2025.5.23-26 C34-NM C34-squarate in activity-based RNA profiling (on live and fixed cells)

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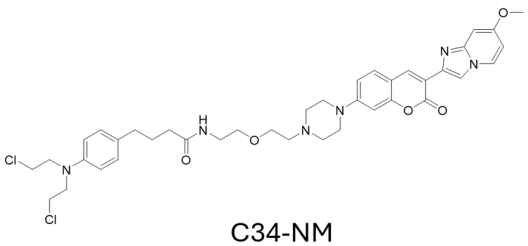
THURSDAY, 5/22/2025

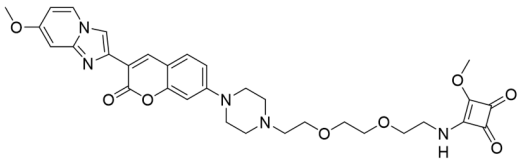
Planned samples - treatment

	#	Covalent modifier	Covalent modifier (final)	Covalent rxn conditions	RT ion
1	76	DMSO (100%)	Ctrl (5% DMSO)	37°C for 2h on live cells	Mn2+ (3mM)
2	77	DMSO (100%)	Ctrl (5% DMSO)	37°C for 2h on live cells	Mn2+ (3mM)
3	78	C34-NM (20mM, stock)	C34-NM (1mM final)	37°C for 2h on live cells	Mn2+ (3mM)
4	79	C34-NM (20mM, stock)	C34-NM (1mM final)	37°C for 2h on live cells	Mn2+ (3mM)
5	80	NM-only (20mM, stock)	NM-only (1mM final)	37°C for 2h on live cells	Mn2+ (3mM)
6	81	NM-only (20mM, stock)	NM-only (1mM final)	37°C for 2h on live cells	Mn2+ (3mM)
7	82	DMSO (100%)	Ctrl (5% DMSO)	37°C for 30min on live cells	Mn2+ (3mM)
8	83	DMSO (100%)	Ctrl (5% DMSO)	37°C for 30min on live cells	Mn2+ (3mM)
9	84	C34-Squarate (20mM, stock)	C34-Squarate (1mM final)	37°C for 30min on live cells	Mn2+ (3mM)
10	85	C34-Squarate (20mM, stock)	C34-Squarate (1mM final)	37°C for 30min on live cells	Mn2+ (3mM)
11	86	Squarate-only (20mM, stock)	Squarate-only (1mM final)	37°C for 30min on live cells	Mn2+ (3mM)
12	87	Squarate-only (20mM, stock)	Squarate-only (1mM final)	37°C for 30min on live cells	Mn2+ (3mM)
13	88	DMSO (100%)	Ctrl (5% DMSO)	37°C for 2h on fixed cells	Mn2+ (3mM)
14	89	DMSO (100%)	Ctrl (5% DMSO)	37°C for 2h on fixed cells	Mn2+ (3mM)
15	90	C34-NM (20mM, stock)	C34-NM (1mM final)	37°C for 2h on fixed cells	Mn2+ (3mM)
16	91	C34-NM (20mM, stock)	C34-NM (1mM final)	37°C for 2h on fixed cells	Mn2+ (3mM)
17	92	NM-only (20mM, stock)	NM-only (1mM final)	37°C for 2h on fixed cells	Mn2+ (3mM)
18	93	NM-only (20mM, stock)	NM-only (1mM final)	37°C for 2h on fixed cells	Mn2+ (3mM)
19	94	DMSO (100%)	Ctrl (5% DMSO)	37°C for 30min on fixed cells	Mn2+ (3mM)
20	95	DMSO (100%)	Ctrl (5% DMSO)	37°C for 30min on fixed cells	Mn2+ (3mM)
21	96	C34-Squarate (20mM, stock)	C34-Squarate (1mM final)	37°C for 30min on fixed cells	Mn2+ (3mM)
22	97	C34-Squarate (20mM, stock)	C34-Squarate (1mM final)	37°C for 30min on fixed cells	Mn2+ (3mM)
23	98	Squarate-only (20mM, stock)	Squarate-only (1mM final)	37°C for 30min on fixed cells	Mn2+ (3mM)
24	99	Squarate-only (20mM, stock)	Squarate-only (1mM final)	37°C for 30min on fixed cells	Mn2+ (3mM)

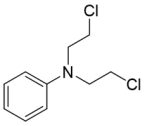
Chemicals



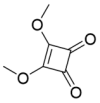
C34-NM



WUXI36
C34-squarate



NM-only



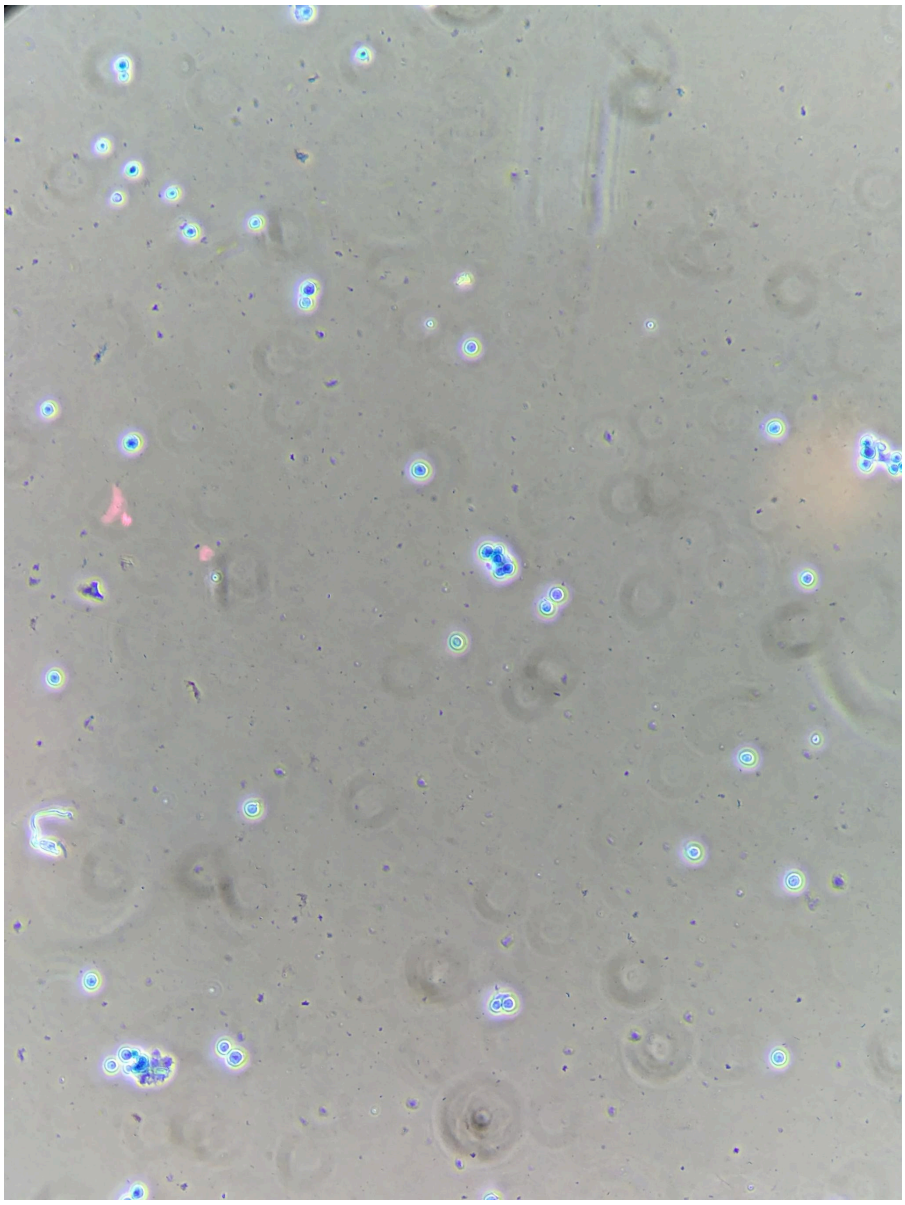
RM26
Squarate-only

FRIDAY, 5/23/2025

DSS fixation testing

- 2x10^5 cells in 50ul PBS, put cells in 1mg/ml DSS and 80% Methanol (both final conc), incubate on ice for 15min, gently invert the tube at every 5min.
- During fixing, there were white precipitation forming, indicating successful fixation. Before fixation, the cell pellet were transparent and mushy; after fixation, the cell pellet were white and solid.
- After fixation, there was no significant changes in terms of cell shapes. There were some clumping, but not to a significant level.

After fixation



- Will use the DSS fixation method for the fixed cell samples.

MONDAY, 5/26/2025

Making buffers right before the experiment:

1x PBS Plus (keep on ice)

- for cell resuspension and fixation

1x PBS Plus

	Component	Vol (1ml)	Vol (15ml)	Final conc
1	1x PBS	1ml	15ml	1x
2	1M MgCl2	3ul	45ul	3mM
3	10% (w/v) BSA/PBS	8ul	120ul	0.08%
4	Total	1ml	15ml	

Fixing buffer (keep on ice)

- for cell fixation
- add at 4:1 ratio to cell suspension, e.g., 600ul fixing buffer to 150ul cells in 1x PBS Plus

Fixing buffer

	Component	Vol	Vol (14x)	Final conc	Note
1	50x DSS	15ul	210ul	1mg/ml	Final conc if added to cell solution at 4:1 ratio
2	Methanol (-20℃)	600ul	8.4ml	80%	Final conc if added to cell solution at 4:1 ratio

Washing buffer (keep on ice)

- for both cell washing and later covalent modification reactions

Washing buffer

	Component	Vol (1ml)	Vol (25ml)	Final conc	E
1	1x PBS	940ul	23.5ml	1x	
2	1M MgCl2	3ul	75ul	3mM	
3	10% (w/v) BSA/PBS	50ul	1250ul	0.5%	
4	10% (v/v) Triton X100	10ul	250ul	0.1%	
5	*RNaseIn*	*1ul*	*25ul*	*0.1%*	*Optional*
6	Total	1ml	25ml		

Cell collection:

- Treat "SARS-CoV2 5UTR"cells (SL5 HEK293) with TrypLE Express at room temp
- Collect and wash cells with **1x PBS Plus**
- Count cells: **4.455x10^6 cells/ml x 5ml**
- Passage at 1:20, and for the extra:

- Prepare **2.8x10^6** cells in **665ul** 1x PBS Plus in a new 1.5ml tube, keep on ice (labeled as "live cell samples")
- Prepare **3.5x10^6** cells in **2.1ml** 1x PBS Plus in a new 15ml tube (labeled as "fixed cell samples")

Cell fixation:

- Add **8.4ml** fixing buffer to the **2.1ml** "fixed cell samples"
- Incubate on ice for 15min, invert tube every 3min
  - While incubating, aliquot "live cell samples" **47.5ul** to 12 tubes, proceed with covalent modifying
- Add **4.5ml** wash buffer, 300xg 3min
- Add **15ml** wash buffer, 300xg 3min
- Resuspend in **665ul** wash buffer
- Aliquot **47.5ul** to 12 tubes

Cell treatment with covalent modifiers:

- Add **2.5ul** DMSO or covalent modifier in DMSO (total volume=50ul) to each sample **at room temp**, pipette to mix
- Incubate at designated temp for **30min or 2h**
- Chill on ice for 2min
- For live cell samples:
  - Add 300ul RLT Plus buffer (total volume=350ul)
  - Vortex for 30s to homogenize
- For fixed cell samples:
  - Add **1.5ul** proteinase K (NEB P8107S), incubate at 37°C for **10min, pipette to mix at 5min**
  - Add 300ul RLT Plus buffer (total volume=350ul)
  - Vortex for 30s to homogenize, keep lysate on ice
- Proceed to RNA extraction

RNA extraction:

- Transfer 350ul lysate to DNA spin column, 8000xg 30s
- Add 350ul 70% EtOH to the flow-through
- Transfer to Zymo Spin IC column, 8000xg 30s, discard the flow-through
- Add 700ul RW1 buffer, 8000xg 15s, discard the flow-through
- Add 500ul 70% EtOH instead of RPE buffer, 8000xg 30s, discard the flow-through
- Add 500ul 80% EtOH, 8000xg 30s, discard the flow-through
- Full speed 2min
- Elute with **12ul** water

RNA yield

	#	RNA conc (ng/ul)	260/280	11ul RNA into X ul water (Dilute to 60ng/ul)	60ng RNA (ul)	Water to 4.4ul (ul)
1	76	236.7	2.06	32.4	1	3.4
2	77	269.9	2.09	38.5	1	3.4
3	78	293.0	2.09	42.7	1	3.4
4	79	289.5	2.08	42.1	1	3.4
5	80	280.7	2.06	40.5	1	3.4
6	81	230.1	2.05	31.2	1	3.4
7	82	194.5	2.03	24.7	1	3.4
8	83	302.3	2.06	44.4	1	3.4
9	84	262.8	2.09	37.2	1	3.4
10	85	289.0	2.09	42.0	1	3.4
11	86	234.5	2.04	32.0	1	3.4
12	87	259.2	2.06	36.5	1	3.4
13	88	222.0	2.01	29.7	1	3.4
14	89	223.3	2.03	29.9	1	3.4
15	90	170.2	2.00	20.2	1	3.4
16	91	227.8	2.04	30.8	1	3.4
17	92	223.9	1.99	30.0	1	3.4
18	93	199.7	1.99	25.6	1	3.4
19	94	131.9	2.01	13.2	1	3.4
20	95	168.6	2.08	19.9	1	3.4
21	96	145.5	2.01	15.7	1	3.4
22	97	164.0	2.01	19.1	1	3.4
23	98	167.6	2.02	19.7	1	3.4
24	99	208.1	2.09	27.2	1	3.4

- Store RNA in -80

Reverse transcription:

- Input: 60ng total RNA

Annealing

	Component	vol (ul)	25x (ul)	Master mix (ul)
1	60ng RNA+water	1	1/EA	
2	CMV-5UTR-SHAPE-Rv (100uM)	0.05	1.25	
3	dNTP (10mM)	0.5	12.5	
4	Water	3.85	96.25	4.4/EA
5	Total	5.4	5.4/EA	

- 65°C 5min, immediately put on ice

Extension

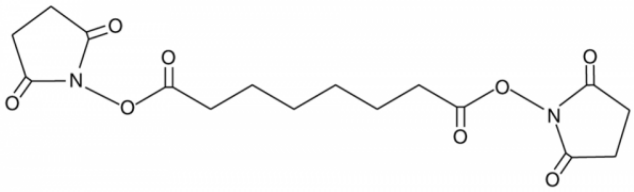
	Component	vol (ul)	25x (ul)	Master mix (ul)
1	Annealing product	5.4	5.4/EA	
2	375mM Tris/500mM KCl (5x buffer)	2	50	
3	100mM DTT (10x)	1	25	
4	Protoscript II	0.5	12.5	
5	RNaseIN	0.1	2.5	4.6/EA
6	30mM MgCl2 or MnCl2	1	25	
7	Total	10	10/EA	

- 42°C 1h, 65°C 20min, 4°C hold
- Store cDNA in -20

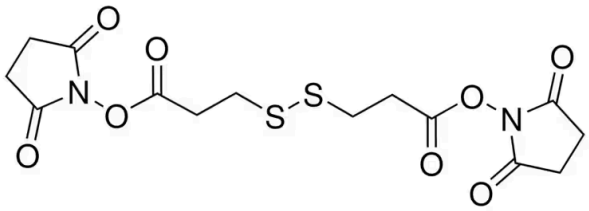
Note:

- DSS is a protein-protein covalent crosslinker.
- DSP, which contains a disulfide bond in the middle, was the original thought for fixing cells. DSP's fixation is reversible by reducing agents such as DTT.
- Because DSS fixation is not reversible, after covalent modification, the fixed cell samples were digested using Proteinase K for 10 min before RNA extraction. This step is to loosen the fixed cellular structure and help with RNA extraction.

DSS.png



DSP.png



- RNaseIn was not used in the Washing buffer in this run for cost consideration.
- The chemicals can be fully dissolved, which was tested by adding 2.5ul chemicals in DMSO into 47.5ul PBS and incubated at 37°C for 30min.
- For fixed cell samples, when added 4.5ml washing buffer for the first wash, the supernatant was white because of the reaction between BSA and DSS; when added 15ml washing buffer for the second wash, the supernatant was transparent.
- For live cell samples, they were covalently modified in 1x PBS Plus buffer. For fixed cell samples, they were covalently modified in Wash buffer, which contains 0.1% Triton X100 for permeabilization.
- Judging by the precipitation during fixation, 1mg/ml DSS seemed to be stronger than 1mg/ml DSP, will use 0.5mg/ml DSS in the future.
- **I will include these samples in the multiplexed library prep (2025.6.5 Amplicon library #2)**