# Role of a Modified Base, 5-Hydroxymethyluracil (5-hmU), in Phase Separation: A Piece in the Puzzle to Finding a Cause for Loss of Structural Integrity in Neurodegenerative Disorders

by Iris


## Abstract

**Background:** Neurons require subcellular compartmentalization to function efficiently and the existence of membrane-less organelles (MLOs) is one of the ways by which proteins and RNA within the neurons aggregate to form compartments. These MLOs are typically formed by liquid-liquid phase separation and are common to many cell types, but some are specific to neuron cells. A number of neurodegenerative diseases are linked to MLOs, especially several proteins implicated in a number of neurodegenerative diseases are found in MLOs. Greater understanding and modulating of the phase-separation process, therefore, may offer therapeutic potential for neurodegenerative diseases. In this project, we studied the presence of 5-hydroxymethyluracil (5-hmU), a post-translational modification (PTM) of the thymidine base, as a possible contributor to the phase-separation process.

**Methods:** (i) *E.coli* plasmid DNA was used as a model to optimize conditions for introducing 5-hmU into DNA using a 2-step PCR (ii) Combination of Base excision and cleavage of AP sites used to quantify % incorporation (of 5-hmU) using Melt Curve analysis (iii) Optimized conditions (salt and Poly-L-Lysine (PLL) concentrations) to simulate phase separation in the modified DNA.

**Results:**

(i) 5-hmU was successfully introduced into a single strand of DNA

(ii) A Melt Curve Analysis successfully quantified the % incorporation of 5-hmU onto the DNA strands of *E. Coli*

(iii) Conditions for phase separation were optimized and phase separation was successfully observed in two DNA samples tested

**Conclusions and Next Steps:** Using *E.coli* DNA as a model, we optimized conditions for studying the phase separation process *in vitro*. We plan to adapt the methods established using *E.Coli* plasmid DNA to a Dinoflagellate model system (which have 30 – 90% naturally occurring modified DNA) and in human *ex vivo* neuronal cells.


## Introduction

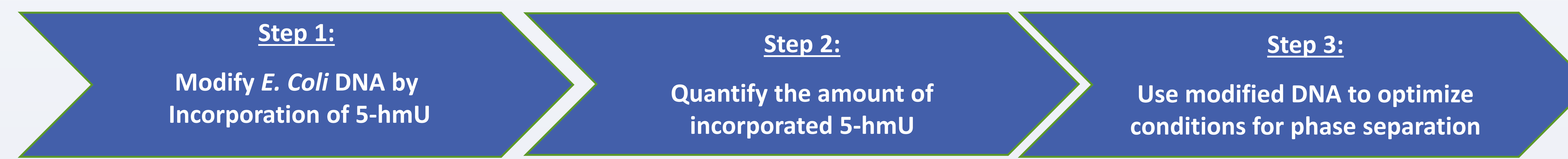
Liquid-liquid phase separation is a common phenomenon in cell biology and has been shown to play a role in many biochemical processes including transcription [1]. In healthy cells, this process helps condensate the transcription machinery into a location near the gene which is being transcribed. Alternatively, aberrant condensates have been shown to be associated with various diseases, including cancer and neurodegeneration [2].

One of the proposed mechanisms for aberrant condensates is a repeat of short DNA stretches [3]. Diseases like Huntington's disease, ALS, fragile-X syndrome, spinobulbar muscular atrophy (SBMA), myotonic dystrophy (DM1) with CTG repeats, and Friedreich's ataxia (FRDA), and others, have been associated with repeat expansion disorders.

Another mechanism which may lead to aberrant phase separation, is the presence of modified bases such as 5-Hydroxymethyluracil (5-hmU). 5-hmU has been reported to affect protein-binding to DNA and it is hypothesized here that a presence of 5-hmU may result in aberrant phase separation of macromolecules within a cell, thereby, affecting cell function [4].

Dinoflagellates are one of the most abundant marine organisms with over 8000 species. The dinoflagellate nucleus contains an extremely large DNA content, a skewed DNA-to-protein ratio of 10:1 (compared to 1:1 in a eukaryotic nucleus), large amounts of Mg2+ and Ca2+ cations in its DNA and most importantly, **a large proportion of nucleotide modifications such as 5-hmU**, which replaces up to 70% of thymine, along with a deficit of histones. These characteristics give Dinoflagellate DNA its permanently condensed chromosomes a liquid crystalline physical appearance. Interestingly, many species of dinoflagellates are known to release neurotoxins. Hence, our far-reaching goal, through this research project is to examine if aberrant phase separation plays a role in release of neurotoxins, and hence, in diseases like neurodegeneration.

## Methods




### Step 1

**Overview:**

In order to replicate the conditions found in Dinoflagellate DNA, the modified base, 5-hmU, was incorporated into *E. coli* DNA. However, unlike Dinoflagellate DNA, 5-hmU was incorporated only on 1 of the 2 DNA strands. This is because quantification of the degree of incorporation of 5-hmU on two strands was nearly impossible with the available resources.

For this, a novel strategy was utilized: 2-step PCR.

Figure 1: Strategy for 2-Step PCR

Step 1: PCR was carried out with standard conditions and a standard dNTP mix (dATP, dCTP, dGTP, and dTTP) for n-1 cycles (34 cycles in this context).

Step 2: For the nth cycle (cycle 35), PCR products were cleaned up and PCR was carried out with standard conditions, but with modified Uracil (dmUTP) nucleotides instead of thymidine (dmUTP vs. dTTP). This strategy allowed for incorporation of 5-hmU on only 1 strand of the amplified DNA.

**Methods and Results:**

**Primer Design:**

The following key conditions were considered while designing the primers necessary to carry out PCR.

- Low GC percentage
- Length 18-22 base pairs
- Melting temperatures at 52-58 bp
- Amplicon length <300 bp
- Melting temperatures at 52-58 bp

Primer pair 10

| | Sequence (5'->3') | Template strand | Length | Start | Stop | Tm | GC% | Self complementarity | Self 3' complementarity |
|---|---|---|---|---|---|---|---|---|---|
| Forward primer | [illegible] | Plus | 21 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| Reverse primer | [illegible] | Minus | 20 | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] | [illegible] |
| Product length | 290 | | | | | | | | |

Figure 2: Forward and Reverse primers (ordered through Primer BioSoft, San Francisco, CA)

**PCR Setup, Step 1:** PCR was carried out for 34 cycles (*Table 1*)

*Source: New England Biolabs, Ipswich, MA

| Reagent* | Concentration | Volume |
|---|---|---|
| Buffer | 10X | 2.5 µL |
| dNTP (A, G, C) | 10 mM | 0.5 µL |
| dTTP | 10 mM | 0.5 µL |
| Forward/Reverse primer | 10 µM each | 0.5 µL + 0.5 µL |
| DNA Template | 2 ng | 1 µL |
| Taq Polymerase | | 0.125 µL |
| water | | 19.375 µL |

Table 1: PCR Reaction Conditions for n-1 cycles

**PCR Cleanup:** After stage 1, the residual primers and nucleotides were removed using a PCR cleanup process.

**PCR Setup, Step 2:** 4 conditions were selected for 5-hmU incorporation:

- 0% (negative control)
- 33% (test group 1)
- 66% (test group 2)
- 100% (positive control)

| %dUTP | 100% | 66% | 33% | 0% |
|---|---|---|---|---|
| dmUTP | 0.5 µL | 0.33 µL | 0.17 µL | 0 µL |
| dTTP | 0 µL | 0.17 µL | 0.33 µL | 0.5 µL |

Table 2: dmUTP:dTTP Ratio for 35th PCR Cycle

PCR was carried out for the 35th amplification cycle using modified dmUTP in replacement of dTTP (5-hmU as opposed to thymidine). Gel electrophoresis was performed to confirm that the PCR reaction was successful.

Figure 3: Ethidium Bromide Stained Agarose Gel (1%) Showing PCR Products Lanes 1: 0%; lane 2: 33%; Lane 3: 100 bp Ladder; Lane 4: 66%; Lane 5:100%

### Step 2

**Overview:**

In order to quantify the actual amount of 5-hmU incorporated into single-stranded *E.Coli* DNA, an innovative combination of methods were utilized as there was no standard protocol available in literature.

Base excision → Cleavage at AP sites

**Base excision:**

The incorporated 5-hmU was excised using the enzyme DNA UDG Glycosylase. This enzyme is typically utilized In DNA repair machinery and excises uracil from DNA, leaving abasic (AP) sites.

Figure 4: Strategy of Base Excision

**Cleavage of the DNA backbone at AP sites:**

The DNA backbone was cleaved wherever UDG excised 5-hmU to ensure that DNA with higher incorporation of dmUTP melts at lower temperatures.

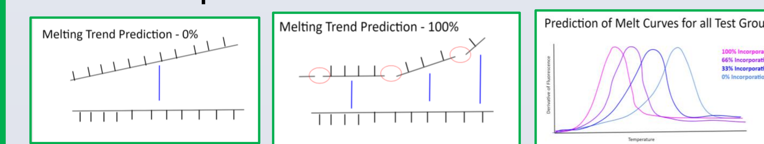


Figure 5: Strategy for Quantifying 5-hmU Incorporation

**Methods and Results:**

**Base Excision and Cleavage at AP sites:**

Standard reaction conditions (Table 3) and 8 different conditions (Table 4) were tested for cleavage of DNA strands at 5-hmU incorporated sites.

| Reagent | Concentration | Volume |
|---|---|---|
| UDG Reaction Buffer | 1 x | 5 µL |
| Water | | 44.6 µL |
| PCR Product | 0.2 µg | 0.2 µL |
| UDG | 1 unit | 0.2 µL |
| Reaction Volume | | 50 µL |

Table 3: Reaction Conditions for Base Excision using UDG

| | KOH (M) | Temp (°C) |
|---|---|---|
| 1 | 0.1 | |
| 2 | 1.0 | |
| 3 | - | 55 |
| 4 | - | 65 |
| 5 | 0.1 | 65 |
| 6 | 1.0 | 55 |
| 7 | 0.1 | 55 |
| 8 | 1.0 | 65 |

Table 4: Optimizing Conditions for DNA Strand Cleavage at AP Sites; No KOH & 37°C were normal conditions (grey cells)

**Melt Curve Analysis:** It is predicted that DNA with higher 5-hmU incorporation will melt at lower temperatures since it is more fragmented as a result of abundant backbone cleavage.

**dmUTP Incorporation Rate:** The linear model: $y = -0.05601 x + 85.022$ was calculated using the negative and positive controls and was used to predict the actual incorporation rate of dmUTP for the remaining two samples.

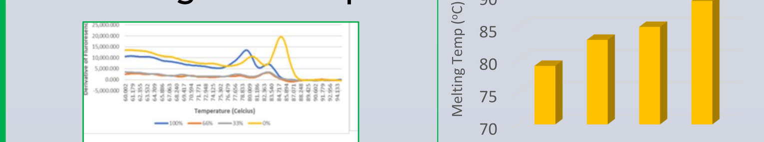



Figure 6: Melt Curves for the 4 DNA Samples (left) and Estimation of Actual 5-hmU Incorporation (right) for Varying 5-hmU Incorporated DNA Strands

Based on Melt Curve analysis, the rate of actual incorporation of 5-hmU was comparative to predicted rates for both test groups

- Test group 1: predicted 33%; actual 37%
- Test group 2: predicted 66%; actual 40%

### Step 3

**Overview:**

In order to induce phase separation within each test sample, conditions within a cell were replicated. As literature for this induction was limited, various conditions were tested within a 5x5 matrix for each test group.

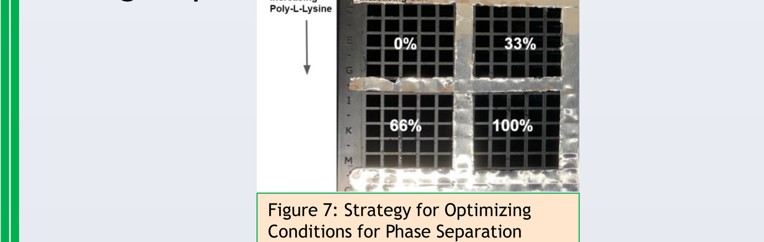



Figure 7: Strategy for Optimizing Conditions for Phase Separation

The matrix was created by laterally increasing the concentration of Poly-L-Lysine and vertically increasing the concentration of NaCl.

**Methods:**

**Matrix Setup:**

The following conditions were used in the 5x5 matrix in a 5.5 µL reaction volume.

| (PLL µM) | (NaCl µM) | | | | |
|---|---|---|---|---|---|
| | 0 µM / 0 µM | 0 µM / 476 µM | 0 µM / 952 µM | 0 µM / 1429 µM | 0 µM / 1905 µM |
| | 0.42 µM / 0 µM | 0.42 µM / 476 µM | 0.42 µM / 952 µM | 0.42 µM / 1429 µM | 0.42 µM / 1905 µM |
| | 0.85 µM / 0 µM | 0.85 µM / 476 µM | 0.85 µM / 952 µM | 0.85 µM / 1429 µM | 0.85 µM / 1905 µM |
| | 1.27 µM / 0 µM | 1.27 µM / 476 µM | 1.27 µM / 952 µM | 1.27 µM / 1429 µM | 1.27 µM / 1905 µM |
| | 1.69 µM / 0 µM | 1.69 µM / 476 µM | 1.69 µM / 952 µM | 1.69 µM / 1429 µM | 1.69 µM / 1905 µM |

Table 5: Optimizing Conditions for Phase Separation Using PLL and NaCl

**Phase Separation Observation:**

It was found that phase separation could best be observed under 0 µL of NaCl while "titrating" Poly – L – Lysine.

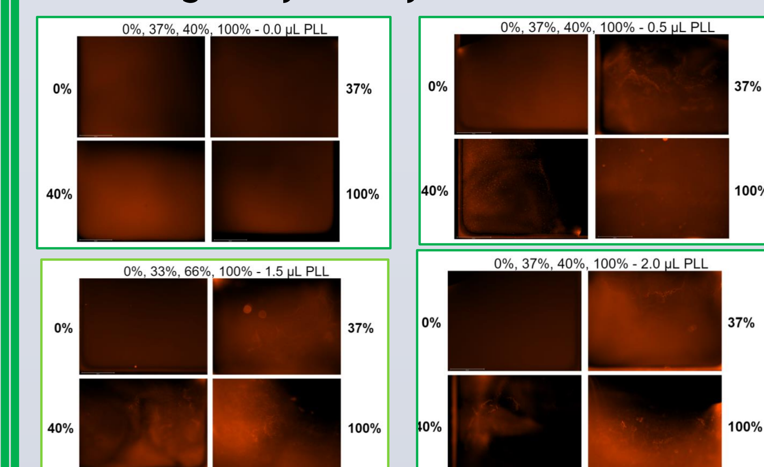



Figure 8: Observation of Phase Separation Within Each DNA Sample Throughout Titration of PLL with a Constant Amount of NaCl
Top Left : DNA Samples Under 0 µL PLL; Top Right : DNA Samples Under 0.5 µL PLL; Bottom Left: DNA Samples Under 1.5 µL PLL; Bottom Right: DNA Samples Under 2.0 µL PLL

There is evidence of an increase in DNA aggregation as the amount of incorporated modified base increases.

## Working with Dinoflagellates

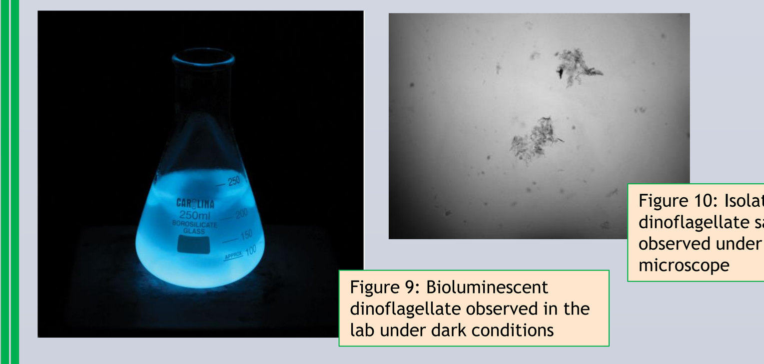


Figure 9: Bioluminescent dinoflagellate observed in the lab under dark conditions

Figure 10: Isolated dinoflagellate sample observed under a microscope

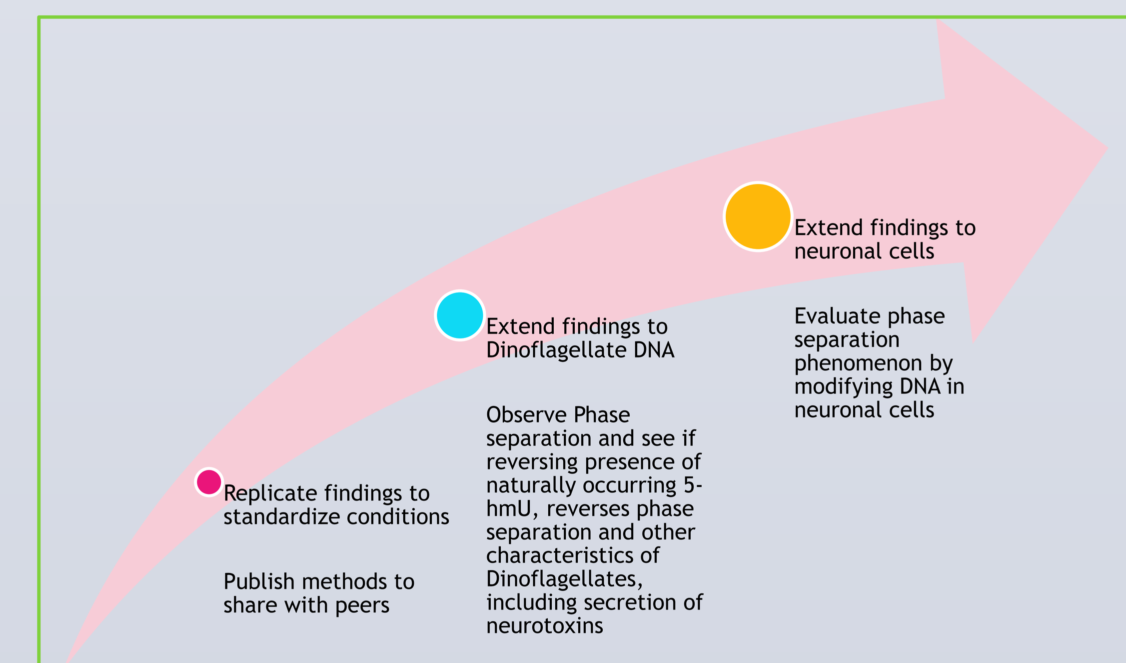
## Summary of Results

- ✓ Modified base 5-hmU was introduced into *E. Coli* DNA
- ✓ Modified base was added only on 1 strand of DNA – using a novel methodology involving a 2-step PCR
  - Step 1 of PCR was performed until the 34th cycle under normal PCR conditions
  - Step 2 was performed on the 35th cycle using different ratios of dmUTP:dTTP in the dNTP mix
  - Gel electrophoresis was used to confirm PCR products
- ✓ The incorporated 5-hmU was quantified using a Melt Curve
  - Conditions designed for 33% 5-hmU incorporation showed actual incorporation of 37%
  - Conditions designed for 66% 5-hmU incorporation showed actual incorporation of 40%
- ✓ Phase separation was observed in *E. Coli*
  - Phase separation was best observed in conditions which had 0 µM of NaCl and 0.42 µM – 1.69 µM of PLL
  - Phase separation was best observed in DNA samples with 40% and 100% rates of 5-hmU incorporation, respectively

## Conclusion and Future Work

Post-translational modifications have been known to regulate phase separation and consequently, contributors to disease pathogenesis. This research project is first step in establishing the relationship between presence of modified bases on DNA, especially, 5hmU, and phase separation.

In this research project, using *E. coli* DNA, we demonstrated that under suitable conditions, phase separation could be observed.




## References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2. Ryan, V. H. and Fawzi, N.L., (2019). Physiological, Pathological, and Targetable Membrane less Organelles in Neurons, *Trends in Neurosciences*, 42, 693-708.
3. Alberti, S. and Dormann, D. (2019). Liquid-liquid phase separation in disease, *Annu. Rev. Genet.*, 53:171-194.