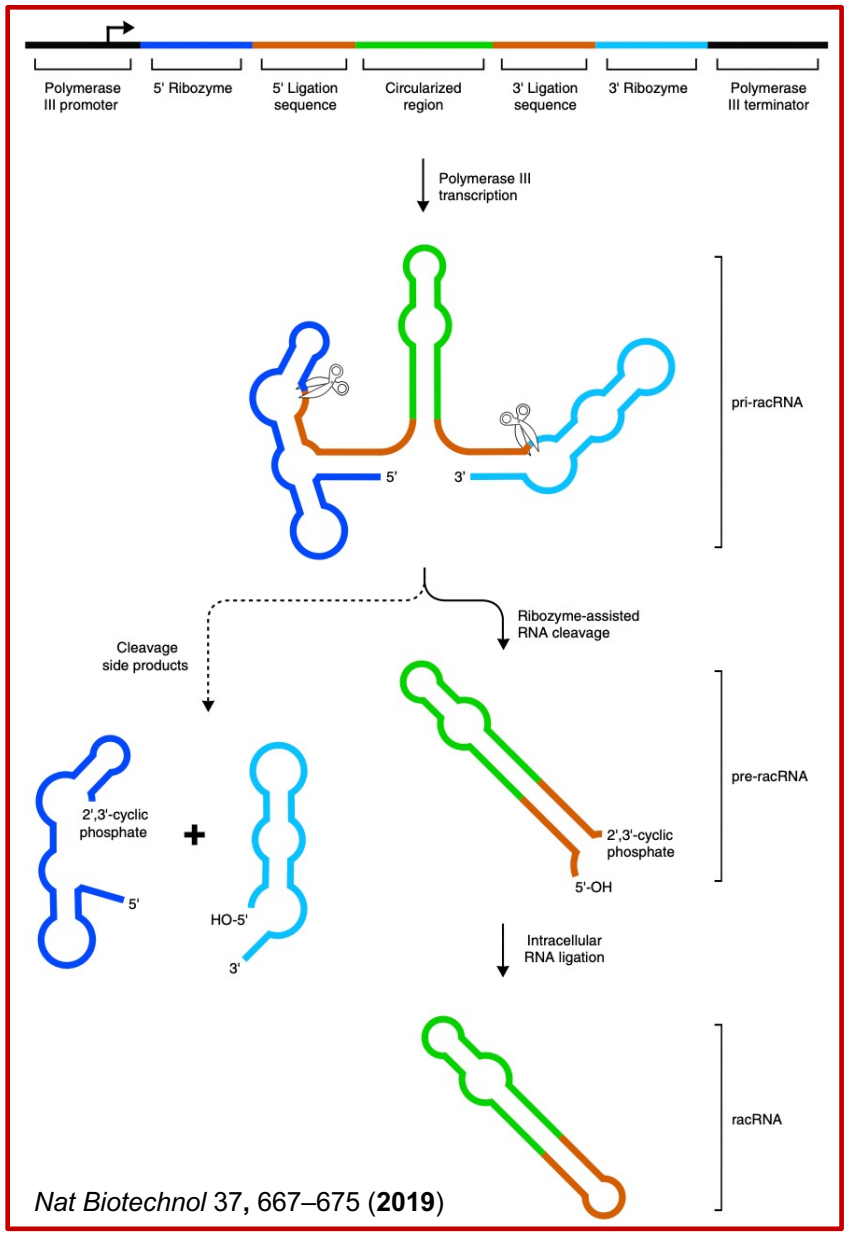




❖ RNA-binding protein mediated circularization



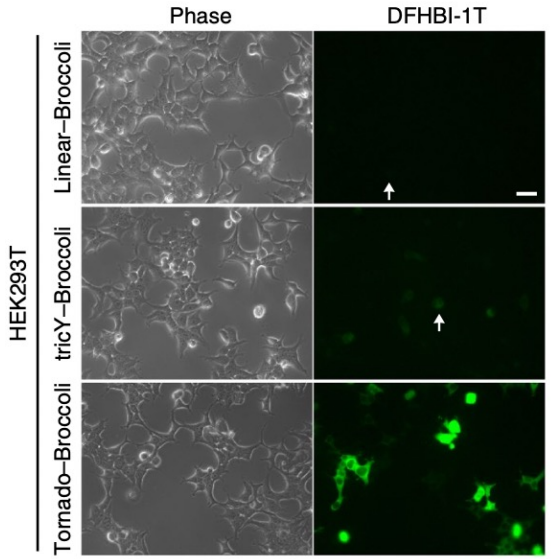
Tornado expression system



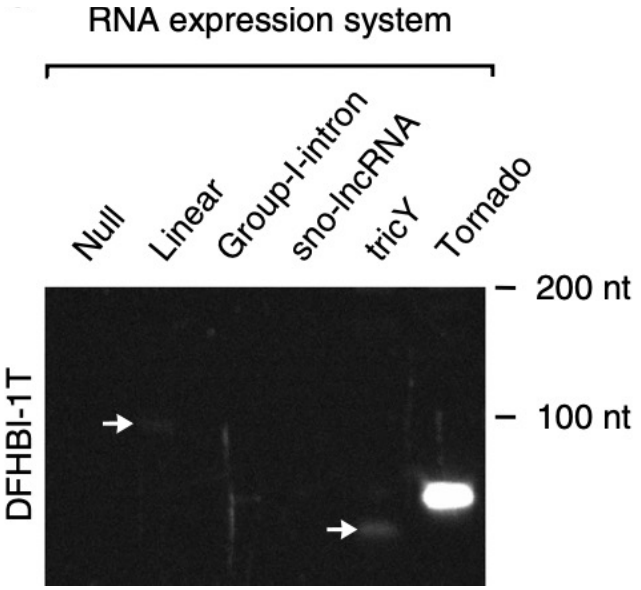
Nat Biotechnol 37, 667–675 (**2019**)

Tornado system encodes circular RNA aptamers

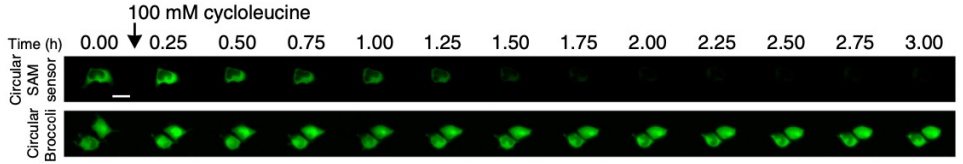
❖ Functionable.



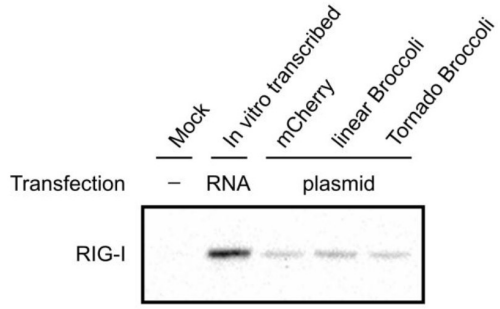
❖ Higher intracellular concentrations.



❖ Longer lifespans.



❖ Non-cytotoxicity.

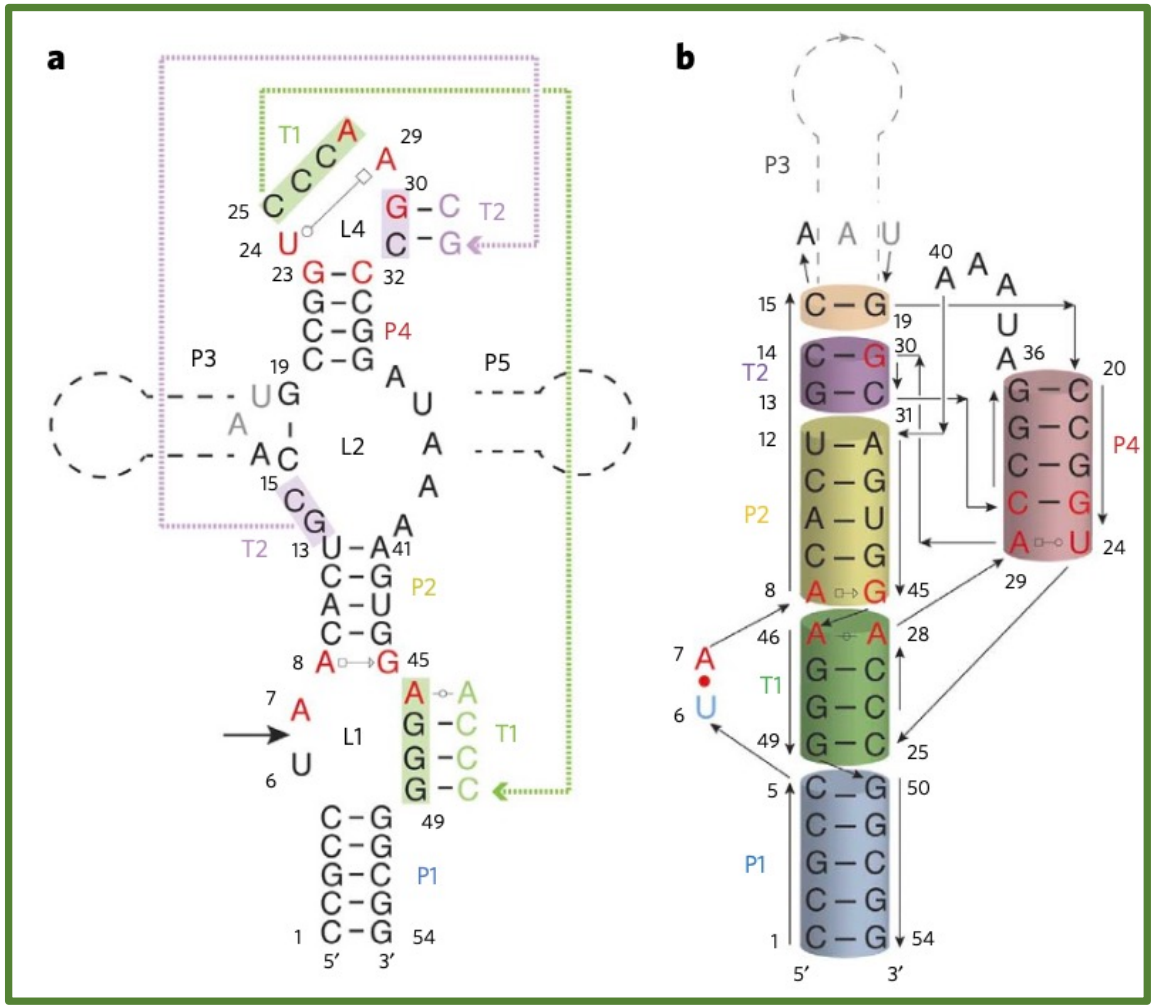


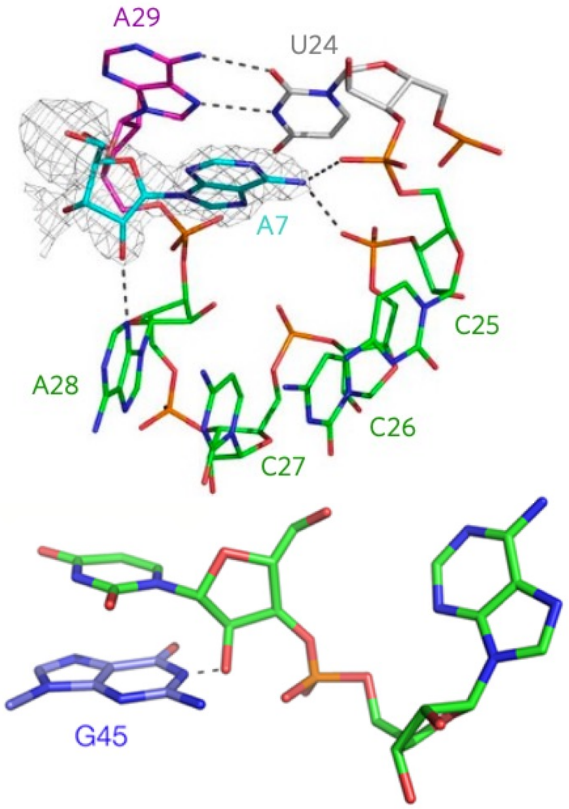
❖ Highly twister-ribozyme dependent.

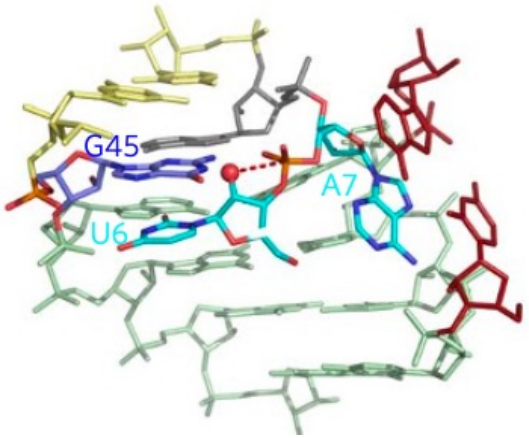
Limitations of Tornado expression system

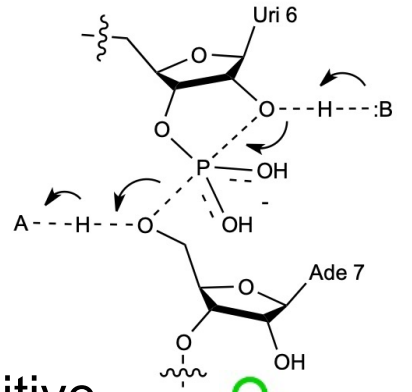
Twister-ribozyme aided self-cleavage --- S_N2-related mechanism.

a. Highly conformational restricted: A stem-loop, two internal loops.





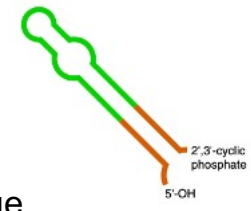




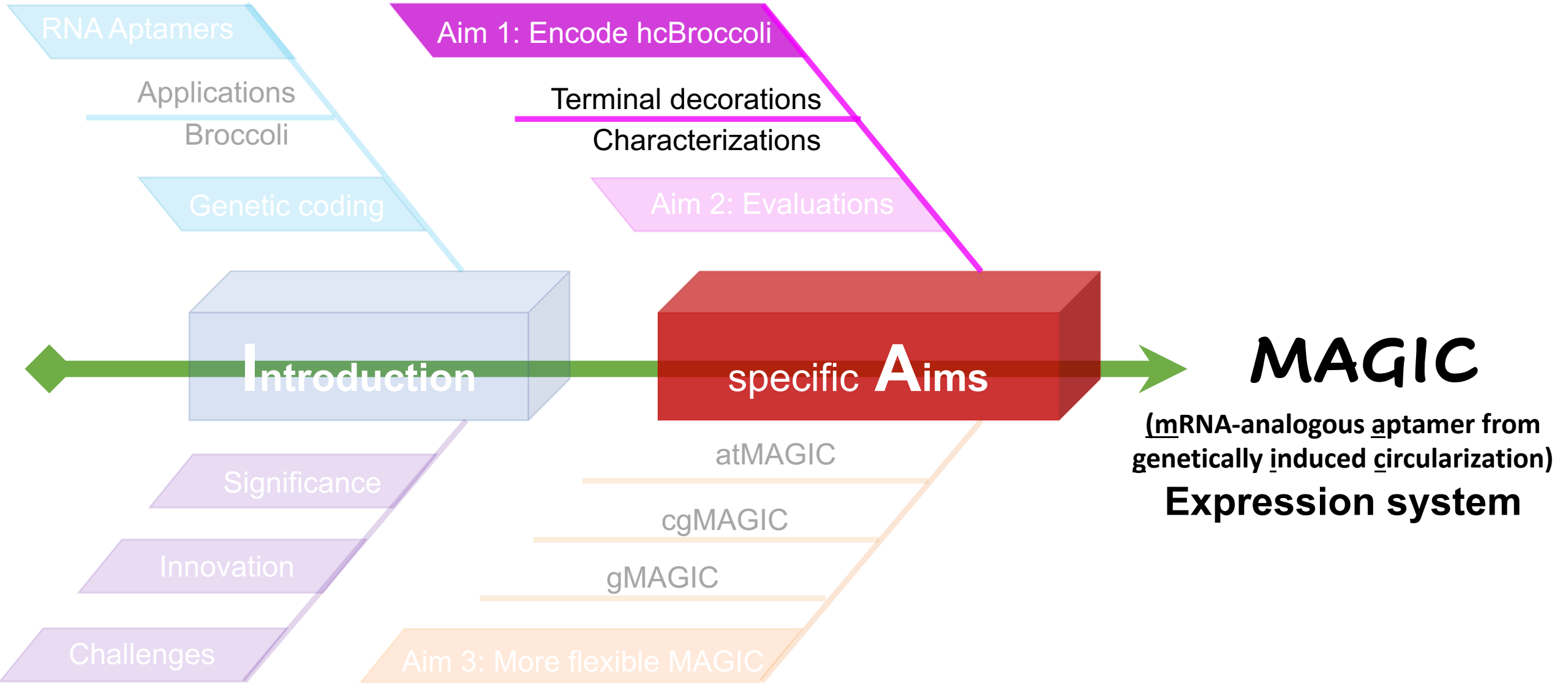
b. Cellular environment sensitive.

c. Kinetics hard-to-control.

K_{cleav1} vs. K_{cleav2} $K_{ligation}$ vs. $K_{cleavage}$

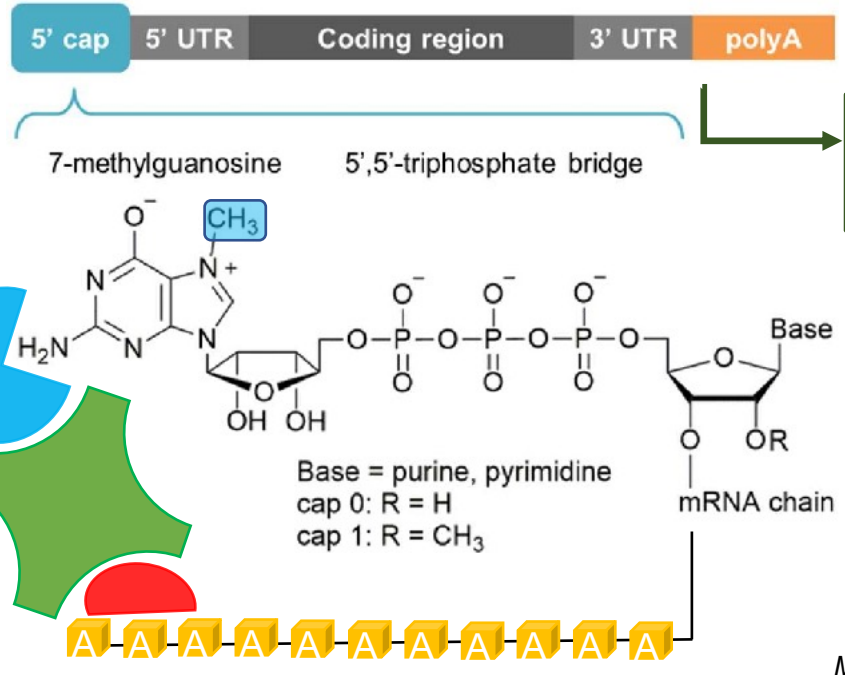


Nat Chem Biol 10, 739–744 (**2014**)
Nat Commun 7, 12834 (**2016**)


RNA Aptamers
Applications
Broccoli
Genetic coding
Aim 1: Encode hcBroccoli
Terminal decorations
Characterizations
Aim 2: Evaluations
Introduction
specific Aims
MAGIC
(mRNA-analogous aptamer from genetically induced circularization)
Expression system
Significance
Innovation
Challenges
atMAGIC
cgMAGIC
gMAGIC
Aim 3: More flexible MAGIC

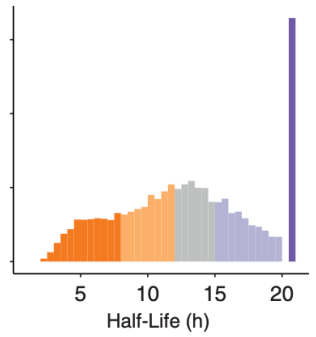
mRNA circularization and analogous MAGIC system

- **mRNA**
  - Average ~10 h half-lifetime.
  - Have m^7G cap group protecting 5’ terminal.
  - Have a poly(A) tail protecting 3’ terminal.
  - Spontaneously cyclize in cytoplasm.

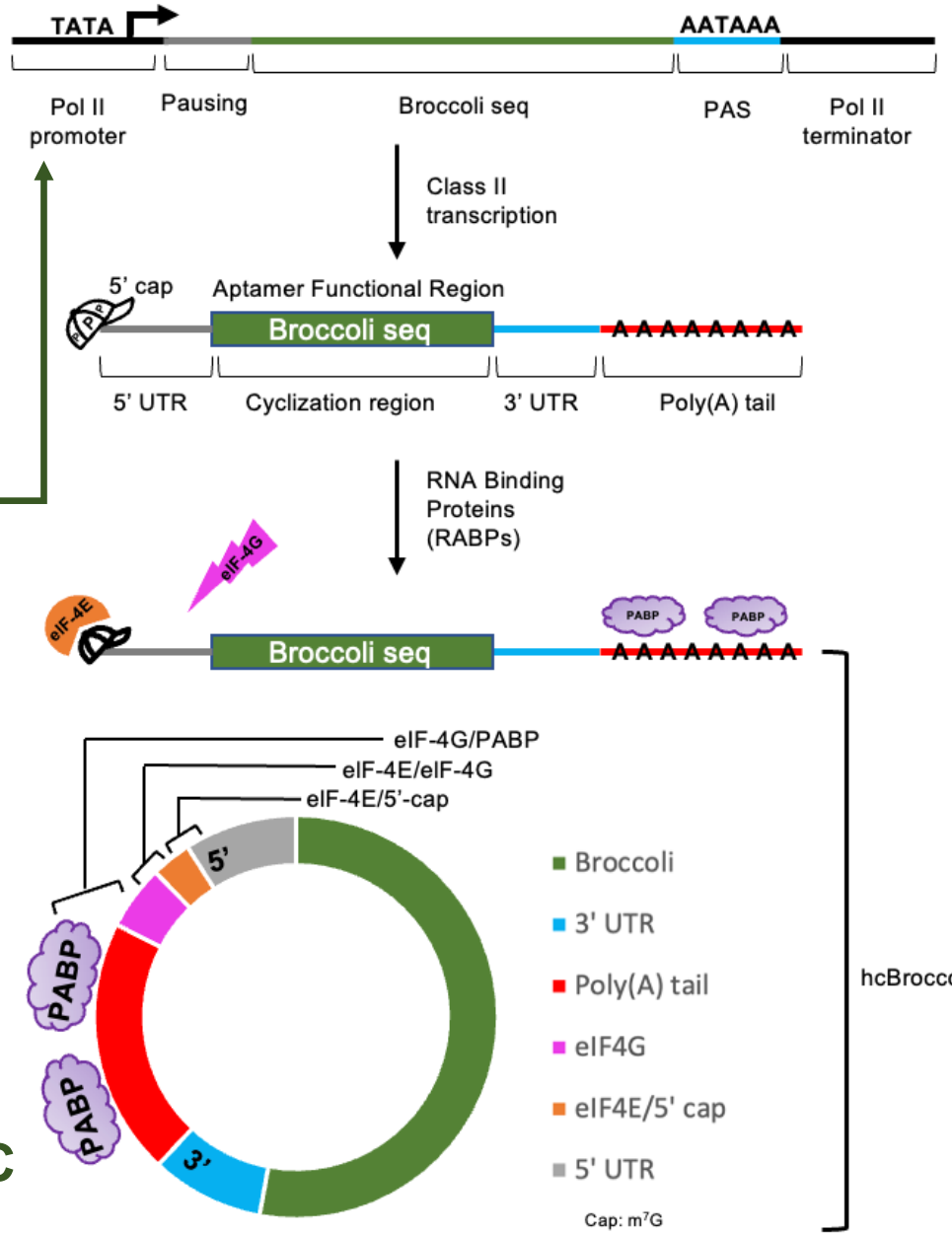


Bioconjugate Chem. **2017**, 28, 7, 1978–1992

Class II transcriptions



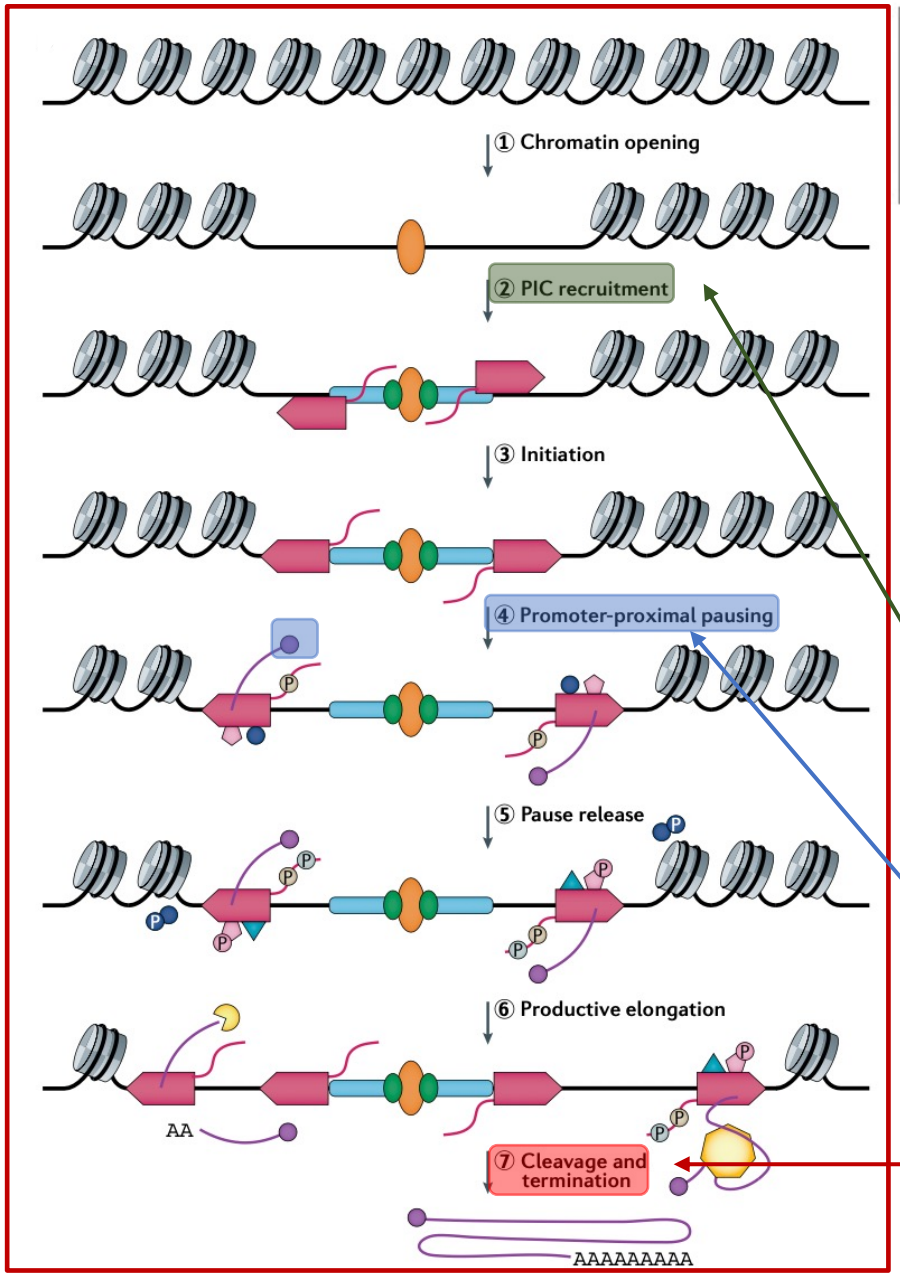
Mol Syst Biol. **2012** Jul 17;8:593

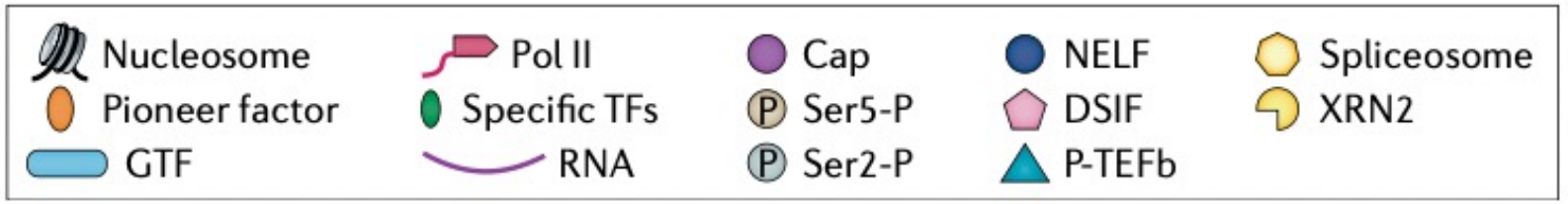


- **Our proposal**

  Develop an mRNA-analogous expression system: **MAGIC**

mRNA biogenesis mechanism: Class II transcriptions





Terminal decoration basis: Class II transcriptions

Co-transcriptional processes:

- Capping
- Polyadenylation

Our objective:

- Initiate Class II transcriptions with ***Promoter II***.

  Recruit the pre-initiation complex (**PIC**).
- Ensure capping.

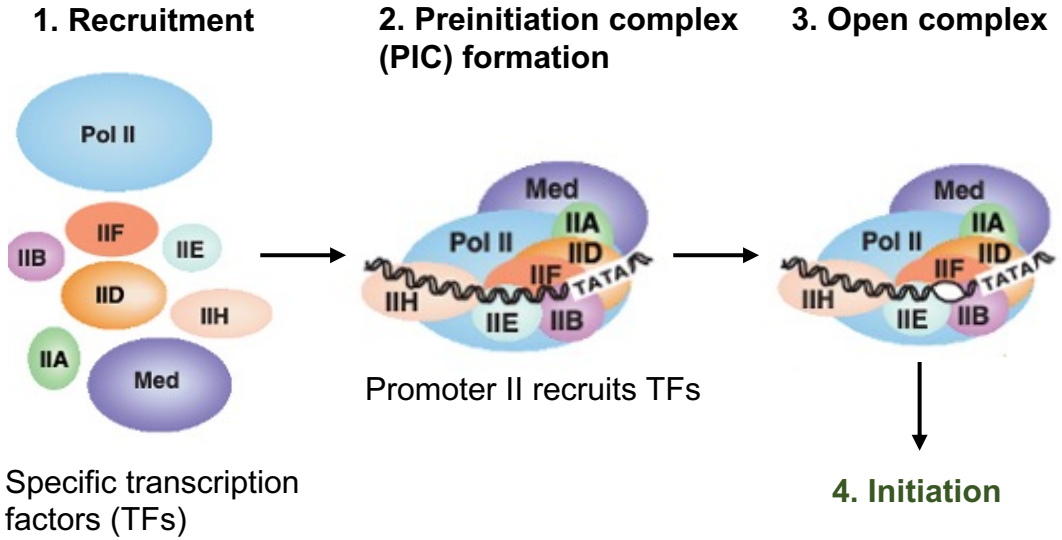
  At pausing stage.
- Ensure polyadenylation.

Nat Rev Genet 20, 705–723 (**2019**)

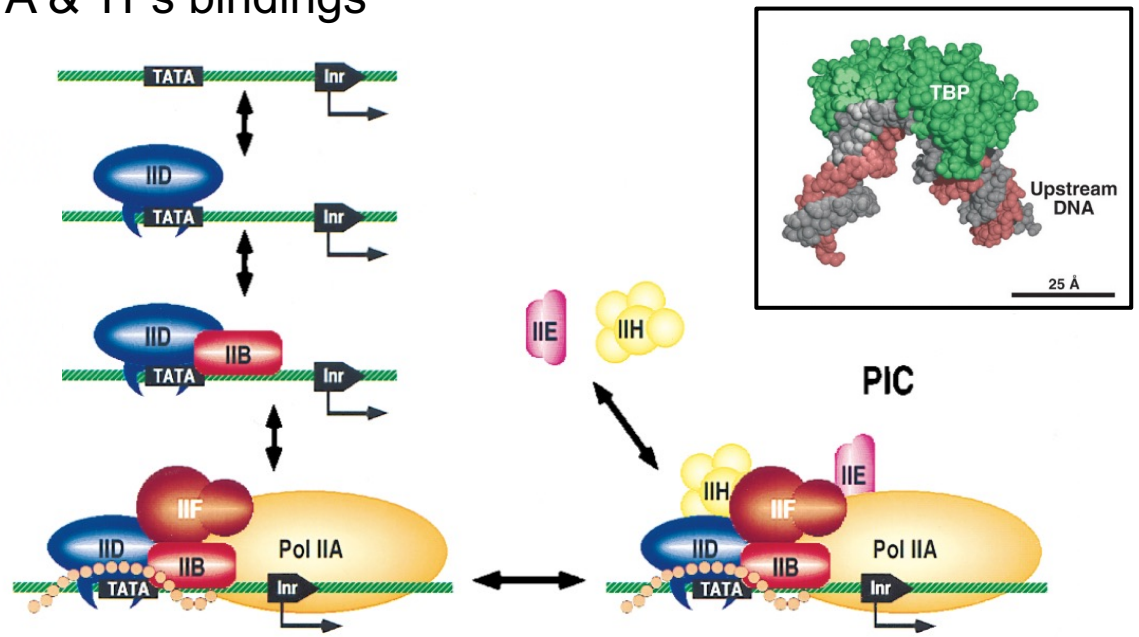
Class II transcription: Initiation (PIC and Pol II recruitment)

Polymerase II promoter: TATA box (~ 20 nt upstream from Initiator)

- The pathway of transcription initiation



- TATA & TFs bindings



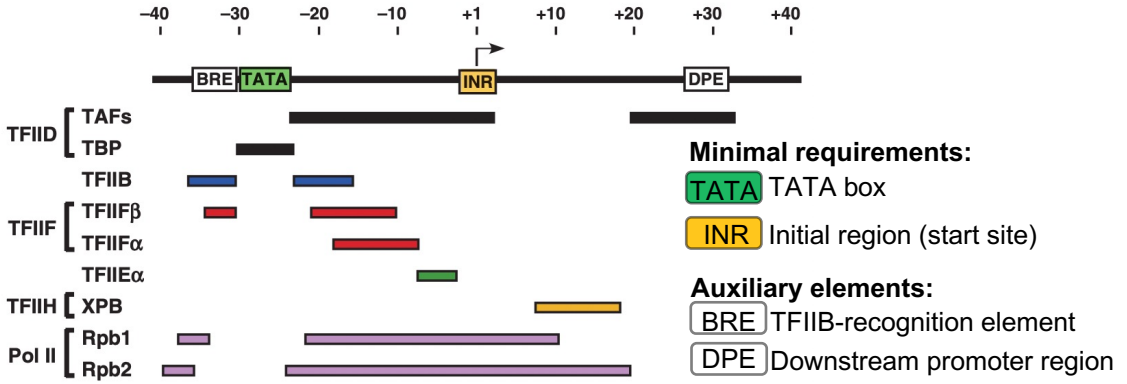
- PIC components & functions

PNAS January 7, **1997** 94 (1) 15-22
Nat Struct Mol Biol 11, 394–403 (**2004**)

Table 1. General class II transcription initiation factors from human cells

Factor		Subunits, kDa	(no.)	Function
TFIID	/TBP	38	(1)	Binds to TATA, promotes TFIIB binding
	\TAFs*	15–250	(12)	Regulatory functions (+ and −)
TFIIB		35	(1)	Promotes TFIIF–pol II binding
TFIIF		30, 74	(2)	Targets pol II to promoter
RNA pol II		10–220	(12)	Catalytic function
TFIIE		34, 57	(2)	Stimulates TFIIH kinase and ATPase activities
TFIIH		35–89	(9)	Helicase, ATPase, CTD kinase activities
All class II GTFs		> 2 MDa	(>42)	

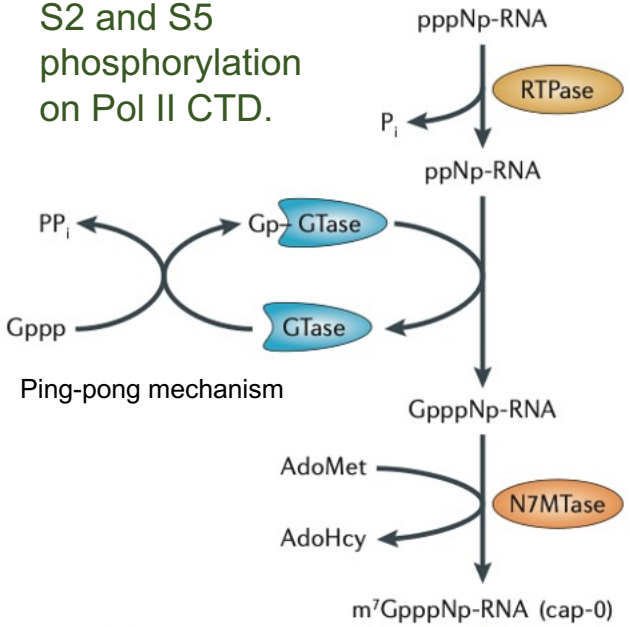
- Element locations



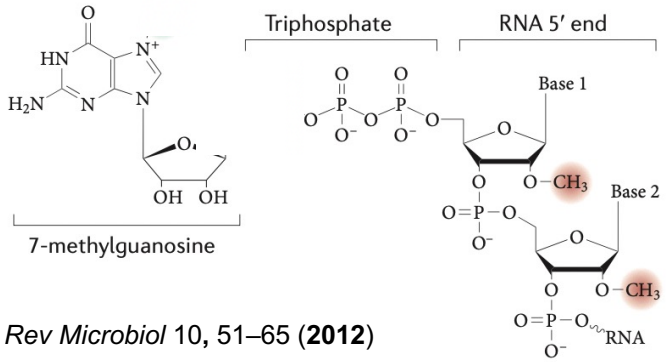
Co-transcriptions: 5’ Capping and 3’ polyadenylation

Capping

- The canonical capping mechanism
  a. Pausing period.
  b. Accompany with S2 and S5 phosphorylation on Pol II CTD.



Ping-pong mechanism

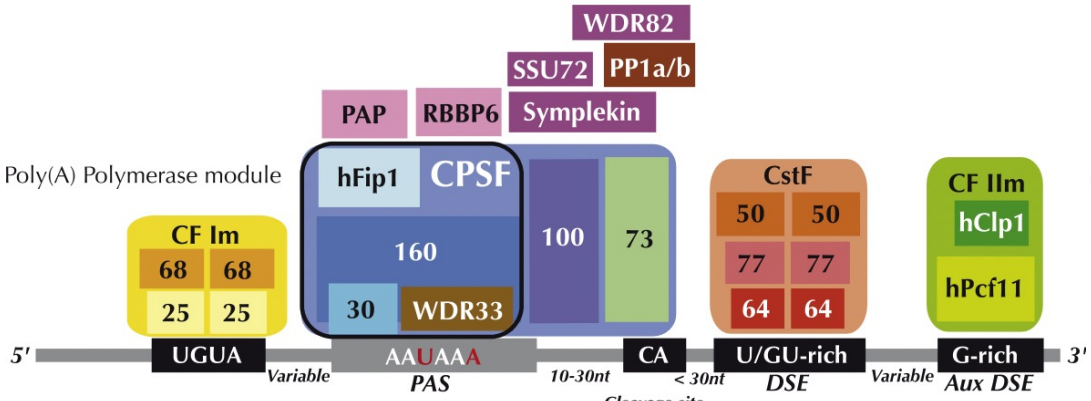


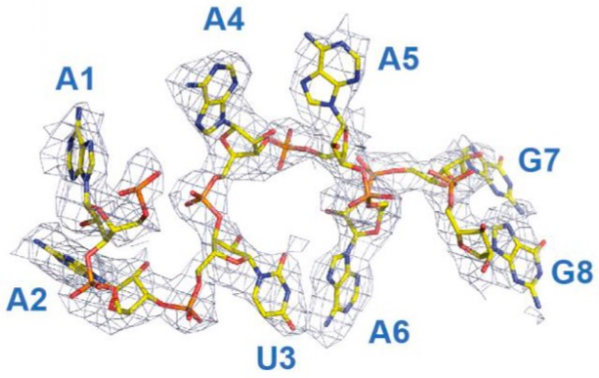
Nat Rev Microbiol 10, 51–65 (**2012**)

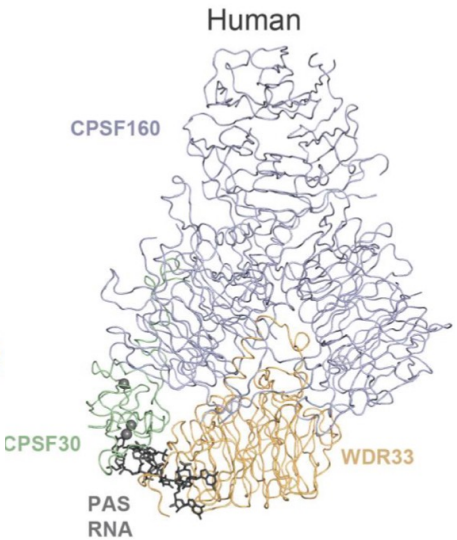
Polyadenylation

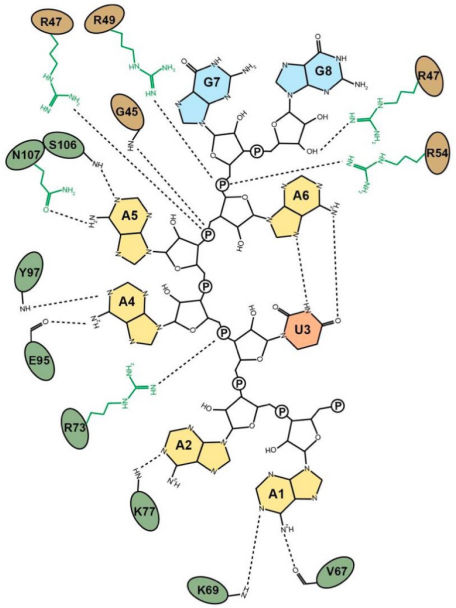
- Minimal requirement: **PAS site**
  a. Final stage, coupled with cleavage.
  b. PAS, AAUAAA, can be specifically recognized by the cleavage and polyadenylation specificity factor (CPSF) complex.

Nat Struct Mol Biol 25, 135–138 (**2018**)
Biochimie. **2019** Sep;164:105-110
FEBS Lett. **2014** Jun 27;588(14):2185-97.









Minimal requirements:
PAS AAUAAA, PAS site
CA Cleavage site (start site)

Auxiliary elements:
UGUA CFIm recognition region
U/GU CFIIm recognition region

AIM1a: Design a template DNA sequence for MAGIC

1. DNA template

❖ Minimal requirements:

---TATA-$N_{(25)}$-AUG-$N_{(5'\ UTR)}$-TATAGAGACGGTCGGGTCCAGATATTCGTATCTGTCGAGTAGAGTGTGGGCTC-
$N_{(3'\ UTR)}$-AATAAA-$N_{(\sim 20)}$-CA---

J. Am. Chem. Soc. **2014**, 136, 46, 16299–16308
Nucleic Acids Research, Volume 38, Issue 21, 1 November **2010,** 7845–7857
Gene. **2007** Mar 1;389(1):52-65.
Molecular and cellular biology 18.7 (**1998**): 3811-3818.
Journal of Biological Chemistry 279.9 (**2004**): 8102-8110.
J Biol Chem. **2017** Jul 14;292(28):11873-11885

❖ Designed simplest DNA template:

24 nt

TCCTGAAGGGGGGCTATAAAAGGGGGTGGGGGCGCGAACCTCTGGCAGGAGCAAAGGCGCCAT

49 nt

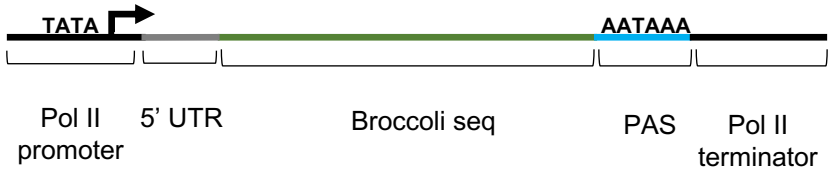
GGCTGTGGAGGGCGGATATAGAGACGGTCGGGTCCAGATATTCGTATCTGTCGAG

TAGAGTGTGGGCTCAGTGCCTCTCCTGGCCCTGGAAGTTGCCTCTCCAGTGCCCACCAGCCTT

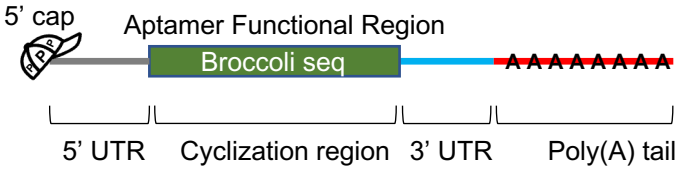
9 nt

GTCCTAATAAAATTAAGTTGCATCATTTTGTCTGACTAGGTGTCCTTCT → 233 nt

❖ Expected transcript: hcBroccoli.



Class II transcription →



AIM1a: Characterizations of Class II transcription initiation

2. Characterize the initiation of class II transcriptions

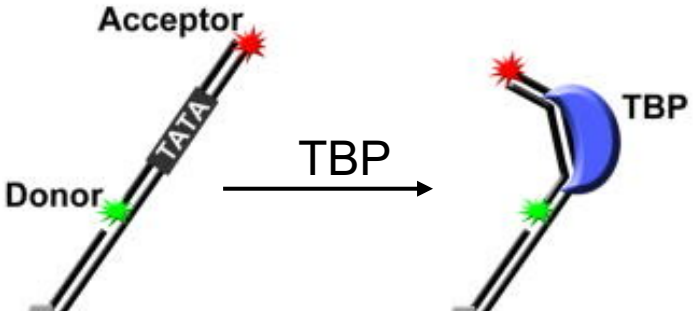
J Biol Chem. **2017** Jul 14;292(28):11873-11885
Biochemistry. **2012** Sep 25;51(38):7444-55.

Method: FRET

(Förster resonance energy transfer)

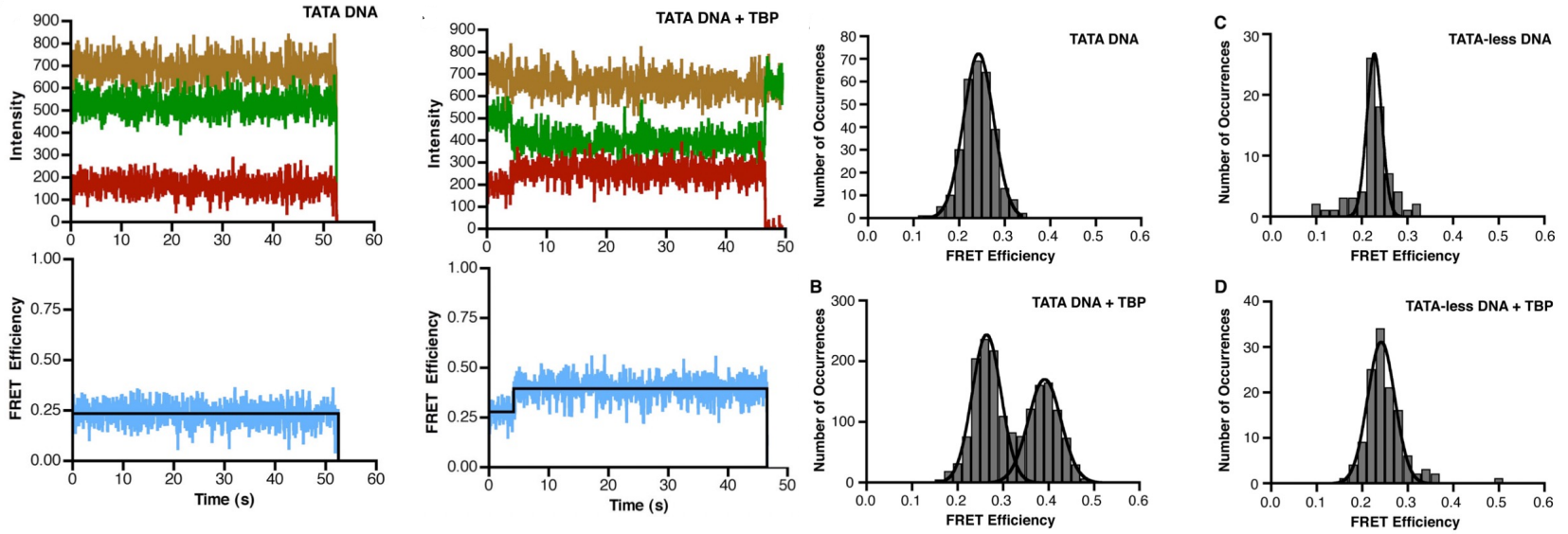
Mechanism:

TBP-induced DNA bending.



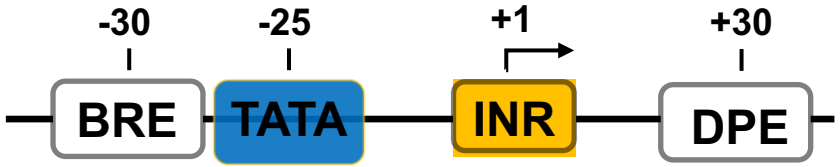
Oligo	Sequence (5′ to 3′)
Consensus TATA donor	CTATAAAAG
Consensus TATA acceptor	CTTTTATAG
Fluorophore Donor:	Alexa 555
Fluorophore Acceptor:	Alexa 647
TATA-less donor	TAGAGTCGG
TATA-less acceptor	CCGACTCTA

Expected outcome: TBP causes an increase in FRET efficiency.



If TBP is not successfully recruited by TATA box solely:

❖ **Promoter optimizations.**



+ BRE: TFIIB-recognition element

+ DPE: Downstream promoter region

TGAASSRCGCCTATAAAAGGGGRRRR-INR-AGACG--
BRE1 BRE2 DPE

- Alternative sequences:

TATA box:	TATAWAAR
DPE:	RGWYG
INR:	RGWCGTG
BRE:	SSRCGCC

N: A, T, C, or G
W: A or T
R: A or G
Y: C or T
S: G or C

AIM1a: Characterization of Capping event

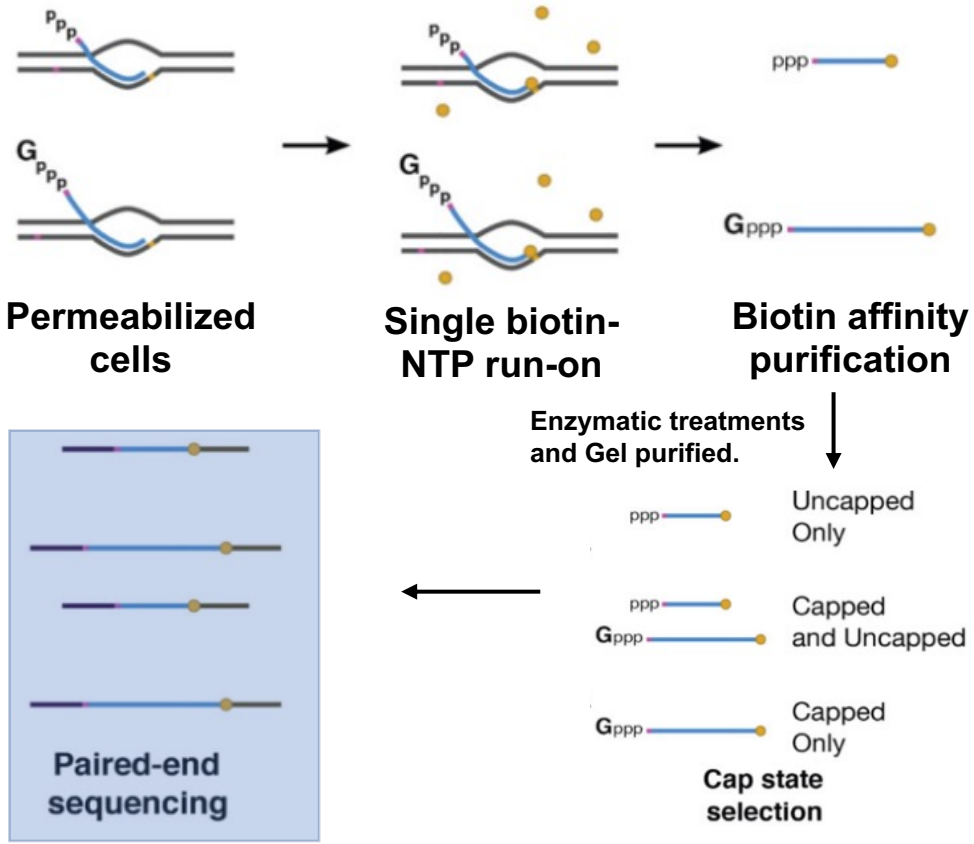
3. Characterize the 5' end capping process

Nat Genet 50, 1533–1541 (**2018**)
Epigenetics & Chromatin 9, 32 (**2016**)
Nat Rev Genet 20, 705–723 (**2019**)

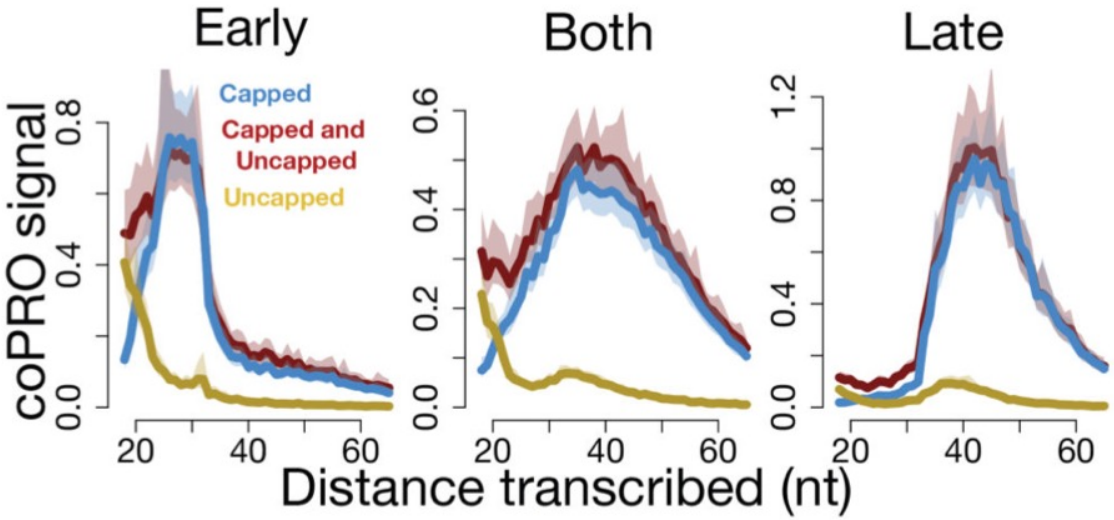
Method: CoPRO

(Coordinated Precision Run-On and sequencing)

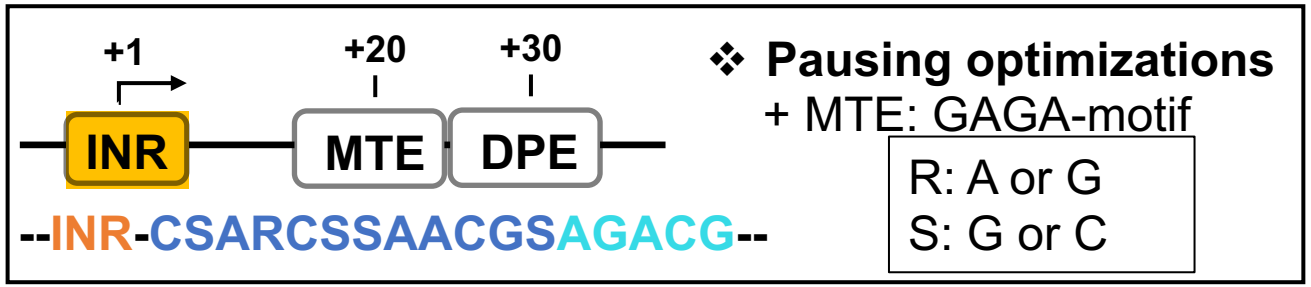
Experimental details:



Expected outcome: Capping CoPRO signal.



If there is no capping event:



Rational: **strengthen the pausing event, elongate the probable capping time for capping-related enzyme complex recruiting.**

AIM1a: Characterization of Polyadenylation

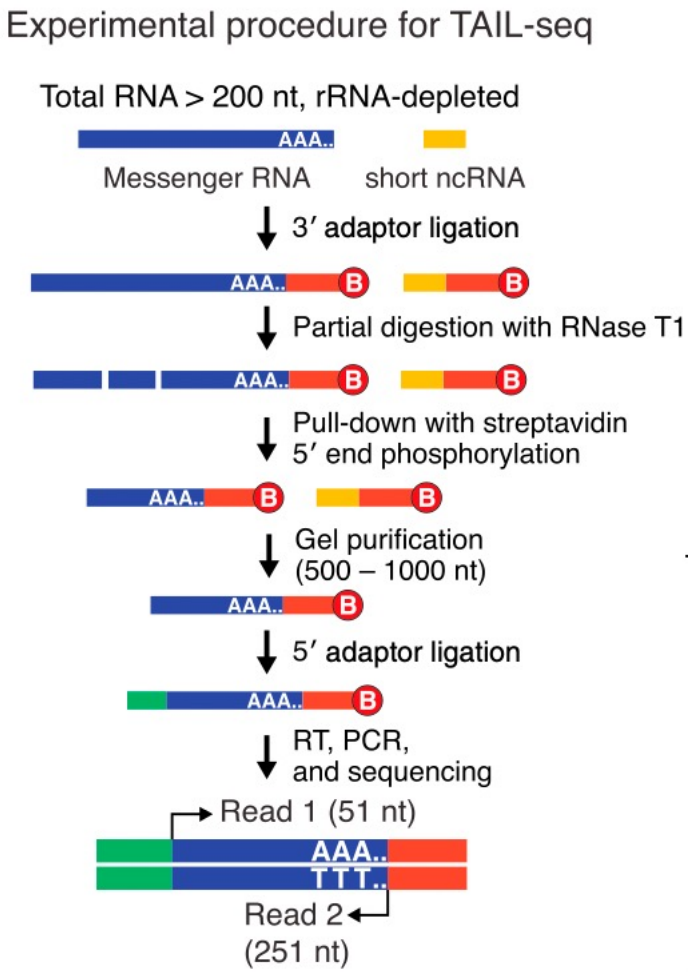
4. Characterize the 3' end polyadenylation

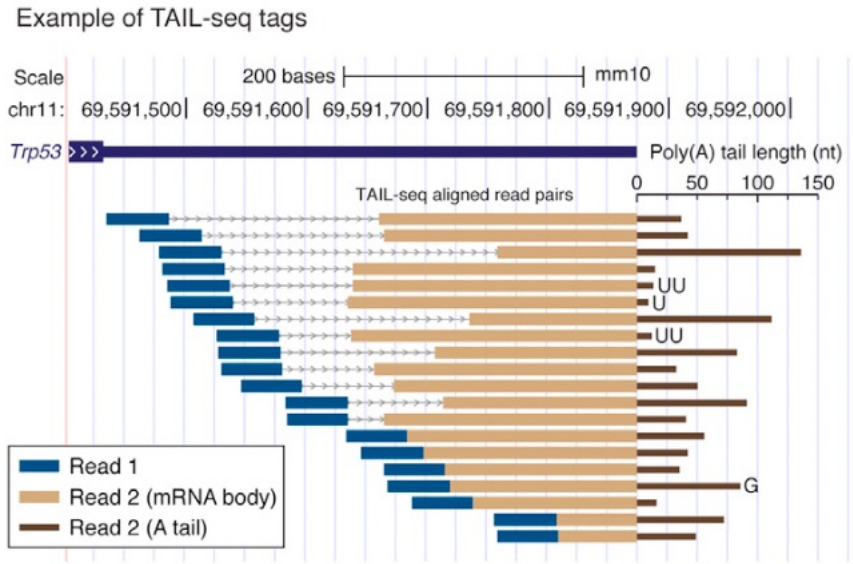
Nat Genet 50, 1533–1541 (**2018**)
Epigenetics & Chromatin 9, 32 (**2016**)

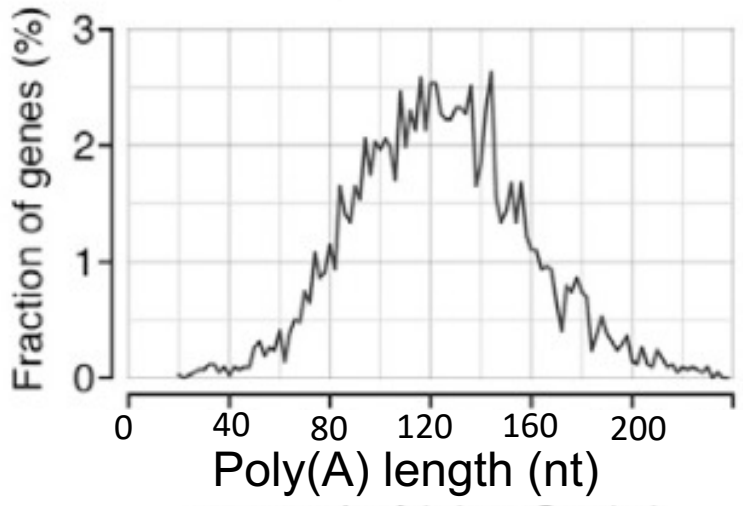
Method: TAIL-seq

Expected outcome: poly(A) tail signal with length > 120 nt.

Experimental details:

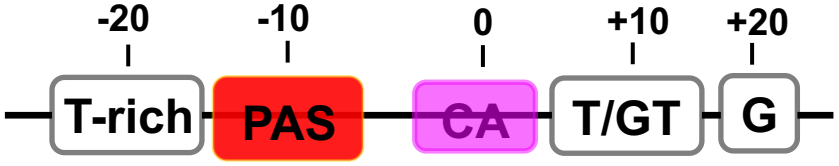






If poly(A) tail is not transcribed with expected length:

❖ **PAS optimizations**
+ T-rich region
+ T/GT rich region
+ G-rich region



--TGTATTAATAAA-CA-GTGTGTTGGTTTTTTGTGTG

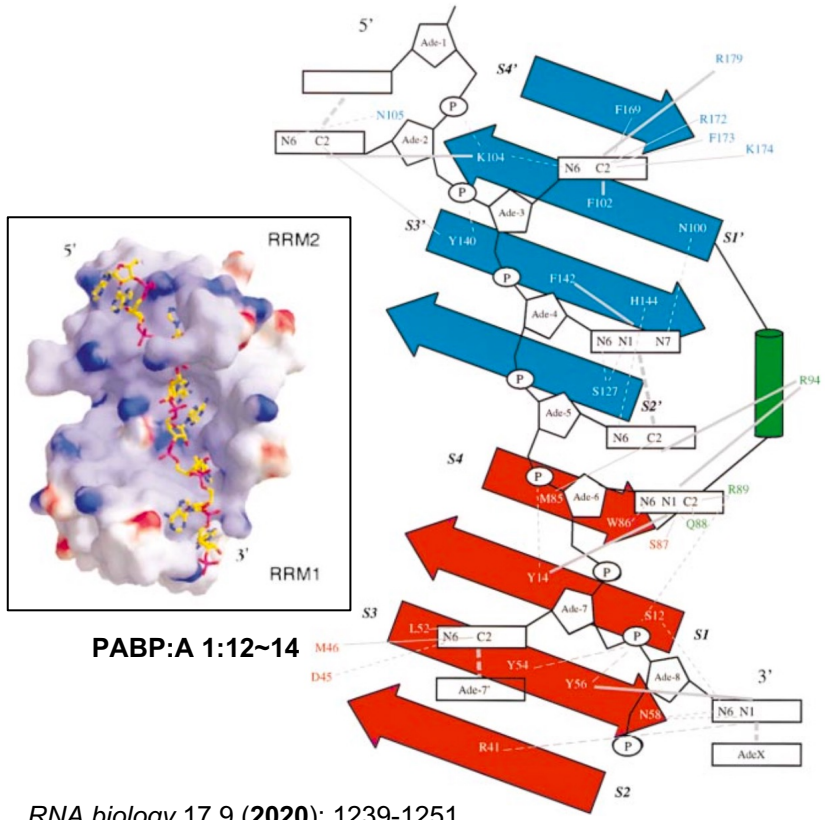
❖ **Append a poly(A) tail on template DNA directly**
Length controllable!

Wiley Interdiscip Rev RNA. **2012** May-Jun;3(3):385-96.
GEnes Dev. **1989** Jul;3(7):1019-25.

hcBroccoli spontaneous circularization

Poly(A)-PABP

Form in nuclear



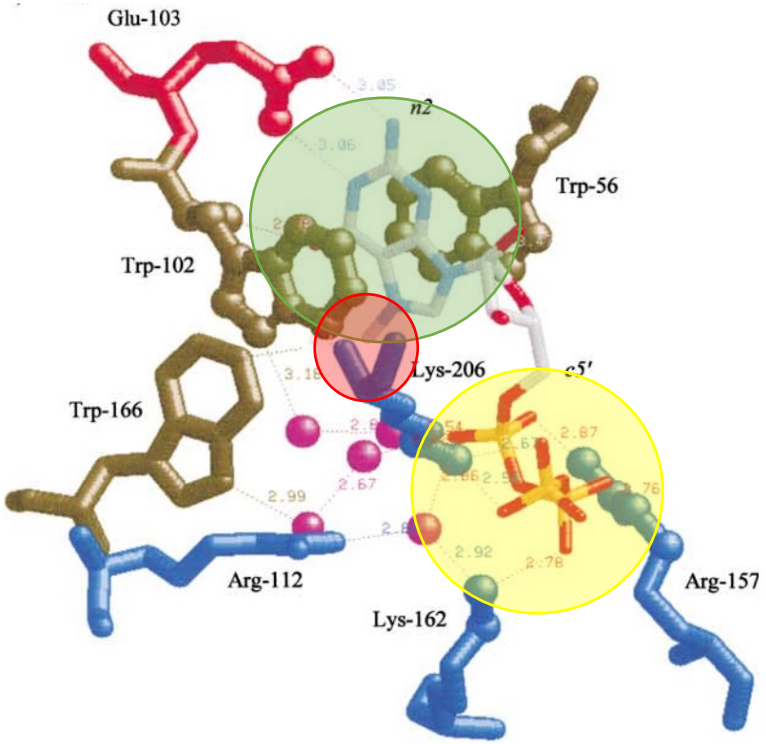
PABP:A 1:12~14

RNA biology 17.9 (**2020**): 1239-1251.
Journal of Biological Chemistry 283.37 (**2008**): 25227-25237.

Biophys J. **2012** Mar 21; 102(6): 1427–1434
Journal of molecular biology 319.3 (**2002**): 615-635.
J Mol Biol. **2002** Jun 7;319(3):615-35
Biochemistry **2004**, 43, 42, 13305–13317

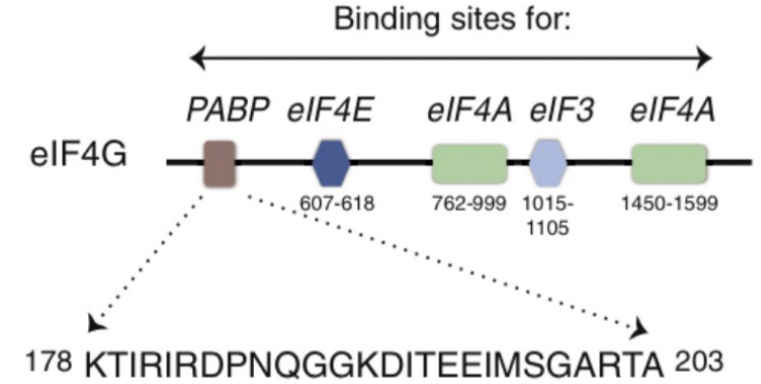
Genome Biol 4, 223 (**2003**)
Journal of Biological Chemistry 283.37 (**2008**): 25227-25237.

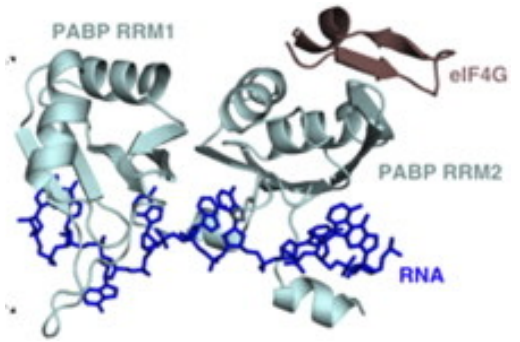
Cap-eIF4E

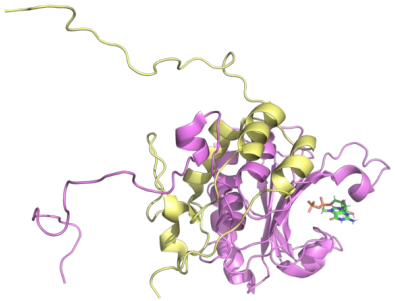


Cap analogue	Human eIF4E K_{as} x 10^{-6} (M^{-1})
m^7GpppG	0.69±0.07
m^7GpppC	0.48±0.05

PABP-eIF4G-eIF4E







RRM-eIF4G K_{as} x 10^{-6} (M^{-1})	eIF4G-eIF4E-m^7G K_{as} x 10^{-6} (M^{-1})
0.32 ± 0.07	4.4 ± 1.3

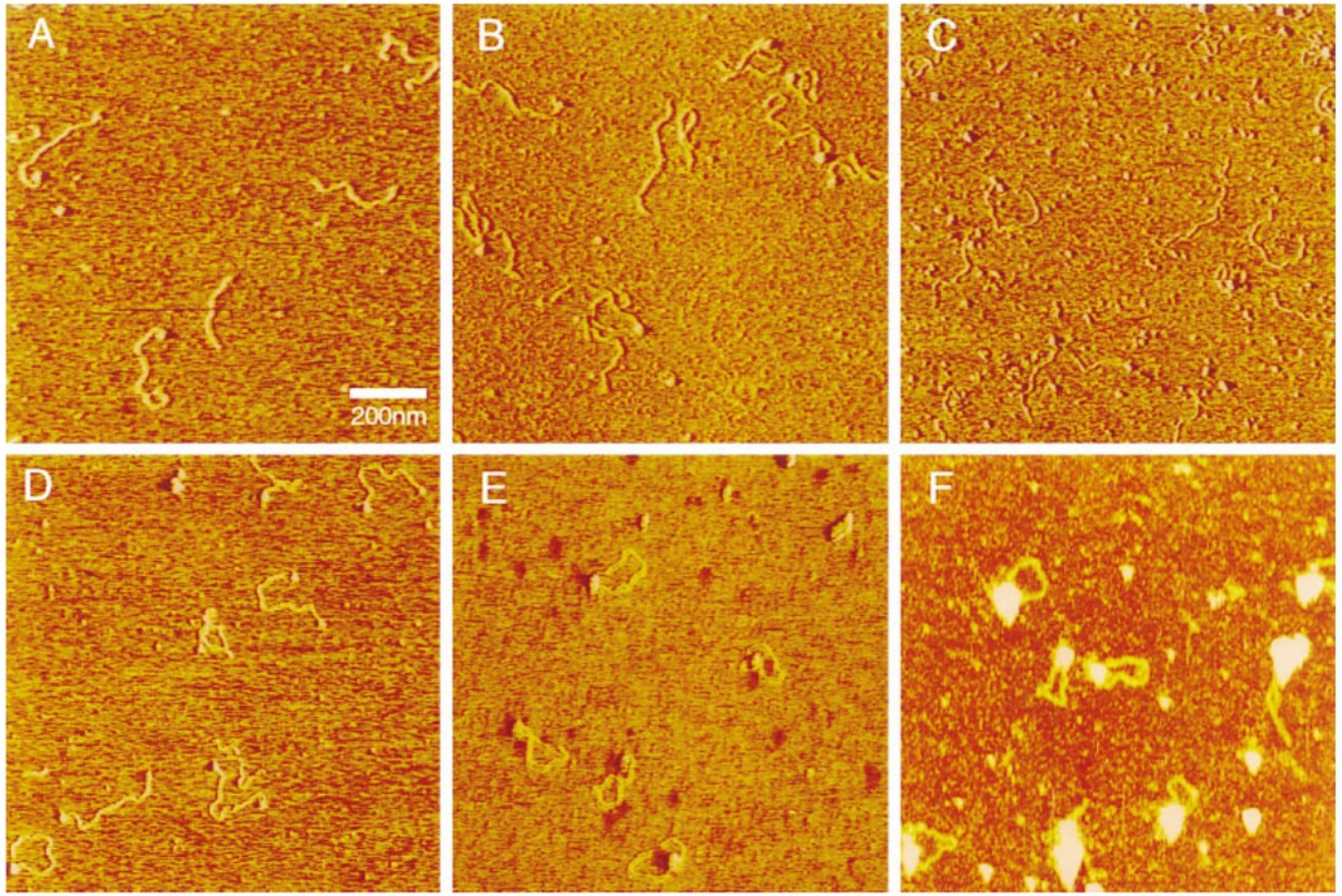
m^7G-eIF4E-eIF4G-PABP compacting rate ***has not*** *been characterized yet.*
But translation can happen **within 1 min**.

AIM1b: Characterization the circularization of hcBroccoli

Proximity characterization

Method: Atomic Force microscope (AFM).

Expected Outcome: Circular RNA/Protein complexes.



Rational:

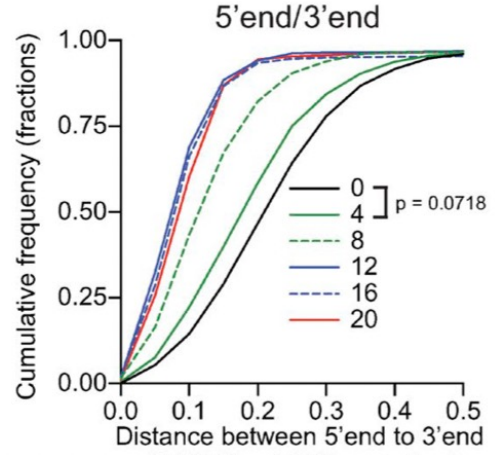
1. **PABP will bind to poly(A) tail in nuclear and help transportation.**
2. **eIF4G-PABP, eIF4E-eIF4G can bind in relative fast rates.**
3. **Human mRNA can translate peptides with a circular form soon after being transported into cytoplasm. (~ s^{-1})**
4. **hcBroccoli has all circularization required region.**

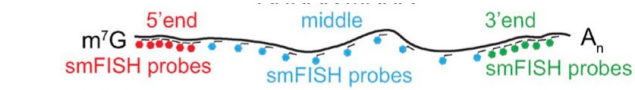
If hcBroccoli doesn't shape in circle:

- **3' – 5' distance adjustment**

+ CG rich at 3' UTR
+ Elongate poly(A) tail

Mol Cell. **1998** Jul;2(1):135-40.
Journal of Cell Biology, 15 Oct **2018**, 217(12):4124-4140





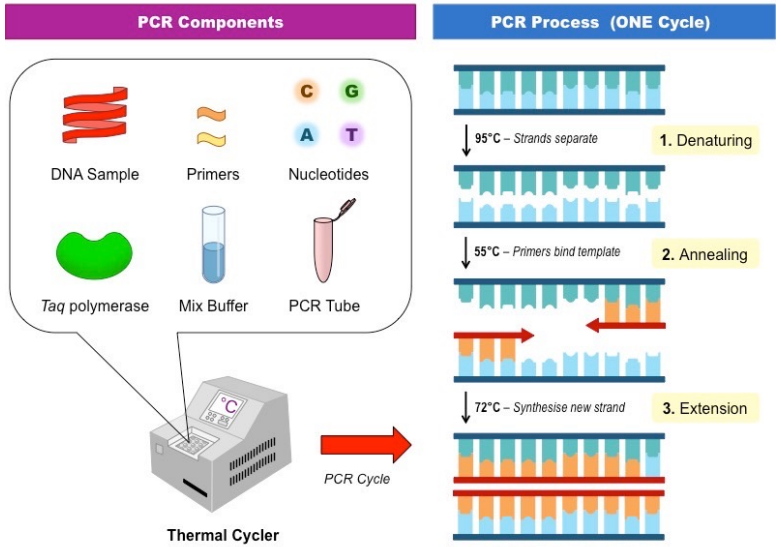
hcBroccoli transcriptions

1. DNA template preparation

5’ ——————————— 3’

PCR

+DNA polymerase



Purification with PCR purification kit

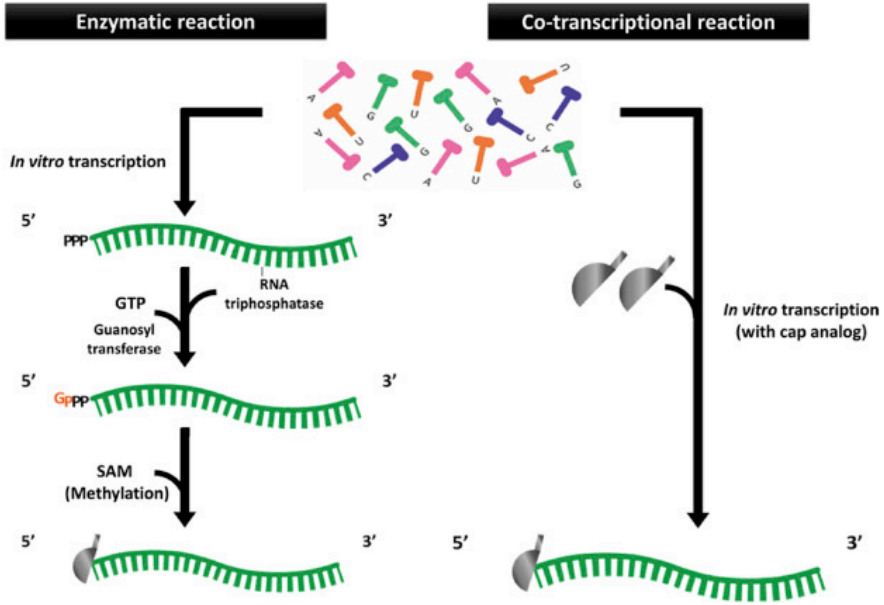
Double strand DNA template

2. Transcriptions

❖ **In vitro**

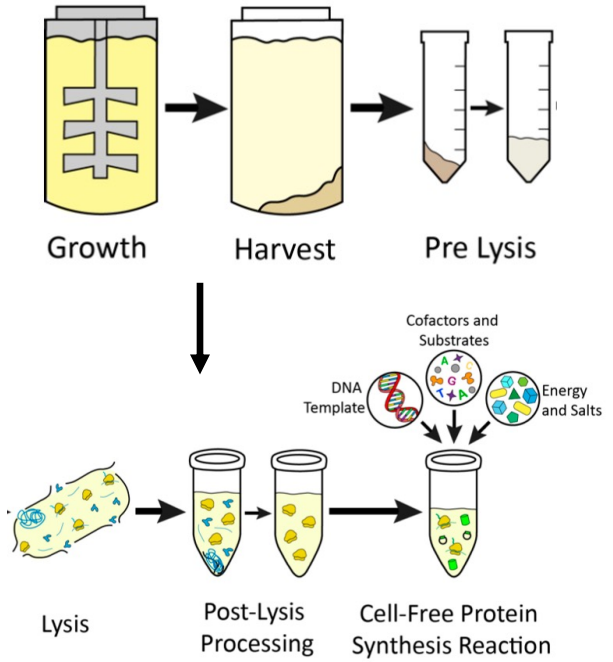
A. PCR

Flash transcription kit



B. MAGIC express system

Cell Lysate



❖ **In cell**

C. MAGIC system genetically encode hcBroccoli.

3. Characterizations: Seq, Gel, AFM…

https://ib.bioninja.com.au/standard-level/topic-3-genetics/35-genetic-modification-and/pcr.html

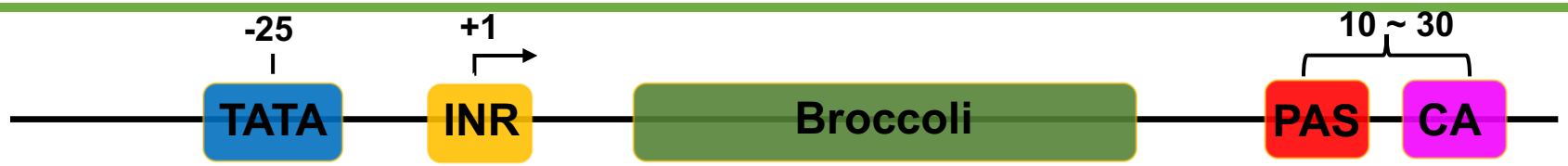
Biomaterials 156 (**2018**): 172-193

Methods and protocols 2.1 (**2019**): 24.

DNA templates designed for MAGIC system

Summary

Simplest DNA template:



5’TCCTGAAGGGGGGCTATAAAAGGGGGTGGGGGCGCGAACCTCTGGCAGGAGCAAAGGCGCC
ATGGCTGTGGAGGGCGGABroccoliAGTGCCTCTCCTGGCCCTGGAAGTTGCCTCTCCAGTGCCC
ACCAGCCTTGTCCTAATAAAATTAAGTTGCATCATTTTGTCTGACTAGGTGTCCTTCT3’

1st optimization for transcription initiation:

5’TCCTGAASSRCGCCTATAWAARGGGRRRRGCGCGAACCTCTGGCAGGAGCAAAGG
CGCCATGGCTGTGGAGGGCGGAAGACGGGGCGGABroccoli-3’

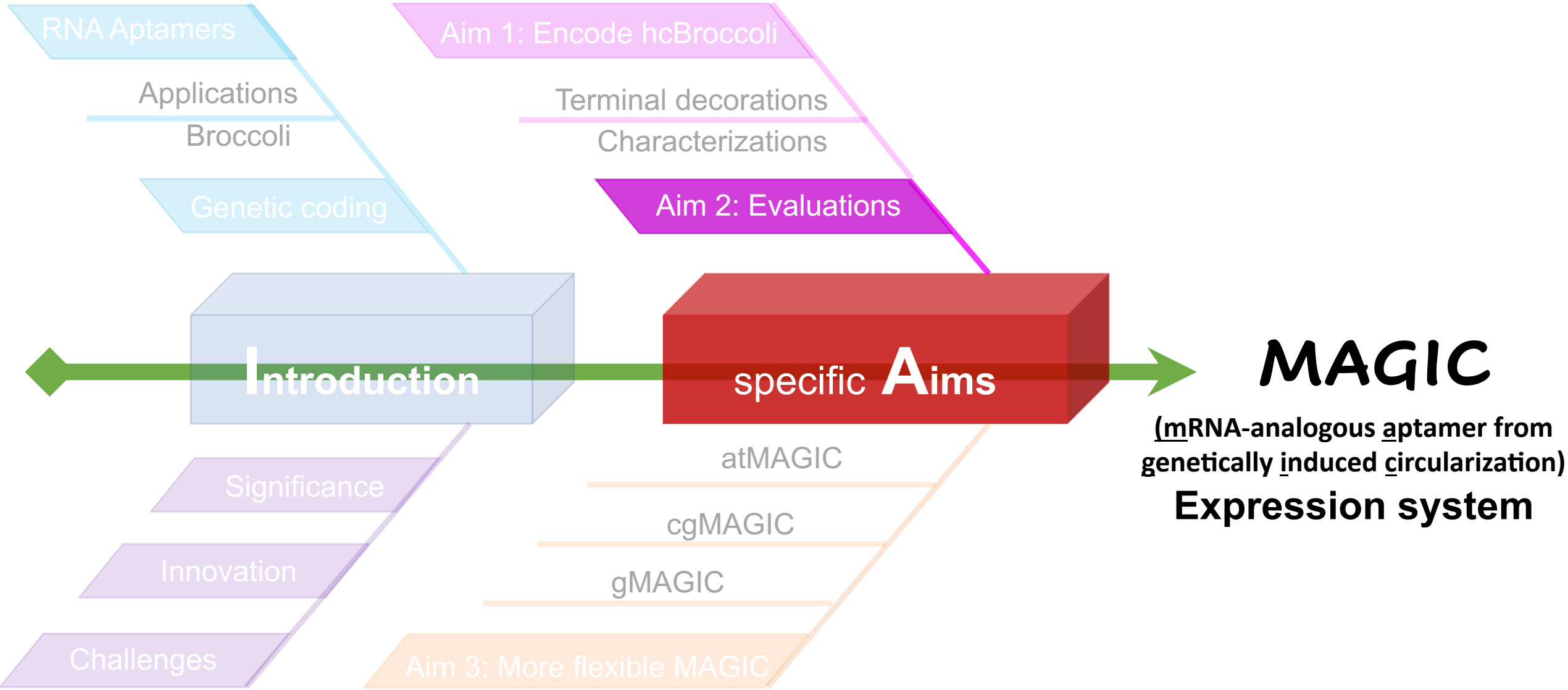
2nd optimization for capping:

5’TCCTGAASSRCGCCTATAWAARGGGRRRRGCGCGAACCTCTGGCAGGAGCAAAGG
CGCCATGGCTGTGGAGGCSARCSSAACGSAGACGGGGCGGABroccoli-3’

N: A, T, C, or G
W: A or T
R: A or G
Y: C or T
S: G or C

3rd optimization for polyadenylation:

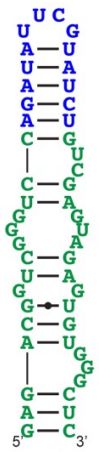
5’TCCTGAASSRCGCCTATAWAARGGGRRRRGCGCGAACCTCTGGCAGGAGCAAAGGCGCCATGGCTG
TGGAGGCSARCSSAACGSAGACGGGGCGGABroccoliAGTGCCTCTCCTGGCCCTGGAAGTTGCCTCTC
CAGTGCCCACCAGCCTTGTCCTTGTATTAATAAAAGATCTTTATTTTCATTAGATCTGTGTGTTGGTTTTTTT
GTGTG

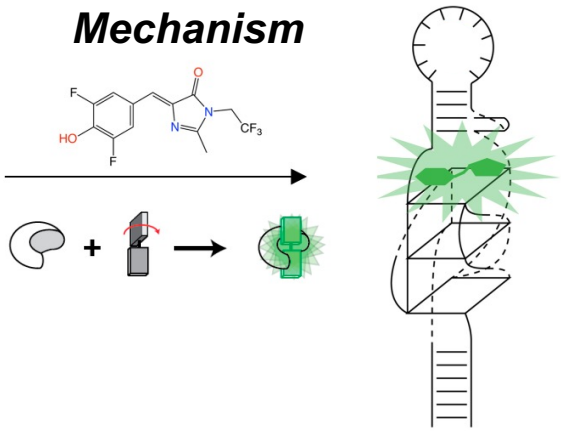

RNA Aptamers
Applications
Broccoli
Genetic coding
Aim 1: Encode hcBroccoli
Terminal decorations
Characterizations
Aim 2: Evaluations
Introduction
specific Aims
MAGIC
(mRNA-analogous aptamer from genetically induced circularization)
Expression system
Significance
Innovation
Challenges
atMAGIC
cgMAGIC
gMAGIC
Aim 3: More flexible MAGIC

AIM2a&2b: Evaluate the functionality&stability of hcBroccoli

A. Evaluate the target binding ability

- ***Rational:*** Broccoli can bind to fluorophore DFHBI-1T and lead to large fluorescence signal.





Testing sets:

Blank Group	Exp Groups
Null (Random sequence)	Linear system Tornado system MAGIC system

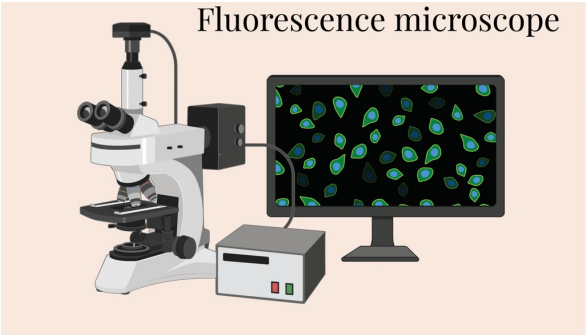
B. Evaluate the stability of hcBroccoli

- ***Rational:*** Circularization and protein coating protections should stabilize the aptamer to achieve a longer functional time.

Camera: CoolSnap HQ2 CCD.
Air objective: a ×20/ × 40.
Microscope: Nikon Eclipse TE2000-E.
Excitation filter: 470 ± 20 nm.
Emission filter: 525 ± 25 nm.
Dichroic mirror: 495 nm.

❖ Evaluation Method:

Fluorescence microscopy



Environment: 37 ℃, 5% CO_2.
Exposure time: 200-500 ms.
Analysis tool: NIS-Elements software.
Fluorescence: ImageJ measurements.

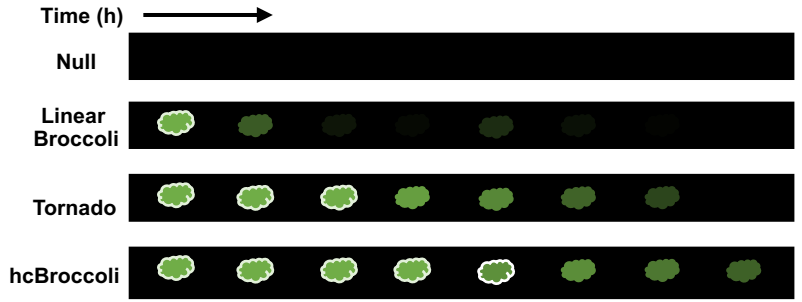
Front. Chem., 28 June **2016**
https://rsscience.com/fluorescence-microscope/

• Experiment designs:

1. Day1: Transfect the HEK293T cells with *different expression systems*.
2. Day 1~2: Subculture the cells.
3. Day 2: Change the medium to FluoroBrite medium containing 40 μM DFHBI-1T.
4. Day 2: Live cell fluorescence images.

• Expected outcomes:

1. Fluorescent signals from all experimental groups.
2. Longer duration time for Tornado system and MAGIC system.



International journal of molecular sciences 19.1 (**2018**): 44.
Nucleic Acids Research, Volume 45, Issue 3, February **2017**, 1404–1415

AIM2b: Evaluate MAGIC system intracellular expression levels

- **Quantification of intracellular RNA concentrations**
- ***Rational:*** The more stable hcBroccoli can maintain their intracellular concentrations at relative higher levels.

NAT Biotechnol. **2019** Jun; 37(6): 667-675
https://www.bio-rad.com/featured/en/flow-cytometer.html

❖ **Evaluation Methods:**

A. In-gel imaging: Transfect -> Culture -> Extraction > Gel image + quantification

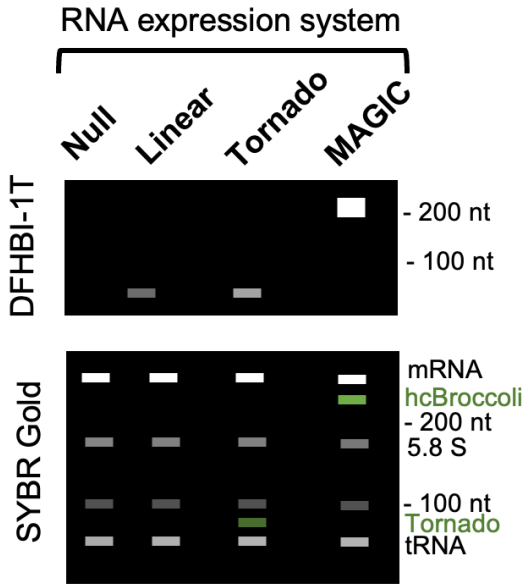
B. Flow cytometry analysis: Transfect -> Culture -> Harvest > LSRFortessa analysis

A. In-gel quantifications

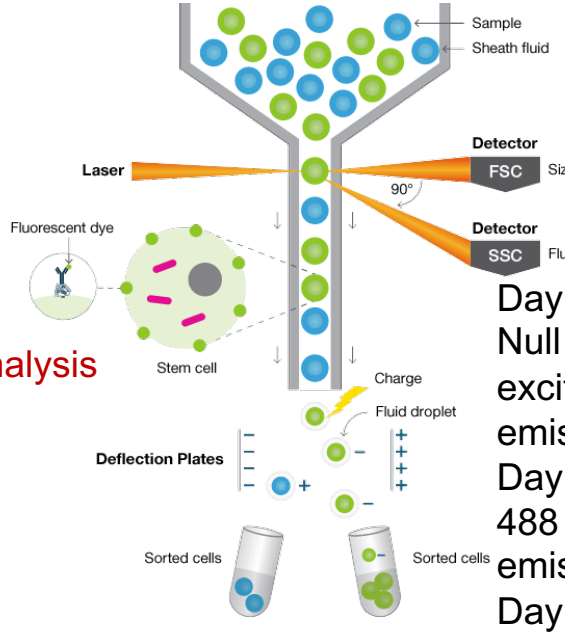
Day 1: Transfect the HEK293T cells with *different expression systems*.
Day 1~2: Subculture the cells for 1~2 days.
Day 3: Add actD 6h before extractions.
Day3: Suspend cells using TrypLE. Quantify the diameter of HEK293T cell.
Day 3: Extract total RNAs using TRIzol LS.
Day 3: 1 μg **Broccoli** run with 10% denaturing PAGE gel.
Day 3: Imaging Broccoli with ChemiDoc MP. (470/30 nm excitation, 532/28 nm emission)
Day 3: Imaging total RNAs with **SYBR stained** Gold gel. (302 nm excitation, 590/110 nm emission)
Day 3: Gel Band intensity is quantified in Image Lab software.

Expected outcome A:

1. Abundant hcBroccoli band.
2. Near endogenous levels



B. Flow cytometry analysis

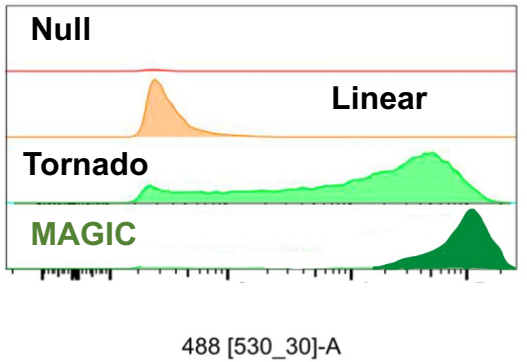


Day 1~3: Cell culture.
Day 3: Harvest with 4% FBS + 40 μM **DHFBI-1T**.
Day 3: DAPI fluorescence for Null group. (355 nm excitation, 455/50 nm emission)
Day 3: Experiment groups, 488 nm excitation, 530/30 nm emission.
Day 3: FlowJo software analysis.

Expected outcome B:

High accumulations for hcBroccoli.

Expression system	Subset	Count
Null	Broccoli	~100
Linear	Broccoli	~5000
Tornado	Broccoli	~26000
MAGIC	Broccoli	~10^4

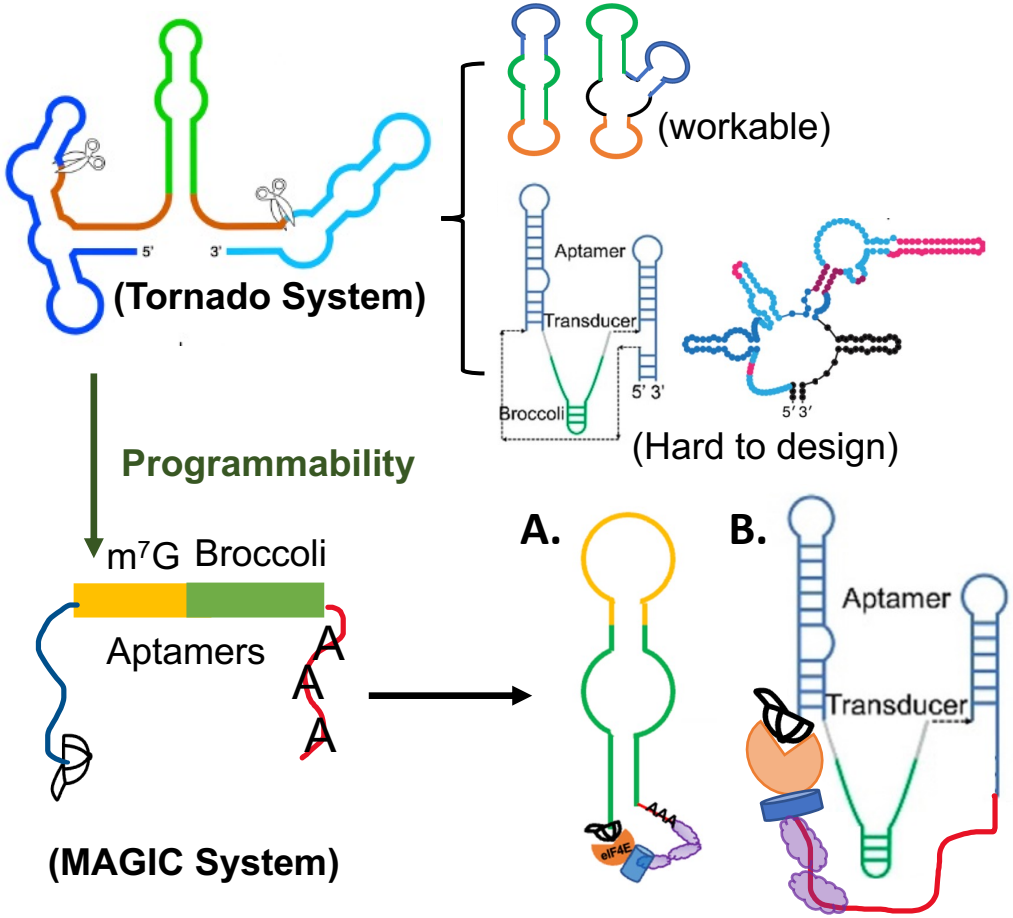


AIM2c: Program MAGIC system for biosensing

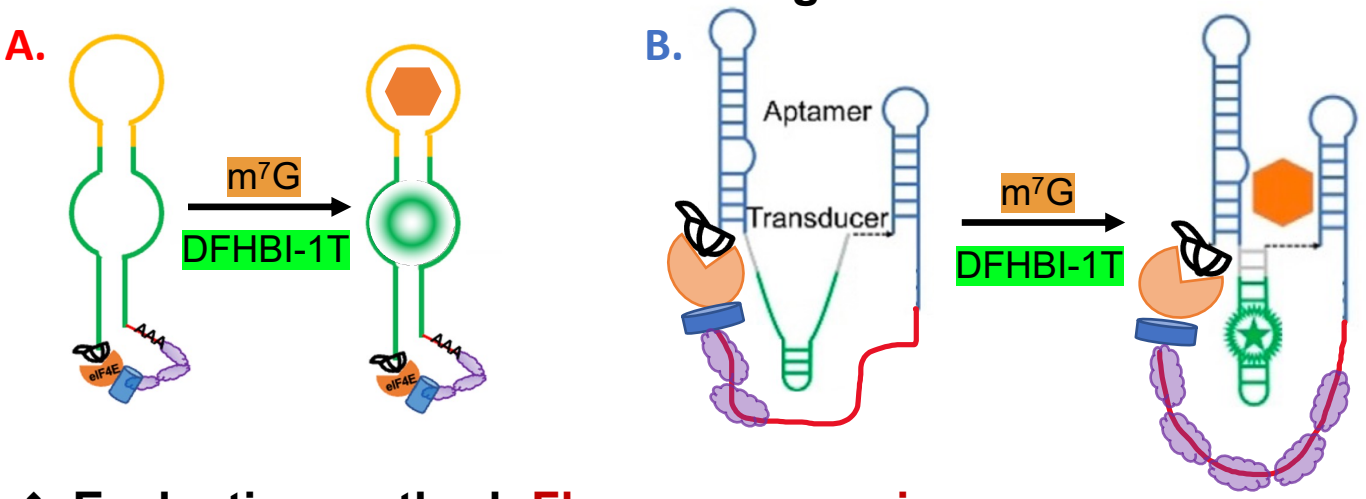
❖ Evaluate hcBroccoli-m^{7}G sensors

- ***Rational:*** Aptamers can be fused together for more diverse intracellular applications.

MAGIC system is able to program more complicated aptamer-based biosensors.



❖ hcBroccoli-m^{7}G biosensor designs:

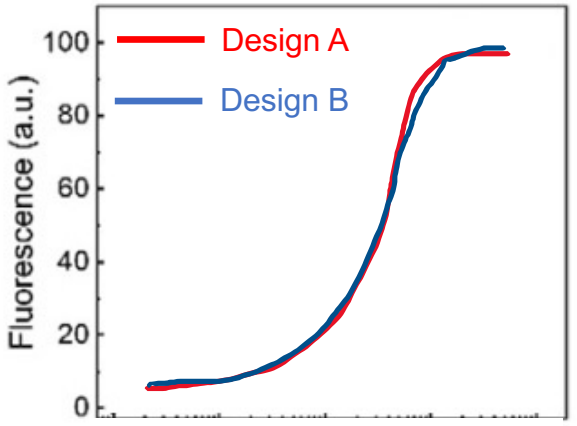


❖ Evaluation method: Fluorescence microscopy

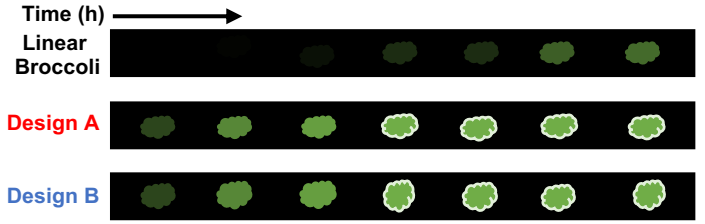
Expected outcome:

NAT Biotechnol. **2019** Jun; 37(6): 667-675
Angewandte Chemie 133.45 (**2021**): 24272-24276.

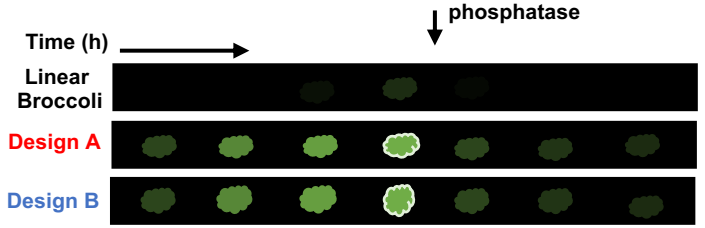
a. Does-dependent behavior



b. Detect m^{7}G



c. Dynamically detect m^{7}G



AIM2d: Examine MAGIC system cytotoxicity

❖ Ensure MGAIC system will not trigger immune response.

- ***Rational:*** The terminal decorations to non-cytotoxic Broccoli are endogenously existing.

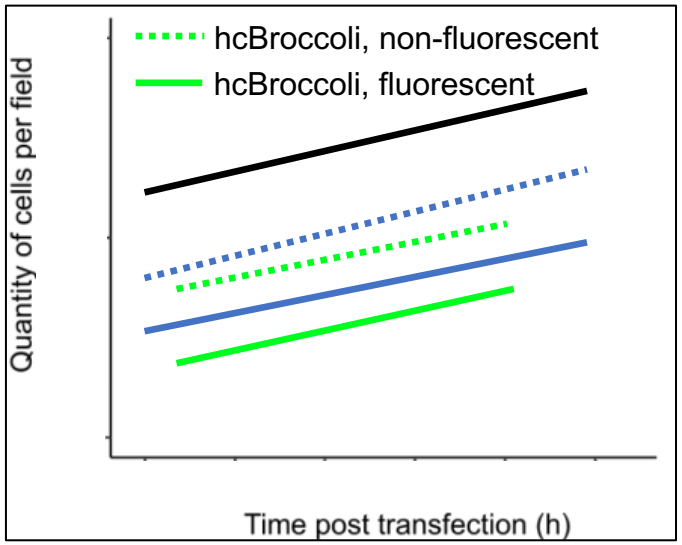
❖ **Evaluation Methods:**

A. Cell proliferation rate characterizations.
B. In-gel retionic acid-inducible genel (RIG-I) analysis.

A. Cell proliferation rate characterizations

1. Day 1-2: Transfect the HEK293T cells with *different expression systems*.
2. Day 3: Subculture the cells with 1:4 and 1:8 onto plates.
3. Day4: count 1:4 cultured fluorescent/non-fluorescent cell numbers.
4. Day5: count 1:8 cultured fluorescent/non-fluorescent cell numbers.
5. Calculate time for cell to growth.

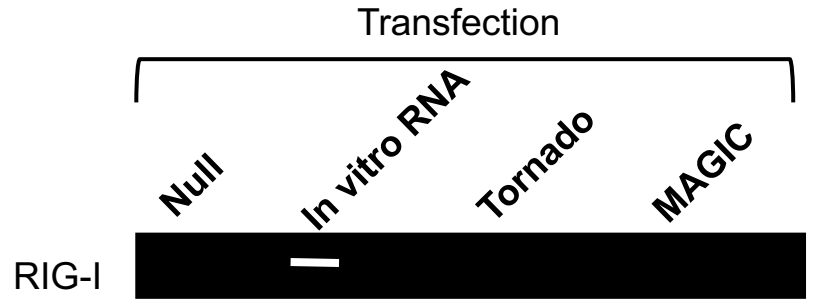
Expected outcome:
low levels of apoptosis.



B. In-gel RIG-I analysis

1. Day 1-2: Transfect the HEK293T cells with *different expression systems*.
2. Day 3: 1 μg mL^{-1} doxorubicin (anti-bacteria).
3. Lyse cells in RIPA buffer with Halt protease. And phosphatase inhibitor cocktail.
4. Separate proteins and analysis by PAGE.

Expected outcome:
Low levels of RIG-I from MAGIC system.

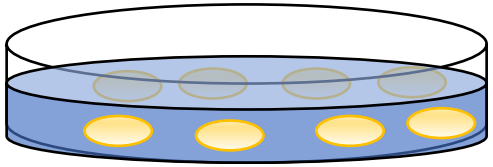


A **Safe** and **High-efficient *MAGIC*** expression system has been developed for diverse applications in mammalian cells!

NAT Biotechnol. **2019** Jun; 37(6): 667-675

Cell culture, transfection and In-gel imaging

• Cell Culture



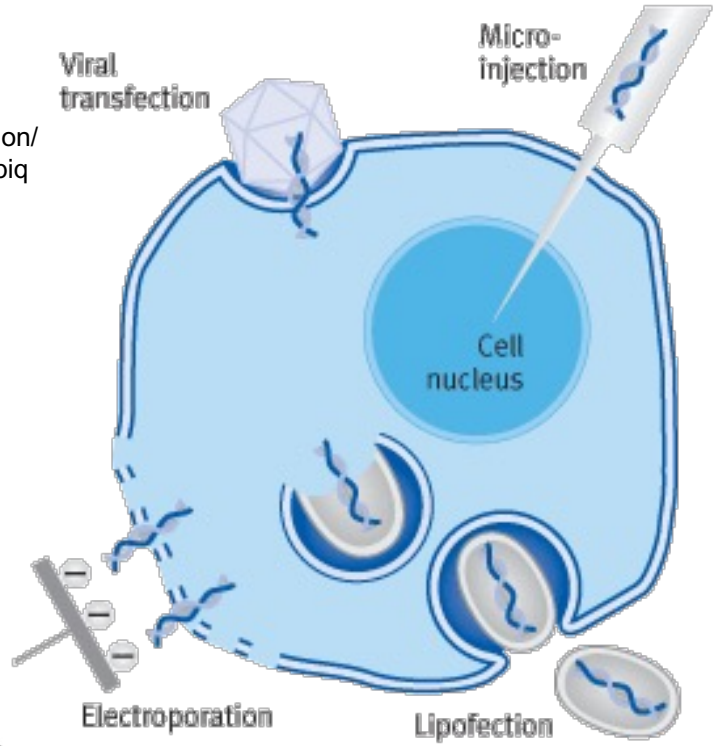
https://www.biontex.com/en/transfection/
https://www.epigentek.com/catalog/epiquik-total-rna-isolation-fast-kit-p-84425.html
https://www.aatbio.com/catalog/gel-electrophoresis

: HEK293T cells

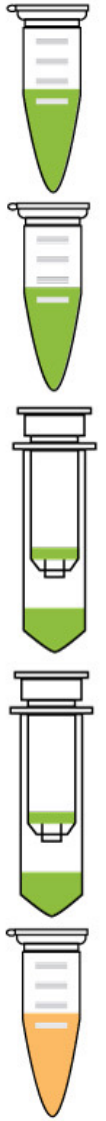
: standard tissue culture conditions

×1 DMEM
10% fetal bovine serum (FBS)
100 U ml^{-1} penicillin
100 µg ml^{-1} streptomycin
50 µg ml^{-1} hygromycin B (anti-bacteria)
5 µg ml^{-1} Actinomycin D (for extraction)

• Cell transfection



• RNA extraction



Mammalian cells or tissues

↓ ×1 phosphate buffered saline (PBS) / TrypLE

Cell lysis

↓ TRIzol LS reagent.

RNA binds to the columns

↓

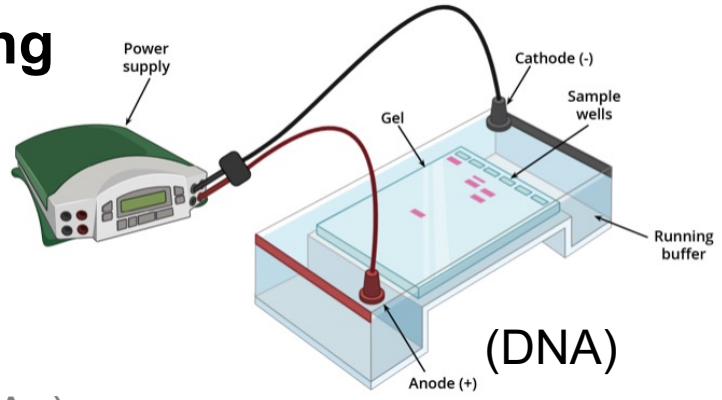
Wash

↓

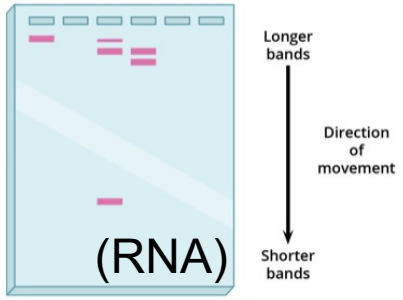
Elute RNA

• RNA in-gel imaging

100 mM KCL
1mM$MgCl_2$
10 µM DHFBI-1T
(For Broccoli imaging)
40 µM HEPES
SYBR stain (for all RNAs)

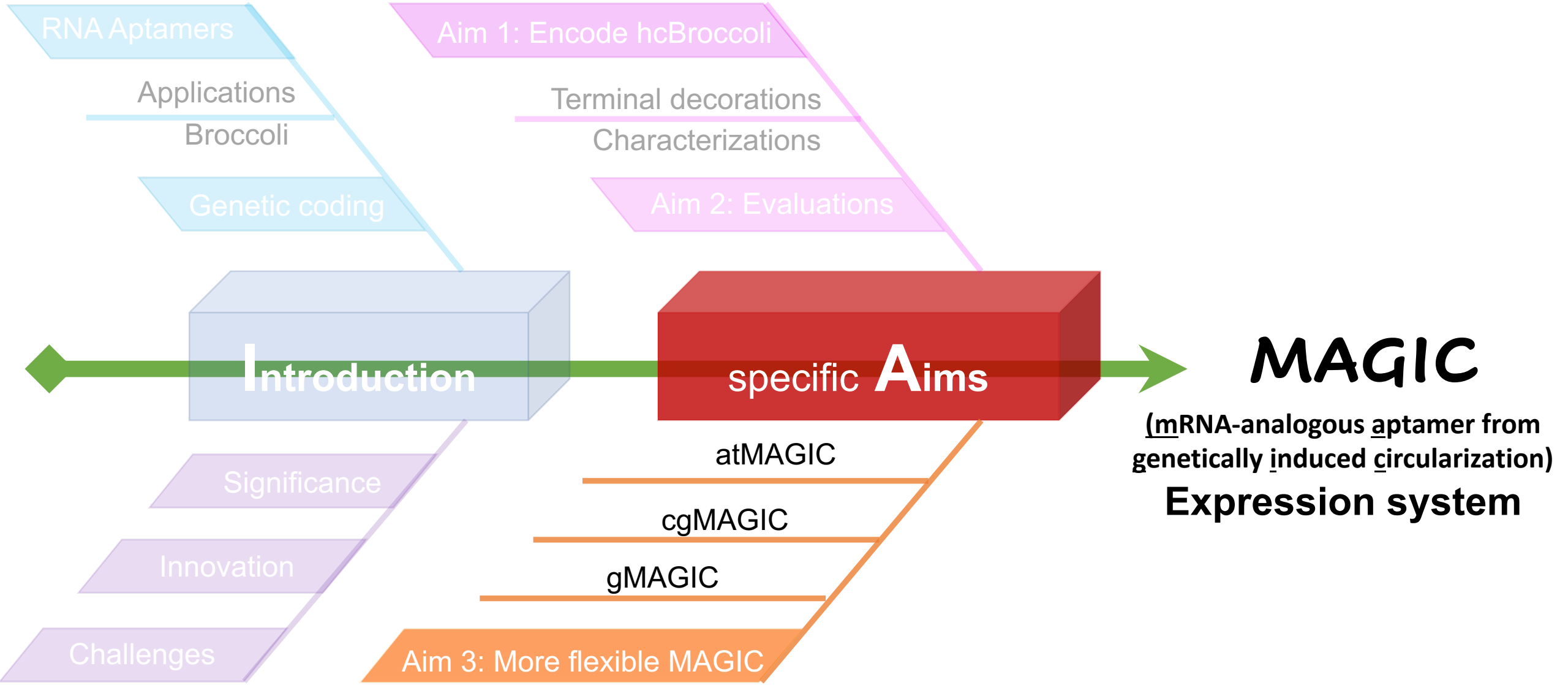


(DNA)



(RNA)

10 % TBE-Urea Gels
270 V in TBE buffer, pH 7.4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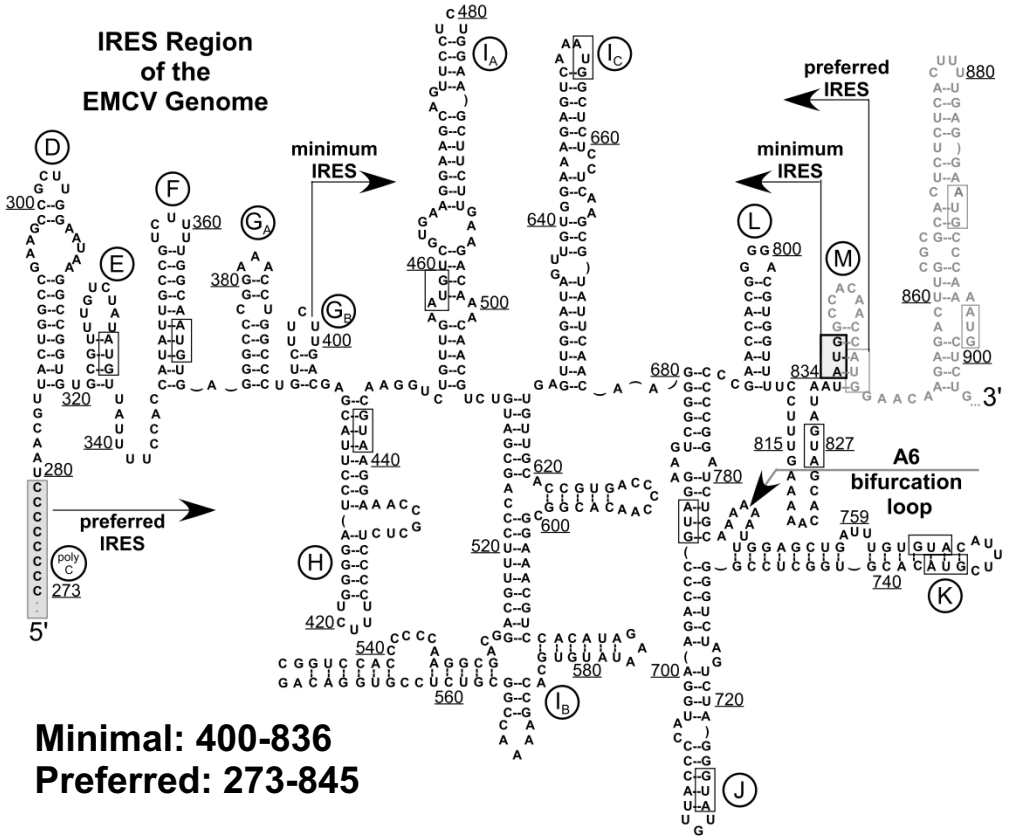
Aim 3: More flexible MAGIC

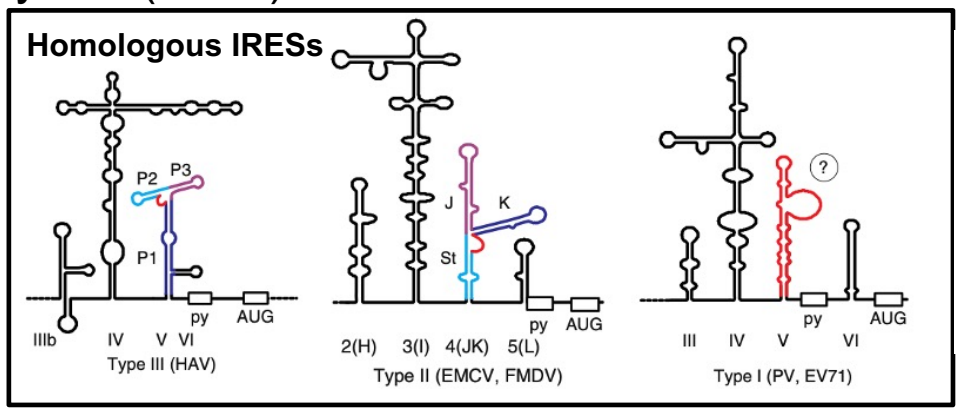
AIM3a: Develop more general MAGAIC systems --- atMAGIC

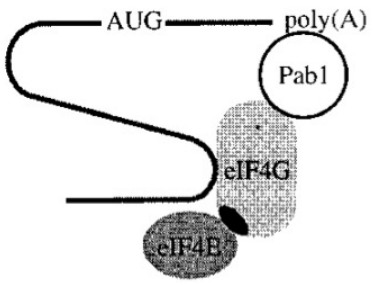
Rational: Natural mRNAs have strong aspiration to shape in a circle in cytoplasm even without 3' poly(A) tail or 5' cap decorations.

A. Cap independent circularization

- Viruses.
- Replace cap-recognition site with a ribosome entry site (IRES).
- Form 3'-PABP-eIF4G-IRES-5' interactions







Pros:

1. Offer a unique opportunity to have cap-independent circularization.
2. May escape from the cap-required class II transcriptions.

Cons:

1. IRESs are super long with the known minimal length is 157 nt, making the 5'-UTR too long.
2. May trigger immune response, a mechanism always adopt by viruses.

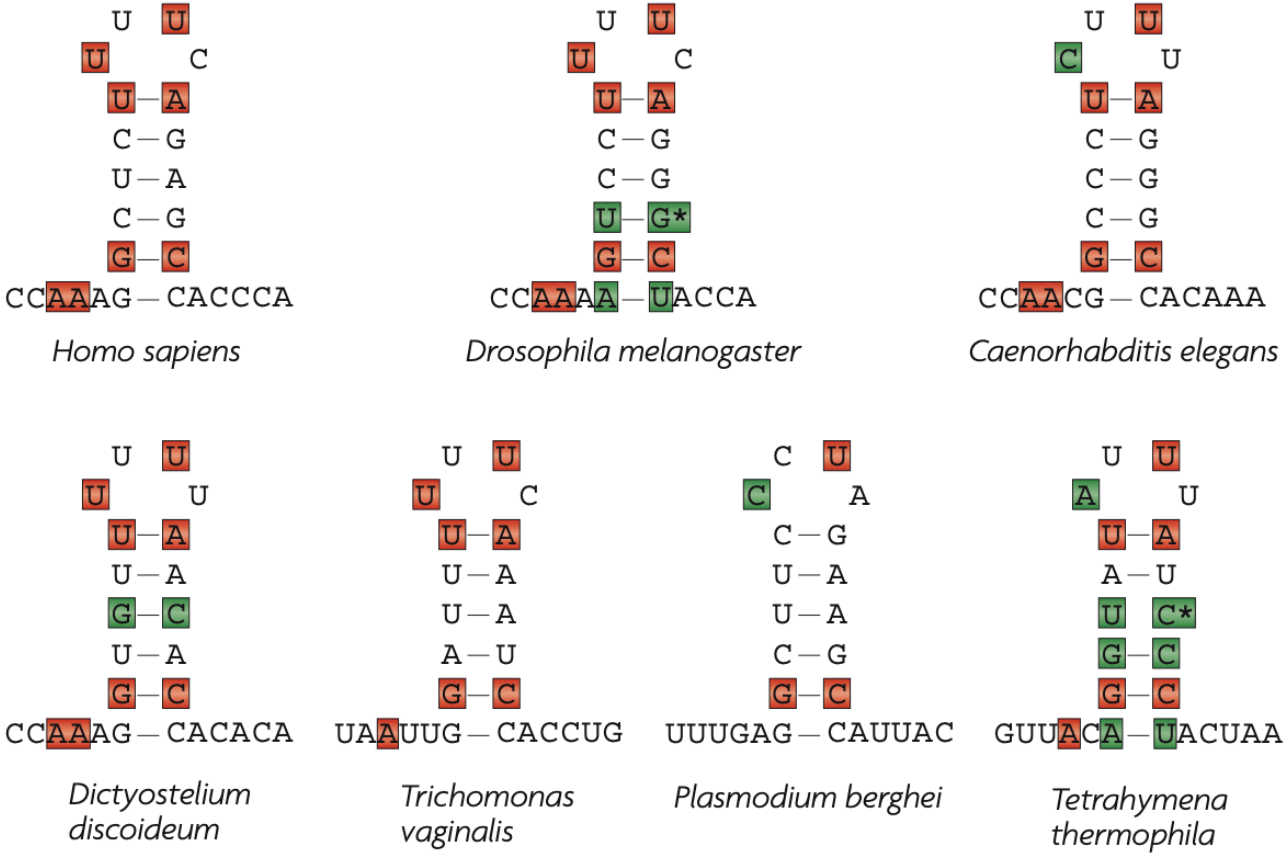
Int J Mol Sci. **2020** Nov; 21(22): 8591.
Biotechniques 41.3 (**2006**): 283-292.

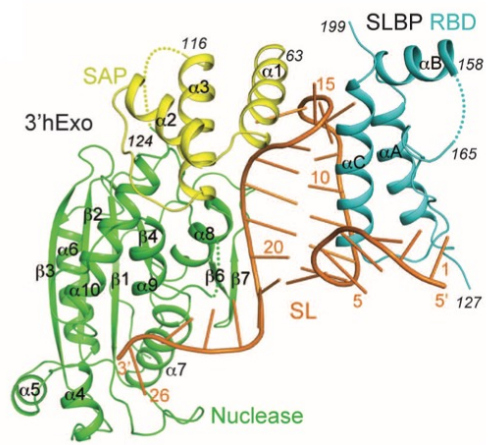
AIM3b: Develop more general MAGAIC systems --- cgMAGIC

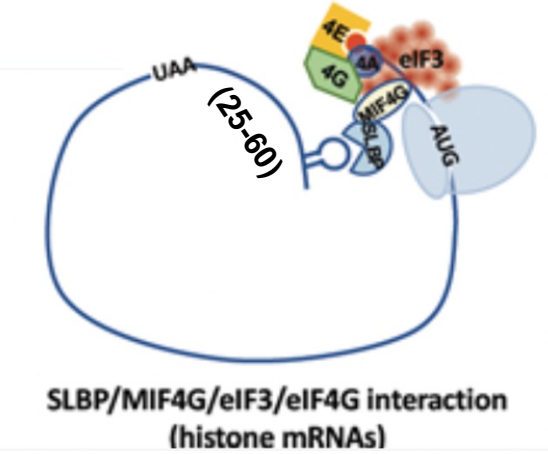
B. Poly(A) independent circularization

- Histone mRNA
- Replace poly(A) tail with a conserved stem-Loop
- Forming 3'-SLBP-MIF4G-eIF3-eIF4F-5' interactions

i.e., 3'-CCAAAGGCTCTTTTCAGAGCCAGGGA-5'







Science. **2013** Jan 18;339(6117)
Biotechniques 41.3 (**2006**): 283-292.

Pros:

1. No need to have a long poly(A) tail at 3' end.
2. No need to optimize the poly(A) tail length.
3. The stem-loop can be engineered to have a couple function with aptamers.
4. Give us an alternative idea that we can also decorate 3'-end with a poly(U) tail.

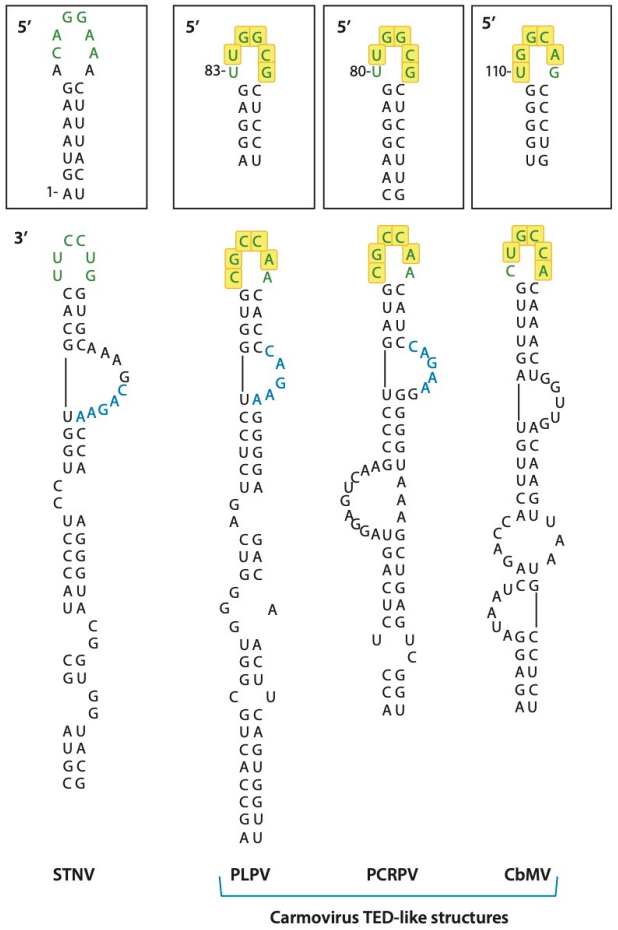
Cons:

1. Always go along with rapid degradations.
2. Maximum accumulation level is SLBP dependent.
3. May not apply to all cell lines.

AIM3c: Develop more general MAGAIC systems --- gMAGIC

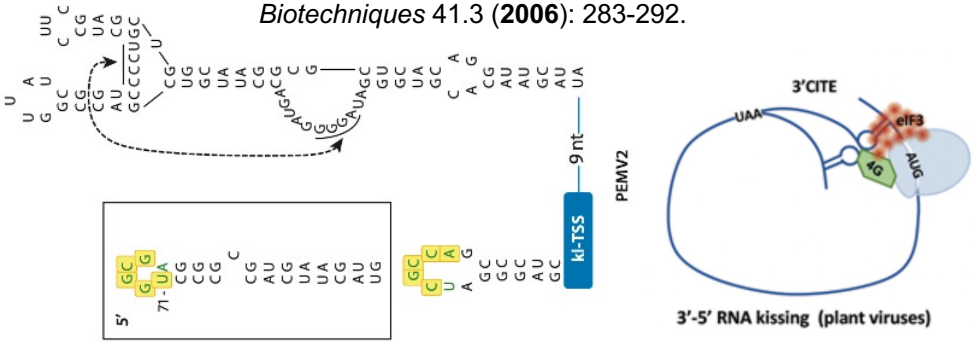
C. RNA-solely circularization

- Plant viruses
- Replace 3' poly(A) with 3' cap-independent translation enhancer
- Replace 5' cap with a hairpin.
- Forming RNA-RNA kissing interactions



Virus	CITE Type	3′ CITE sequence[a]	5′ hairpin sequence[a]	5′ hairpin location
		Carmovirus		
SCV	PTE	CUGCCA	UGGCAG	5′ ORF
PFBV	PTE	CUGCCA	UGGCAG	5′ ORF
CarMV	PTE	CUGCCA	UGGCGG	5′ terminus
HnRSV	PTE	CUGCCA	UGGCAG	5′ ORF
HCRSV	PTE	GCCA	UGGC	5′ terminus
GaMV	PTE	UUGGCG	CGCCAA	5′ terminus
PSNV	PTE	UUGGCG	GCCA	5′ UTR
MNSV	ISS	UGGCU	AGCCA	5′ ORF
TGP-carmo	ISS	CGGCAA	UUGCCG	5′ terminus
CbMV	TED-like	CUGCCA	UGGCAG	5′ ORF
PLPV	TED-like	CGCCAA	UUGGCG	5′ ORF
PCRPV	TED-like	CGCCAA	UUGGCG	5′ ORF
		Umbravirus		
PEMV	kl-TSS	UCGCCA	UGGCGA	5′ ORF
		Panicovirus		
PMV	PTE	UUGCAG	CUGCAA	5′ terminus
CMMV	PTE	UUGCCG	CGGCAA	5′ terminus
		Necrovirus		
STNV	TED	UUCCUG	CAGGAA	5′ terminus
TNV-D	BTE	UGGU	ACCA	5′ terminus
OLV-1	BTE	UGGUG	UACCA	5′ terminus
LWSV	BTE	UGGU	ACCA	5′ terminus

Annual review of microbiology 67 (**2013**): 21-42.
Biotechniques 41.3 (**2006**): 283-292.



Pros:

1. No protein-mediation required.
2. No decoration required. May escape from the class II transcriptions.
3. The conserved sequences are not very long.

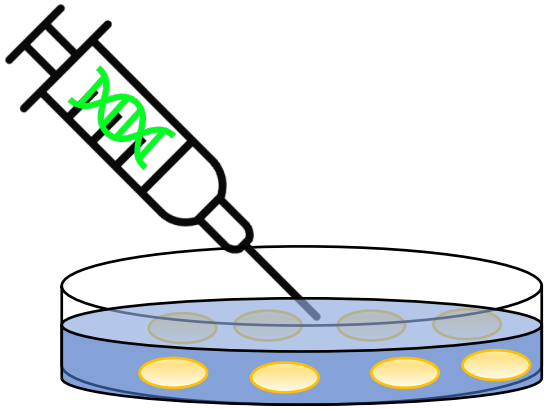
Cons:

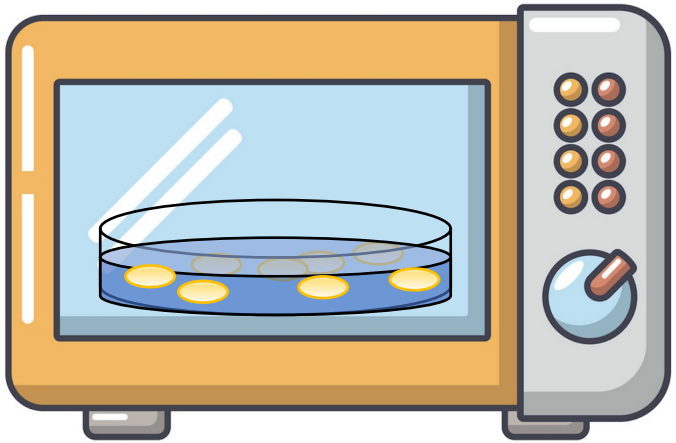
1. Only find in plant viruses.
2. With no protein protections, and no poly(A) tail or cap decorations, lack stability.
3. May cause immune response.

(All extended MAGIC systems can be characterized follow Aim1 and Aim2 protocols.)

Overall, easy-implement MAGIC expression system!

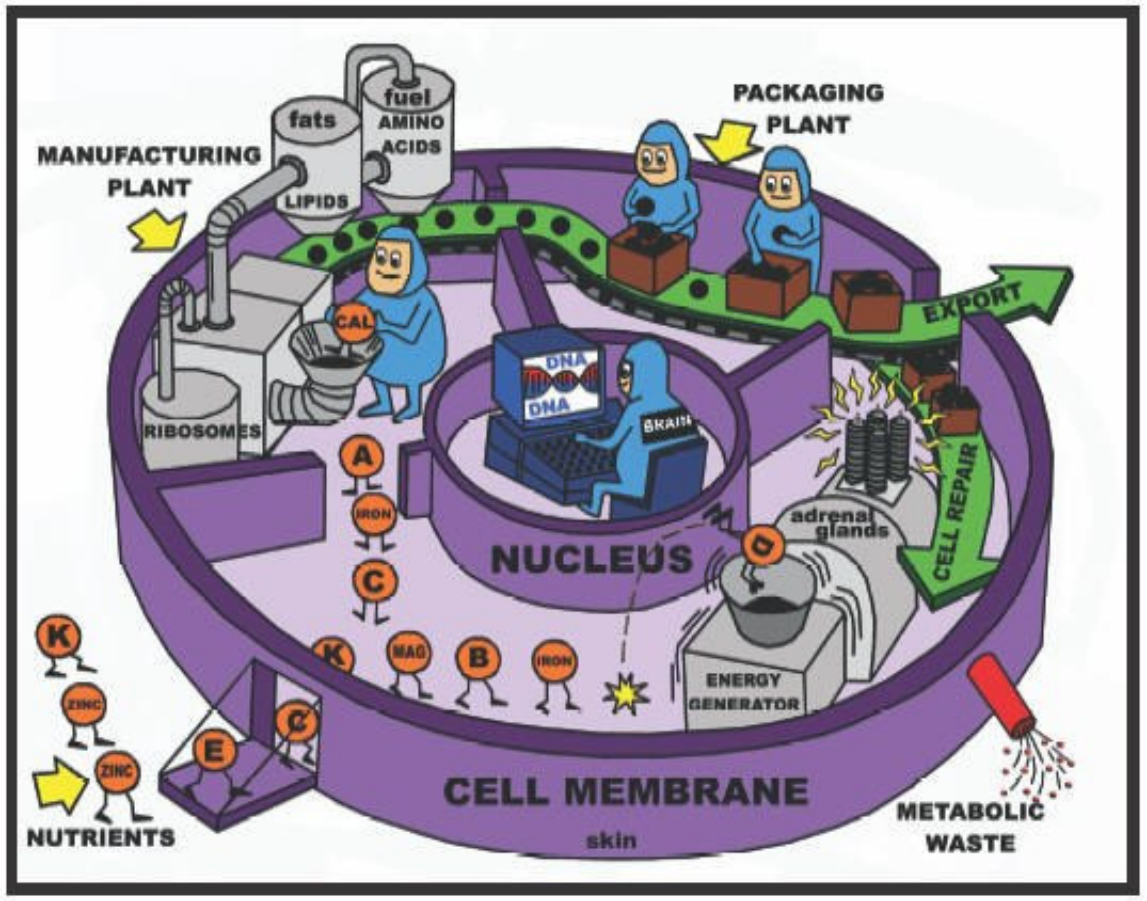
1. Transfection





...

MAGIC Cellular Expression Factory



2. Characterizations.

https://www.vectorstock.com/royalty-free-vector/microwave-oven-icon-cartoon-style-vector-19673800
https://slideplayer.com/slide/17928800/

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- Jian Huang
- Samantha Schultz
- Ryan Pham

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- Ru Zheng