Development of an ultrasensitive transcriptomic mapping platform to study opioid-regulated circuits in neuronal tissue

Morgan Oatley[1], James T. Anderson[1], Allison R Hickman[1], Alexandra C. Nowlan[2], Lu Sun[1], Zachary B. Gillespie[1], Matthew J. Meiners[1], Marcus A. Cheek[1], Zoe A. McElligott[2], Michael-Christopher Keogh[1], & **Bryan J. Venters[1]**

[1]Epicypher, Inc., Research Triangle Park, NC 27713, USA. [2]Department of Psychiatry, University of North Carolina, Chapel Hill, NC, USA.

Background

Detecting acute transcriptional changes can provide significant biological insight into the immediate effects of transcription factors and environmental stimuli. Here, we propose a new approach to directly and quantitatively profile chromatin-engaged RNA polymerase II (Pol II) as a proxy for nascent RNA transcription. We developed a novel Pol II CUT&Tag assay utilizing phospho-specific Pol II antibodies and a Tn5 transposase fusion (pAG-Tn5) to identify sequences with transcriptionally-engaged polymerase. In addition, we designed and produced novel DNA-barcoded nucleosome spike-in controls with Pol II CTD epitopes (CTD-dNucs) for in-assay antibody validation, technical monitoring, and quantitative cross-sample comparisons. We then optimized the technique for different sample inputs, demonstrating its capacity to work with nuclei from fresh, frozen and fixed tissues.

(A)

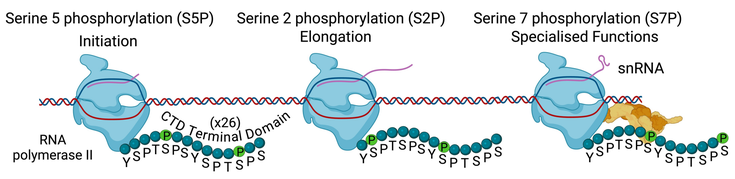


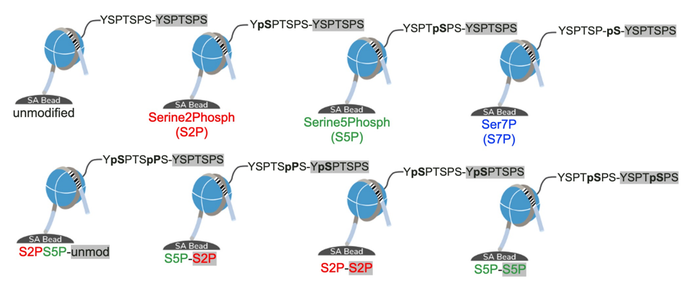
Figure 1. Schematic of RNA pol II phosphorylation sites in the C-Terminal Domain (CTD) and their relationship to different stages of transcription.

Table 1. Techniques for measuring nascent gene transcription

Assay	Technique	Measure	Strength	Weakness
mRNA-seq	Sequencing poly-A transcripts	Poly-A transcripts only	Widely used & inexpensive	Time insensitive, measures stable transcripts only
SLAM-seq	Metabolic labelling of RNA	All actively transcribed transcripts	Commercially available & compatible with single cell	Not compatible with all sample types
PRO-seq	Nuclear run-on	All actively transcribed transcripts	Single nucleotide resolution	Difficulty mapping short, repetitive transcripts
CUTANA-RNA pol II	**CUT&Tag**	**RNA-pol-II associated DNA**	**High resolution, high throuput & diverse sample input**	**New technology**

RNA pol II antibody validation with CTD spike-in controls

(A)



(B)

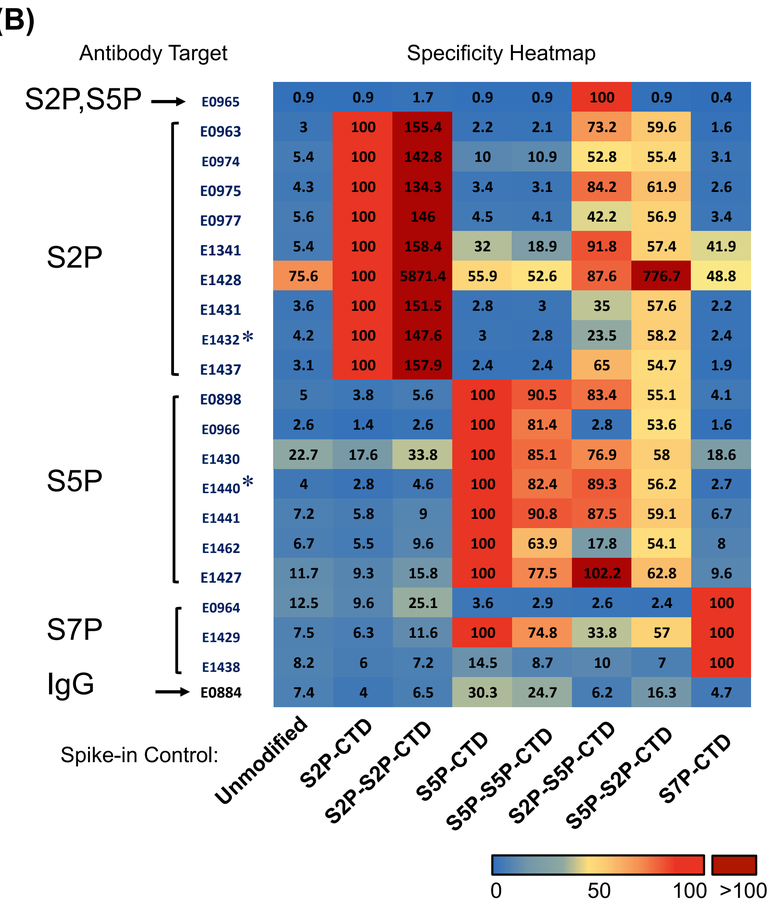
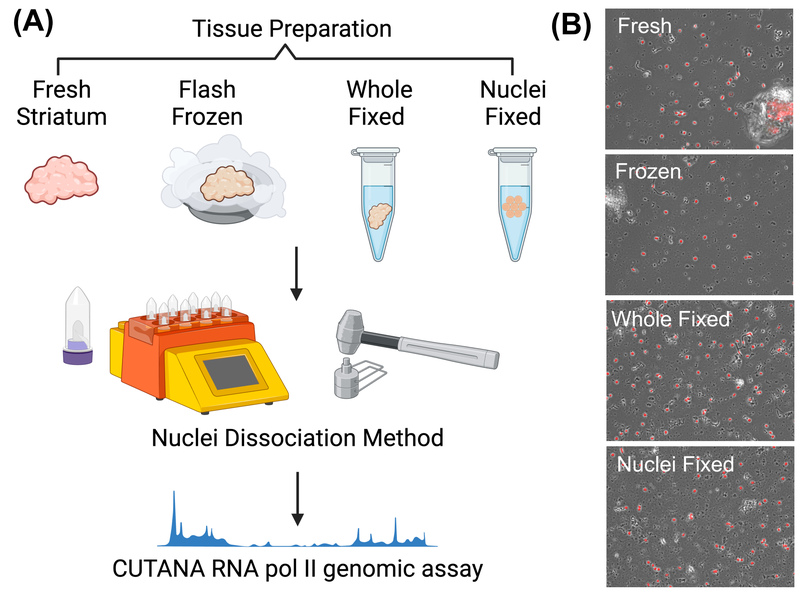


Figure 2. (A) Design of Pol II CTD dNucs spike-in panel used for in-assay quality control. **(B)** Specificity Heatmap of commercial antibodies (specific catalogue and lot numbers designated by unique E-number), tested in RNA pol II CUT&Tag workflow with Pol II CTD dNuc spike-in controls. Values were normalized to the on-target spike-in nucleosome and displayed as a percentage with red indicating high specificity and blue indicating low specificity. Asterisks indicate best performing antibodies selected for down-stream experiments.

Tissue Preparation Optimization



(C)

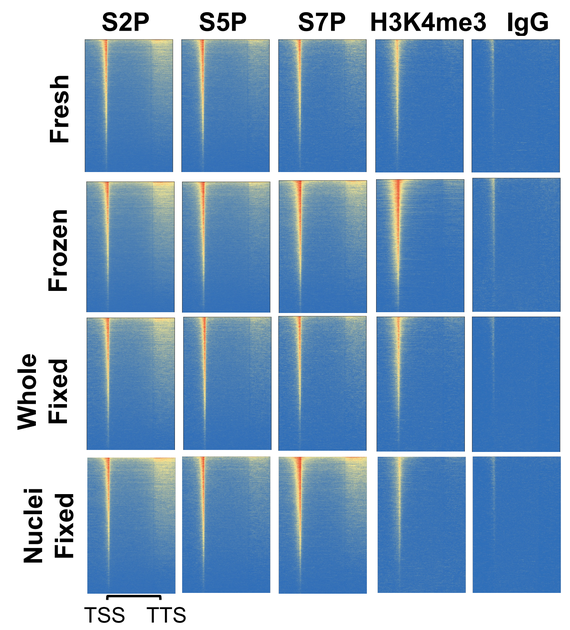


Figure 3. (A) Outline of method for brain tissue dissociation. **(B)** Bright-field images with propidium iodide staining of different nuclei preparations. **(C)** Gene body heatmaps displaying transcriptional signal between the TSS and TTS across different RNA pol II CUT&Tag antibody assays with different sample inputs. Ranked by H3K4me3 intensity where red indicates enrichment and blue indicates background.

Measuring phospho-Pol II binding dynamics in primary mouse neurons

Fentanyl-related overdose deaths have risen sharply in the US since 2013.[1] While fentanyl has rapid behavioural effects, blocking pain perception and causing hyperlocomotion, little is known about the transcriptional changes that occur in neurons after exposure to the drug.[2] Opioids are known to impact on the medium spiny neurons of the striatum.[3] To better characterize the immediate effects fentanyl has on the transcription of striatal neurons we designed a pilot experiment comparing transcriptional changes detected by our novel RNA pol II CUT&Tag approach with traditional mRNA sequencing.

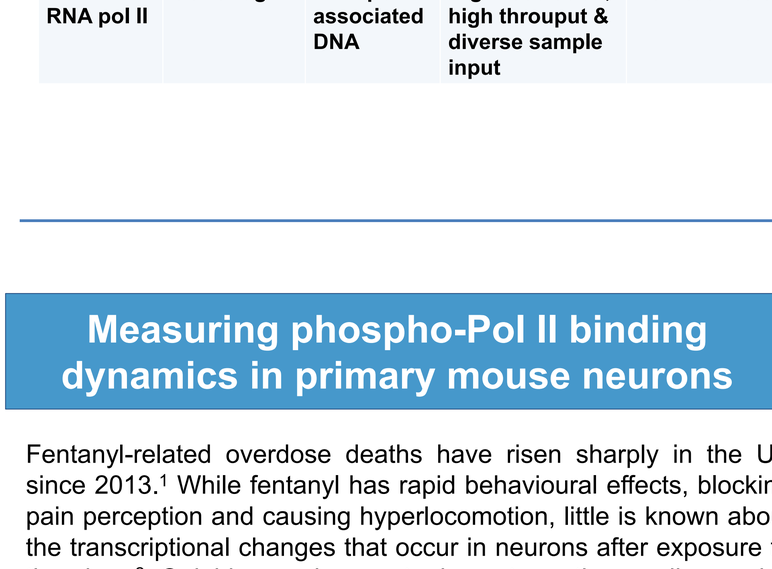


Figure 4. Schematic of pilot study of RNA pol II CUT&Tag assay on mouse striatum. A time-course was performed whereby the mouse brain was immediately harvested and the striatum dissected 10 minutes, 30 minutes or 60 minutes after an intraperitoneal injection of either fentanyl (1mg/kg) or equivalent saline.

Created in BioRender. v23n319

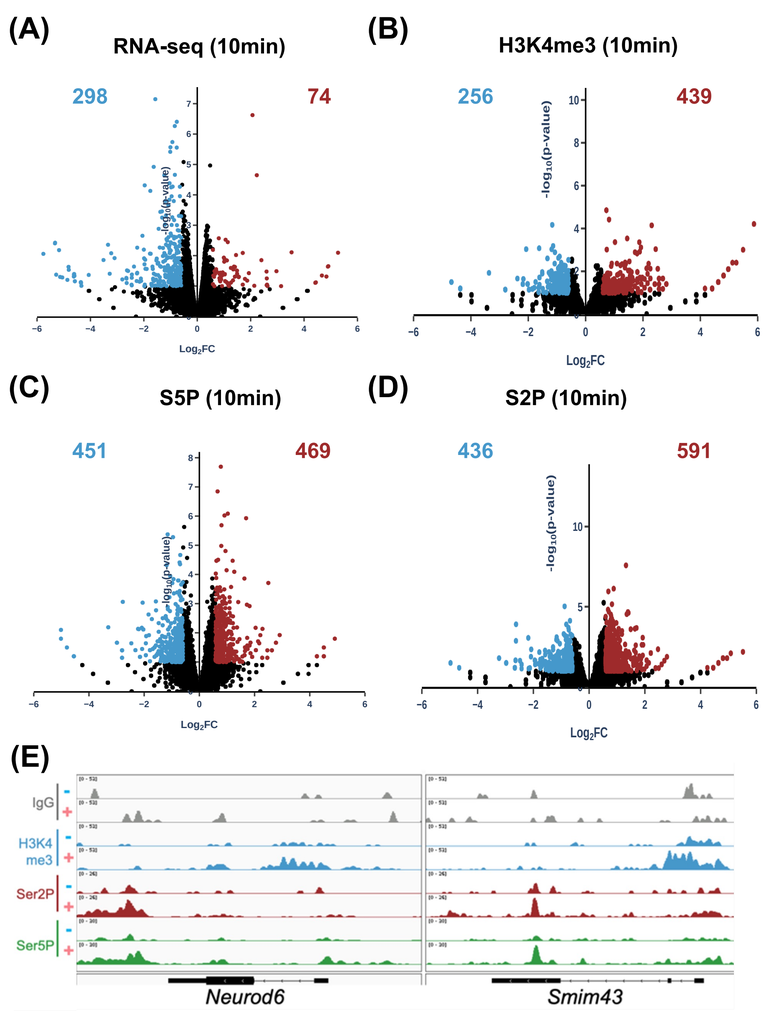


Figure 5. Sequencing data of pilot opioid stimulation of mouse primary neurons, harvested 10 minutes post injection with either saline or fentanyl. Volcano plots of differential gene analysis between saline and fentanyl treated conditions of **(A)** RNAseq, **(B)** H3K4me3, **(C)** S5P RNA pol II and **(D)** S2P RNA pol II. Down-regulated genes are highlighted in blue and up-regulated genes are highlighted in red based on an adj. p-value <0.1 and a log-2-fold changed of >1.5. **(E)** Gene loci demonstrating immediate change in RNA pol II engagement in response to fentanyl treatment.

Conclusions

- Development of an RNA pol II CTD spike-in panel has enabled screening for highly specific, quality reagents.
- RNA pol II CUT&Tag is compatible with fresh, frozen and fixed tissue preparations.
- This new methodology has enabled the interrogation of the immediate effects of fentanyl treatment on neuronal biology in an *in vivo* setting.

RNA pol II CUT&Tag is a new highly sensitive approach for measuring phosphorylated RNA pol II engagement with DNA

References

1. Han Y, Yan W, Zheng Y, Khan M Z, Yuan K, Lin L. (2019) "The rising crisis of illicit fentanyl use, overdose and potential therapeutic strategies." *Translational Psychiatry* ***9***
2. Varshneya N B, Walentiny D M, Moisa L T, Walker T D, Akinfiresoye L R, Beardsley P M. (2021) "Fentanyl-related substances elicit antinocoception and hyperlocomotion in mice via opiod receptors." *Pharmacology Biochemistry & Behaviour,* ***208***
3. Wang W, Xie X, Zhuang X, Huang Y, Tan T, Gangal H, Huang Z, Purvines W, Wang X, Stefanov A, Chen R, Rodriggs L, Chaiprasert A, Yu E, Vierkant V, Hook M, Huang Y, Darcq E & Wang J. (2023) "Striatal μ-opioid receptor activation triggers direct-pathway GABAergic plasticity and induces negative affect ." *Cell Reports* ***42(2)***