CBC | CHICAGO BIOMEDICAL CONSORTIUM

CBC Catalyst Award Proposal Title Page

Title of the proposal *(no more than 100 characters including white space)*

Novel tools for quantitative mapping of epigenetic and structural remodeling of eukaryotic chromatin

Name, degree, title, institution, and contact information including the email address of each PI

Dr. Jan-Hendrik Spille,
Assistant Professor, Departments of Physics and Chemistry,
University of Illinois at Chicago
(617) 417-2618
jhspille@uic.edu

Alexander Ruthenburg, PhD
Associate Professor, Department of Molecular Genetics and Cell Biology
University of Chicago
(773) 702-1067
aruthenburg@uchicago.edu

Do you have any current or pending grant applications that potentially overlap with this application?
Check ONE:

YES ☐ **NO** ☒

If YES, please identify them.

(ENTER THE OVERLAP HERE)

Explain the overlap in the Biosketch section.

Does the proposed research involve animal subjects?	**Check ONE:**	**YES** ☐	**NO** ☒
Does the proposed research involve human subjects?	**Check ONE:**	**YES** ☐	**NO** ☒
Does the proposed research involve embryonic stem cells?	**Check ONE:**	**YES** ☒	**NO** ☐

(Note: if the data entered exceeds one page, it is acceptable to submit it as two pages.)

Project Lay Summary | It is widely appreciated that chromosome structure controls gene expression, and that changes to chromosomal architecture are critical features of development and disease etiology, but the molecular mechanisms relating structure to function remain poorly understood. We propose development of two complementary technologies to further advance the quantitative accuracy and spatial resolution of chromosomal architecture and dynamics studies. This collaborative proposal integrates the unique super-resolution imaging and biophysical analysis expertise of the Spille lab (UIC) with pioneering quantitative epigenomics methods of the Ruthenburg lab (UChicago), to uncover the causal sequence of events in epigenetic rewriting and structural remodeling of the *Hoxb* gene cluster during the differentiation of mouse embryonic stem cells towards neuronal cells. This gene cluster will serve as a test bed for these novel assays, which we anticipate will have broad application in studying how genome architecture is reconfigured.

Impact | The proposed work is highly significant because it addresses an unsolved question underlying gene activation in developmental and disease-associated cell state transitions: Do epigenetic chromatin modifications lead to structural changes that activate gene transcription, or do these modifications follow structural remodeling of chromatin? Further, we seek to define the precise order of events distinguishing large-scale chromosomal movements from short-range promoter-enhancer contact dynamics. It is high risk because it requires adaptation of highly sophisticated, technically challenging novel assays. If successful, we will have established an assay to make any chromatin conformation capture technique quantitative on an absolute scale that enables facile comparison, developed a complementary single-cell imaging technique to provide readouts that combine chromatin structure with protein coverage, and elucidated how both aspects of gene regulation work together in the activation of a model locus with central importance in developmental biology. Moreover, we anticipate that the development of these two transformative methods will bridge the substantial gap between ensemble high-resolution genomics views (average chromosomal conformation and epigenetic modification landscape), and the more idiosyncratic single-cell measurements of chromosomal architecture which may be intimately tied to cell-autonomous distinctions in histone mark patterns.

Nature of inter-institutional collaboration and long-term funding plan | Although we have an active collaboration ongoing, this proposal is centered on a new collaborative project between the Ruthenburg and Spille labs. The Ruthenburg lab uses ensemble quantitative genomic methods largely developed in-house to elucidate the interplay of epigenetic modifications and chromatin structure. The Spille lab uses single molecule super-resolution microscopy to investigate biophysical mechanisms that drive organization of the cell nucleus. In essence, our expertise represents two distinct, yet complementary approaches, wherein clear synergies and cross-validation will arise from tackling the same question with two different experimental modalities. Our two labs began collaborating on a project investigating the role of nuclear actin in chromatin organization because a graduate student in the Ruthenburg lab, Jelena Scepanovic, required super-resolution microscopy expertise for her thesis project. Although we do not have any joint grants, our labs have shared resources and reagents, held several joint meetings to discuss progress on this project, and Ms. Scepanovic works closely with a student in the Spille lab to acquire super-resolution microscopy data. This proposal leverages both group's complementary expertise to initiate a new research direction. We lack sufficient preliminary data for a strong NIH application. Catalyst funding will support the proof-of-concept experiments necessary for a compelling NIH R01 application (NHGRI) in the October 2023 or February 2024 cycle.

Description of Proposed Research | *Background and Significance*. Cis-regulatory elements (CREs) including promoters, enhancers, and silencers, together with transcription factors, regulate spatiotemporal gene expression. Due to their decisive influence on transcription initiation, enhancers have been studied extensively in a many biological models. While our knowledge about active enhancer and promoter associated histone marks (*1-3*), enhancer grammar (*4, 5*), and cell-type specificity of enhancer-promoter cont is extensive (*6, 7*), our mechanistic understanding of the activation of and downstream transcriptional regulation by these elements remains limited. Furthermore, multiple studies have reported conflicting findings regarding the role of enhancer-promoter looping in transcriptional initiation (*8-12*), as well as the timing of histone modifications (*13*), emphasizing the present gap in mechanistic understanding of developmental enhancer-dependent gene activation. To address these seminal questions, we will study the order and causality of *i)* histone mark deposition, *ii)* extrusion of the *Hoxb* cluster from its repressed territory, *iii)* enhancer-promoter interaction (EPI) establishment, and *iv)* transcription initiation using a powerful combination of super-resolution

chromatin imaging (*14*) and fine-resolution chromosome conformation capture (*15*) in a well-established gene expression model—*Hoxb* cluster activation by retinoic acid (RA)-induced ectodermal differentiation of mouse embryonic stem cells (mESCs). Upon RA stimulation, a subset of *Hoxb* genes loop out of their chromosome territory (*16*), acquiring stereotyped histone marks which roughly accompany transcriptional activation (*17*). At the resolution of current technology, the order of events remains unclear, markedly limiting our ability to develop a precise mechanistic model. Here, we will scrutinize the progression of structural and functional changes of regulatory chromatin elements in *Hoxb* activation with novel sequencing (**Aim 1**) and single cell imaging technologies (**Aim 2**) to simultaneously provide quantitative measurements of 3D chromatin organization and histone mark spatial organization during the initial 2-4h of the activation time (*17*). Similar locus displacements have been reported for many other genes during cell differentiation and development, underscoring the importance of deciphering underlying mechanisms. We expect to generate data with unprecedented detail and spatiotemporal resolution that will be powerful hypothesis generators for causal relationships between histone mark deposition, large-scale chromosomal movement, enhancer-promoter looping, and transcriptional initiation in the process of gene activation upon cell state transition.

Approach | Aim 1: ***To develop technology to transform proximity ligation from relative scaling to absolute scaling with internal standard calibration.*** To interrogate fine-scale 3D chromosome structure changes in response to RA stimulation, we will develop a new calibrated version of micro-C, a recently described genome-wide proximity ligation method with significantly enhanced resolution compared to prior methods (*15*). The higher level of structural detail afforded by micro-C is essential to ascribe contact points and loops to underlying functional DNA elements, necessary to link architectural features to molecular mediators. Yet a major drawback of micro-C, and indeed any similar proximity ligation assay (PLA), is that the relative contract frequency scaling applies the reference frame within a single experiment and cannot be applied to others. To enable comparative studies, normalization of the relative contact frequencies from two 3D genome datasets is required. All current methods rely on the assumption that the differences between the two datasets are not widespread, but localized to a small minority of genomic loci. In our first forays into micro-C, we have become concerned that normalization choices can completely alter the experimental interpretation of perturbations, and likely blunt quantification of biological effect sizes. Because we anticipate the architectural changes upon RA-stimulation to be large scale and not restricted to the *Hoxb* cluster (*17, 18*), this normalization assumption, common to all of these techniques may be flawed, rendering clear interpretation of experimental outcomes problematic (*19-21*).

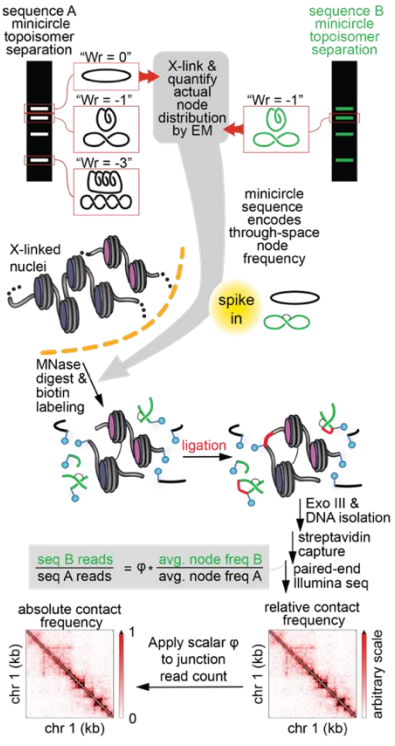


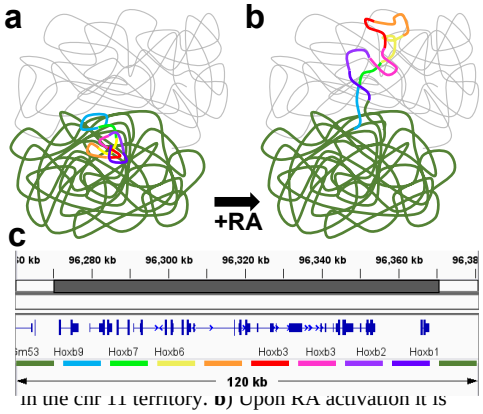
Fig. 1 | Proposed method to calibrate micro-C with DNA minicircles.

To experimentally address this issue, we propose to pioneer the internal standard-calibration of PLA, as we have with ChIP-seq (*20-22*). We will design internal standards to spike into micro-C so we can express data on a universal, more biologically meaningful scale of absolute pairwise contact frequencies, as opposed to relative contact frequencies particular to each individual experiment and thus not readily comparable unless little has changed between the two conditions. We envision using minicircle DNA, whose topoisomers can be isolated by chloroquine gels(*23*), to create DNA-through space crossovers (nodes) of defined number. We will use equivalently sized minicircles with completely orthogonal sequences to encode a series of different absolute through-space contact standards (**Fig. 1**). These DNA minicircle standards will be designed with no sequence homology to mouse or human genomes to enable facile discrimination of sequencing reads, the actual distributions will be measured by AFM on crosslinked material after purification. Characterized topoisomer standards can be aliquoted and frozen, then spiked into the normal Micro-C workflow which should restrict the recovery to cross-linked nodal cross-overs **(Fig. 1)**. Post sequencing and alignment to the mouse genome concatenated with the standard sequences, the counts of each standard (with a discontinuity indicative of cleavage and ligation) can ratiometrically be compared amongst standard members. Even if standardization

does not furnish unambiguously absolute contact frequency, this could nevertheless resolve the normalization problem. This *t*opoisomer *i*nternally-calibrated micro-*C* (TI-C) method could be transformative—it could be broadly applicable to all PLA-type experiments to enable facile and pitfall-free quantitative comparisons, furnish more biologically meaningful absolute contact frequencies (directly comparable to single-cell oligoPaint), and be commercialized to promote widespread adoption, as we have with ICeChIP.

Using this new method, we will track the RA induction of Hox cluster movements with a time course spanning the first 4 hours of activation, where these changes are known to occur (*17*) We will first examine the absolute contact frequency using TI-C with then monitor their changes upon induction with RA. Having absolute ensemble contact frequencies from TI-C should enable direct comparison to oligoPaint-based super-resolution measurements in Aim 2, which will also be critical for focusing our choices of one or two time points where the changes are the most drastic. At matching time points, we will assess active enhancer- and promoter-associated histone mark levels (H3K4me3, H3K4me1, H3K27ac as well as S2p-Pol II) using the ICeChIP quantitative method we pioneered (*20, 22*) to gain insight into the activation status of these CREs.

Aim 2: ***Mapping mechanisms of Hox activation with super-resolution in single cells.*** We expect that calibrated micro-C will provide a quantitative, fine-scale, nucleosome resolution view of chromatin contact evolution during Hox activation. This resolution will allow us to identify molecular agents mediating the process by comparison to ChIP-seq profiles at the corresponding timepoints. But micro-C measures only direct chromatin contacts within a narrow capture radius of tens of nanometers. Any larger-scale locus rearrangements go undetected. Furthermore, micro-C is an ensemble technique requiring the use of genetic material from millions of cells per replicate. Therefore, we propose to complement TC-C assays in Aim 1 with a chromatin-tracing approach, allowing single-cell resolution. Only recent developments in super-resolution microscopy provide the nanoscale resolution to recapitulate ensemble chromatin contact data with single cell optical methods (*14, 24, 25*).



in the chr 11 territory. **b**) Upon RA activation it is activated and translocated out of the chr 11 territory. **c**) We will tile the array of hox genes spread out over ~100kb with 2-5kb resolution oligoPaint probes.

These cutting-edge technologies improve volumetric resolution of DNA FISH 1000-fold and push sequence resolution to genomic levels of 2-5kb (*24*). Multiplexed imaging with probe exchange overcomes the limitation to 2-3 color channels and allows full tracing of the chromatin fiber in single alleles. We will design an oligoPaint library tiling 200kb of the *Hoxb* locus and adjacent chromatin in 2-5kb increments. We will complement the *Hoxb* library with probes that highlight the chromosome 11 territory. By tracing locus topology with nanometric resolution of up to 100 chromatin tiles before and after RA induction, we expect to not only recapitulate short-range micro-C contact maps, but gain a global picture of the progression of locus topology changes and translocation during activation.

Identifying molecular drivers of structural changes requires a concurrent protein-level readout in single cells akin to ensemble ChIP-seq data. We will use a novel assay developed in the Spille lab to integrate oligoPaint chromatin tracing with antibody-based mapping of epigenetic marks at genomic resolution. Briefly, histone modifications at specific chromatin elements will be read out via DNA-barcoded antibodies as part of the super-resolution imaging process. This combined readout of chromatin structure and histone modification with single allele resolution will allow us to stratify data by locus topology and determine if histone marks precede structural changes, or if they are only acquired by those locus topologies that have already undergone fine-scale (EPI) or large-scale (translocation) topological changes. Experiments of this type require the acquisition of large single allele ensembles enabled by an automated imaging and probe exchange system in the Spille lab as well as sophisticated data analysis and visualization strategies that we will develop as part of this proposal.

Measures of success | We will construe success by *i*) publication of a high-profile collaborative paper to showcase the newly developed technology, *ii*) R01-level funding for further mechanistic and technology development, and *iii*) wide adoption of the methods developed in this proposal.

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Program Director/Principal Investigator (Last, First, Middle): Spille, Jan-Hendrik

DETAILED BUDGET FOR INITIAL BUDGET PERIOD
DIRECT COSTS ONLY

FROM 06/01/2023 THROUGH 05/31/2025

List PERSONNEL *(Applicant organization only)*
Use Cal, Acad, or Summer to Enter Months Devoted to Project
Enter Dollar Amounts Requested *(omit cents)* for Salary Requested and Fringe Benefits

NAME	ROLE ON PROJECT	Cal. Mnths	Acad. Mnths	Summer Mnths	INST.BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Jan-Hendrik Spille	PD/PI			1.00	99,395	1,988	739	2,727
Jonah Galeota-Sprung	GRA		9	3	42,467	32,559	2,380	34,939
Filmon Medhanie	Tech	1.5			41,600	5,200	410	5,610
SUBTOTALS →						39,747	3,529	43,276

CONSULTANT COSTS

EQUIPMENT *(Itemize)*

SUPPLIES *(Itemize by category)*

oligoPaint library with auxiliary adapter and readout probes, PCR and purification kits, antibodies, cell culture, imaging media and slides — 30,000

TRAVEL

INPATIENT CARE COSTS

OUTPATIENT CARE COSTS

ALTERATIONS AND RENOVATIONS *(Itemize by category)*

OTHER EXPENSES *(Itemize by category)*

Publication cost (year 2)

CONSORTIUM/CONTRACTUAL COSTS	DIRECT COSTS	
SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD *(Item 7a, Face Page)*		$ 73,276
CONSORTIUM/CONTRACTUAL COSTS	FACILITIES AND ADMINISTRATIVE COSTS	
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD		$ 73,276

Program Director/Principal Investigator (Last, First, Middle): Spille, Jan-Hendrik

BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS	INITIAL BUDGET PERIOD *(from Form Page 4)*	2nd ADDITIONAL YEAR OF SUPPORT REQUESTED	3rd ADDITIONAL YEAR OF SUPPORT REQUESTED	4th ADDITIONAL YEAR OF SUPPORT REQUESTED	5th ADDITIONAL YEAR OF SUPPORT REQUESTED
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*	43,276	43,551			
CONSULTANT COSTS					
EQUIPMENT					
SUPPLIES	30,000	7,173			
TRAVEL					
INPATIENT CARE COSTS					
OUTPATIENT CARE COSTS					
ALTERATIONS AND RENOVATIONS					
OTHER EXPENSES		1,000			
DIRECT CONSORTIUM/ CONTRACTUAL COSTS					
SUBTOTAL DIRECT COSTS *(Sum = Item 8a, Face Page)*	73,276	51,724			
F&A CONSORTIUM/ CONTRACTUAL COSTS					
TOTAL DIRECT COSTS	73,276	51,724			

TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD	**$** 125,000

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

see attached budget justification document

Program Director/Principal Investigator (Last, First, Middle): Ruthenburg, Alexander

DETAILED BUDGET FOR INITIAL BUDGET PERIOD
DIRECT COSTS ONLY

FROM: 06/01/2023 THROUGH: 05/31/2025

List PERSONNEL *(Applicant organization only)*
Use Cal, Acad, or Summer to Enter Months Devoted to Project
Enter Dollar Amounts Requested *(omit cents)* for Salary Requested and Fringe Benefits

NAME	ROLE ON PROJECT	Cal. Mnths	Acad. Mnths	Summer Mnths	INST.BASE SALARY	SALARY REQUESTED	FRINGE BENEFITS	TOTAL
Alexander Ruthenburg	PD/PI	0				0		
Jelena Scepanovic	GRA	10				32,130	7,165	39,295
SUBTOTALS →						32,130	7,165	39,295

CONSULTANT COSTS	
EQUIPMENT *(Itemize)*	
SUPPLIES *(Itemize by category)*	
See detailed Budget Justification (year 1-- Micro-C, tissue culture, general supplies)	21,205
TRAVEL	1,000
INPATIENT CARE COSTS	
OUTPATIENT CARE COSTS	
ALTERATIONS AND RENOVATIONS *(Itemize by category)*	
OTHER EXPENSES *(Itemize by category)*	
Publication	1,000

CONSORTIUM/CONTRACTUAL COSTS	DIRECT COSTS	
SUBTOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD *(Item 7a, Face Page)*		$ 62,500
CONSORTIUM/CONTRACTUAL COSTS	FACILITIES AND ADMINISTRATIVE COSTS	
TOTAL DIRECT COSTS FOR INITIAL BUDGET PERIOD		$ 62,500

Program Director/Principal Investigator (Last, First, Middle): Ruthenburg, Alexander

BUDGET FOR ENTIRE PROPOSED PROJECT PERIOD
DIRECT COSTS ONLY

BUDGET CATEGORY TOTALS	INITIAL BUDGET PERIOD *(from Form Page 4)*	2nd ADDITIONAL YEAR OF SUPPORT REQUESTED	3rd ADDITIONAL YEAR OF SUPPORT REQUESTED	4th ADDITIONAL YEAR OF SUPPORT REQUESTED	5th ADDITIONAL YEAR OF SUPPORT REQUESTED
PERSONNEL: *Salary and fringe benefits. Applicant organization only.*	39,295	39,295			
CONSULTANT COSTS					
EQUIPMENT					
SUPPLIES	21,205	21,205			
TRAVEL	1,000	1,000			
INPATIENT CARE COSTS					
OUTPATIENT CARE COSTS					
ALTERATIONS AND RENOVATIONS					
OTHER EXPENSES	1,000	1,000			
DIRECT CONSORTIUM/ CONTRACTUAL COSTS					
SUBTOTAL DIRECT COSTS *(Sum = Item 8a, Face Page)*	62,500	62,500			
F&A CONSORTIUM/ CONTRACTUAL COSTS					
TOTAL DIRECT COSTS	62,500	62,500			

TOTAL DIRECT COSTS FOR ENTIRE PROPOSED PROJECT PERIOD	**$** 125,000

JUSTIFICATION. Follow the budget justification instructions exactly. Use continuation pages as needed.

see attached budget justification document

Detailed Budget Justification

Personnel:

Jan-Hendrik Spille, Assistant Professor of Physics and Chemistry, UIC: 0.18 summer months (2% effort) x 2 years + fringe = **$5,476**. Dr. Spille will have overall responsibility for research conducted at UIC. Dr. Spille will oversee work conducted by a graduate assistant and a research technologist in his lab.

Jonah Galeota-Sprung, Graduate Assistant (9 academic months 50% effort, 3 summer months 80% effort x 2 years + fringe = **$69,949**). Mr. Galeota-Sprung is an expert in statistical analysis of super-resolution microscopy data and will be responsible for the acquisition, analysis, and interpretation of oligoPaint chromatin and protein super-resolution datasets (Aim 2).

Filmon Medhanie, Research Technologist ($20/h, 5h/week x 2 years = **$11,402**). Mr. Medhanie is the expert for any molecular biology and biochemical assays in the Spille Lab. He will assist with oligoPaint library maintenance, cell culture, and sample preparation.

Alexander Ruthenburg, Associate Professor, (2 academic months, no salary = **$0**). Dr. Ruthenburg will oversee the work conducted and provide general oversight, technical advice, analysis, assistance in writing papers. When needed, Dr. Ruthenburg will support Jelena's many experiments in the lab.

Jelena Scepanovic, Graduate Student, (10 academic months, 2 years + fringe = **$78,590**). Ms. Scepanovic is one of the key driving forces of this project, she helped conceive the project and established the collaboration with the Spille lab. Ms. Scepanovic will culture mESCs, generate and characterize the DNA minicircles, and perform and analyze *t*opoisomer *i*nternally-calibrated micro-*C (TI-C)* and ICeChIP experiments (Aim 1).

Supplies:

We will purchase an *oligoPaint library* tiling up to 100 individual chromatin elements in the HoxB locus with high sequence resolution (down to 2kb). We will include an additional channel covering the entire chromosome 11 to demarcate the chromosome territory. The library consists of a set of primary probes directly binding genomic DNA, each appended by functional overhangs for library amplification and binding of secondary probes. For a cost-efficient assay, we will use unlabeled secondary probes with a toehold displacement anchor and a common tertiary probe carrying the fluorescent label. The total cost for a renewable library and auxiliary probes is **$16,000**. The library needs to be maintained using in vitro amplification and purification kits. We budget a total of **$4,000** ($2,000/year) for library amplification and verification by NGS. *Protein level super-resolution microscopy* compatible with oligoPaint requires primary antibodies tagged with a DNA barcode and detected via binding of a fluorescently labeled readout oligonucleotide. Custom barcoded antibodies are available from Massive Photonics for $700 per target. We budget for 4 different protein targets (H3K27ac, H3K4me1, H3K4me3, S2p-PolII). Total cost: **$2,800**.
The expenses for *consumables in the Spille Lab* are about $600/month. This covers all tissue culture supplies, including serum, media, sterile plates and pipettes, as well as reagents for sample preparation including imaging buffers. We request $7,200 in year 1 and $7,173 in year 2 (=**$14,373**). **Spille Lab total: $37,173**.

In the Ruthenburg lab, we will generate DNA minicircles for TI-C calibration using two commercial kits (10 preps each, available from System Biosciences) whose total cost is **$3,620**. The DNA minicircle structure will be validated using atomic force microscopy (AFM). We budget **$380** for AFM training for Ms. Scepanovic, and **$150**/quarter ($600/year) for the user facility access fee for the UofC Materials Research Center which hosts AFM. This is a sequencing heavy grant, and we request **$14,175** for 3 SP flow cells (880 million read pairs each, $1,675/flow cell), allowing initial sequencing runs for library quality assessment, and 2 S2 Nova-Seq flow cells (4 billion read pairs each, $4,575/flow cell), which will enable high sequencing depth and the resulting fine scale ICe-C resolution. Finally, we request **$2,430** for expenses for *tissue culture consumables in the Ruthenburg Lab*. **Ruthenburg Lab total: $42,410** ($21,205/year).

We request **$3,000** for **publication cost in a high impact journal** and **$2,000** ($1,000/year) for Ms. Scepanovic to present results at a national conference. Additional funds needed for this will come from other sources. **Total budget requested for 2 year period = $250,000**.

BIOGRAPHICAL SKETCH

Provide the following information for the Senior/key personnel and other significant contributors.
Follow this format for each person. **DO NOT EXCEED FIVE PAGES.**

NAME: Spille, Jan-Hendrik

eRA COMMONS USER NAME (credential, e.g., agency login): JSPILLE

POSITION TITLE: Assistant Professor

EDUCATION/TRAINING *(Begin with baccalaureate or other initial professional education, such as nursing, include postdoctoral training and residency training if applicable. Add/delete rows as necessary.)*

INSTITUTION AND LOCATION	DEGREE *(if applicable)*	Completion Date MM/YYYY	FIELD OF STUDY
University of Wuerzburg, Germany	B. Sc.	11/2007	Physics
University of Wuerzburg, Germany	M. Sc.	09/2009	Physics
University of Bonn, Germany	Ph.D.	04/2014	Biophysical Chemistry
University of Bonn, Germany	Postdoc	03/2015	Biophysical Chemistry
Massachusetts Institute of Technology, Cambridge	Postdoc	05/2019	Biophysics

A. Personal Statement

The overall goal of my research program is to decipher mechanisms of self-organization of biomolecules and chromatin in the cell nucleus and how biological function arises from this process. As a postdoc, while attempting to directly observe transient assemblies of RNA Pol II [a] using light sheet microscopy, I discovered that some assemblies persisted for tens of minutes and merged upon contact. That observation provided first experimental evidence of phase-separated transcription condensates and opened up the field to experimentally probe these assemblies and their role in gene regulation [b]. The small size of transcription condensates and many other membraneless nuclear organelles prevents a rigorous characterization of condensate properties with established tools, leading to frequent use of qualitative descriptors such as “liquid-like” behavior of condensates, and missing morphological complexity. Another shortcoming in the condensate field is the lack of consideration for the ubiquitous underlying chromatin scaffold in the nucleus. As a physicist by training I am particularly interested in how this scaffold can regulate biomolecular condensate formation [c]. The biophysical mechanisms underlying this process are poorly understood. My strong background in developing novel quantitative fluorescence imaging assays with single molecule sensitivity puts me in a unique position to overcome these limitations. A better quantitative and conceptual understanding of condensate regulation is essential for integrating them into paradigms of biological function in transcription and beyond.
I started my own lab in the Physics Department at UIC in January 2020. The COVID-19 pandemic significantly impacted my efforts to set up the lab and hire personnel, but we have now reached the productive stage and produced exciting preliminary data. Specifically, we have implemented multi-color super-resolution assays to resolve previously unknown substructure in transcription condensates. As part of an NIH-supported (NIAID R21) project with Dr. Amy Kenter’s group (UIC Immunology) [d], we established immunoFISH protocols to study chromatin loci and condensate structure. A strong focus in the lab is now to enable concomitant 3D chromatin tracing and mapping protein content along chromatin topologies. I expect that this technological advancement will lead to paradigm-shifting revelations about chromatin structure-function relationships that we are beginning to see already with single cell techniques that resolve only one of the two modalities. This Catalyst application together with Dr. Ruthenburg presents an early use case of our efforts.
Through other active interdisciplinary collaborations, my group has access to world-class experts in genomics assays such as sequencing-based chromatin conformation capture technology (Dr. Kenter), chromatin

modeling (Dr. Jie Liang, UIC Bioengineering), and in vitro as well as computational methods to study condensates (Dr. Huan-Xiang Zhou, UIC Physics/Chemistry). Collaboration with their groups and knowledge transfer will complement our imaging-based single cell views.
Affiliations with the Chemistry and Biomedical Engineering Departments have allowed me to form a multi-disciplinary team of trainees with background in physics, biology, biochemistry, and bioinformatics. To foster interdisciplinary exchange, I initiated a bi-weekly Biophysics Seminar series with graduate student/postdoc and faculty presentations. Undergraduate research including Honors College Capstone Thesis projects is an important component of our work and students have received Honors College Research Awards (5x) and prestigious LASURI award distinctions (3x). I have won fellowship support for proposed projects, planned project timelines, directed research teams including students and outside collaborators, and prepared manuscripts as corresponding author. Our creative efforts to establish ground-breaking new assays were recently recognized by two Scialog (Chemical Machinery of the Cell) awards.

a. Cho, W.K., Jayanth, N., English, B., Inoue, T., Andrews, J., Conway, W., Grimm, J., Spille, J.-H., Lavis, L., Lionnet, T., Cisse, I. **RNA Polymerase II cluster dynamics predict mRNA output in living cells.** Elife 5, e13617 (2016). PMCID: PMC4929003
b. Cho, W.K.* & Spille, J.-H.*, Hecht, M., Lee, C., Li, C., Grube, V., Cisse, I. **Mediator and RNA Polymerase II clusters associate in transcription-dependent condensates.** Science 361 (6400), 412-415 (2018). *contributed equally PMCID: PMC6543815
c. Agbleke, A.A., Amitai, A., Buenrosto, J.D., Chakrabarti, A., Chu, L., Hansen, A.S., Koenig, K.M., Labade, A.S., Liu, S., Nozaki, T., Ovchinnikov, S., Seeber, A., Shaban, H.A., Spille, J.-H., Stephens, A.D., Su, J.-H., Wadduwage, D. **Advances in Chromatin and Chromosome Research: Perspectives from Multiple Fields.** Mol Cell. (2020). *All authors contributed equally. PMCID: PMC7888594
d. Watson, C.T., Kenter, A.L., Spille, J.-H. **Igh Locus Polymorphism May Dictate Topological Chromatin Conformation and V Gene Usage in the Ig Repertoire.** Front. Immunol. 12:682589 (2021). PMCID: PMC8167033

B. Positions, Scientific Appointments, and Honors

Positions and Employment

2019-2020 Visiting Research Assistant Professor, Department of Physics, UIC, Chicago, IL
2020- Assistant Professor, Department of Physics, UIC, Chicago, IL
2020- Assistant Professor, Department of Chemistry, UIC, Chicago, IL
2021- Affiliate Member, Center for Bioinformatics and Quantitative Biology, Department of Bioengineering, UIC, Chicago, IL

Other Experience and Professional Memberships

2011- German Society for Biophysics
2016- Biophysical Society
2021- American Physical Society

Honors

2005-2009 Undergraduate Study Scholarship, German Academic Scholarship Foundation (Studienstiftung)
2010-2012 PhD Fellowship, German Academic Scholarship Foundation (Studienstiftung)
2012,2013 Travel Award, German Academic Exchange Service (DAAD)
2014 Edmund-ter-Meer Dissertation Award, University Society Bonn
2017-2019 Postdoctoral Fellowship, German National Academic Foundation (DFG)
2020-2021 Scialog Fellow: Chemical Machinery of the Cell (RCSA/Moore Foundation)
2021 Scialog (Chemical Machinery of the Cell) Research Award (2x)

C. Contributions to Science

1. 3D single molecule localization. I developed and built a light sheet microscope for 3D single molecule localization with the ability to automatically focus on a freely diffusing mRNA particle (mRNP) in the cell nucleus.[a] Diffusing particles would typically move out of the focal plane of a microscope within fractions of a

second. The automated tracking capability with rapid and precise 3D particle localization enabled single molecule observations for more than 10 seconds, increasing the observation time of individual particles by two orders of magnitude. Careful analysis allowed for direct observation of transient changes in mRNP mobility corresponding to interactions with nuclear structures, and characterization of target search modes for mRNA trafficking.[b] Approaches used for these assays will be of high importance for this proposal, e.g. mRNPs were tagged with fluorescently labeled oligonucleotides similar to those we will use here for multiplexed chromatin tracing and molecules were localized in 3D with nanometric precision. Building a light sheet microscope was essential for enabling single molecule localization and long-term tracking with minimal photobleaching and phototoxicity.

a. Spille, J.-H., Kaminski, T., Koenigshoven, H.P., Kubitscheck, U. **Dynamic three-dimensional tracking of single fluorescent nanoparticles deep inside living tissue.** Optics Express, 20 (18), 19697-19707 (2012). DOI: 10.1364/OE.20.019697
b. Spille, J.-H., Kaminski, T., Scherer, K., Rinne, J., Heckel, A., Kubithscheck, U. **Direct observation of mobility state transitions in RNA trajectories by sensitive single molecule feedback tracking.** Nucleic Acids Research, 43 (2), e14 (2015). PMCID: PMC4333372

2. Quantitative single molecule imaging. Quantitative analysis of singe molecule and super-resolution microscopy data is complicated by the need to account for emitter properties. Through careful characterization of those properties it becomes possible to estimate actual protein copy numbers in subdiffraction-sized assemblies detectable by super-resolution imaging. I initially used this approach to investigate the pore-forming activity of the lantibiotic Nisin in model membranes and bacteria.[a] A more sophisticated analysis of the photo-switchable fluorescent protein Dendra2 commonly used in live cell super-resolution imaging allowed me to estimate the number of RNA Pol II copies in clusters at an induced gene locus.[b] The analysis methods were included in an open source data analysis platform (qSR) for enabling quantitative super-resolution analysis by a wider user base.[c] We used this technology to extract quantitative thermodynamic properties of misfolded protein aggregates associated with neurodegenerative disease.[d] These works document the technical foundation for performing single molecule localization microscopy in the cell nucleus and my dedication to developing methods to extract quantitative spatial and temporal information with resolution well below the diffraction limit from biological systems.

a. Scherer, K., Spille, J.-H., Sahl, H.-G., Grein, F., Kubitscheck, U. **The lantibiotic Nisin induces Lipid II aggregation, causing membrane instability and vesicle budding.** Biophys J 108, 1114-1124 (2015). PMCID: PMC4375720
b. Cho, W.K., Jayanth, N., English, B., Inoue, T., Andrews, J., Conway, W., Grimm, J., Spille, J.-H., Lavis, L., Lionnet, T., Cisse, I. **RNA Polymerase II cluster dynamics predict mRNA output in living cells.** Elife 5, e13617 (2016). PMCID: PMC4929003
c. Andrews, J., Conway, W., Cho, W.K., Narayanan, A., Spille, J.-H., Jayanth, N., Inoue, T., Mullen, S., Thaler, J., Cisse, I. **qSR: a quantitative super-resolution analysis tool reveals the cell-cycle dependent organization of RNA Polymerase I in live human cells.** Scientific Reports, 8 (1), 1-10 (2018). PMCID: PMC5943247
d. Narayanan, A., Meriin, A., Andrews, J., Spille, J.-H., Sherman, M., Cisse, I. **A first order phase transition underlies protein aggregation in mammalian cells.** Elife 8, e39695 (2019). PMCID: PMC6361590

3. These earlier works paved the way for a quantitative analysis of higher order protein assemblies in the cell nucleus. I found that numerous factors of the transcription regulation machinery (e.g. Mediator, Pol II, BRD4) are concentrated in biomolecular condensates, providing experimental evidence for a new paradigm of transcription organization.[a] These transcription condensates are stable for tens of minutes, yet most constituents turn over on the timescale of seconds. Biochemical evidence indicates that condensates form at chromatin structures containing clustered enhancer elements (“super enhancers”) that control transcription of master transcription factor genes. In particular, the enhancer-associated chromatin mark H3K27ac appears to play a pivotal role in condensate formation, and inhibition of BRD4 binding to acetylated histones using the small molecule BET inhibitor JQ1 dissolved condensates entirely. We

showed that transcription condensate composition is controlled in part by post-translational modifications[b] and that upon stimulation, signaling factors get recruited rapidly to condensates.[c] To quantify functional output of transcription at arbitrary gene loci in live cells I developed a genome editing approach to reliably insert a labeling cassette for nascent RNA.[a] Transcription condensates nucleated by super enhancers present a new paradigm in our understanding of transcription regulation, with important implications for the role of chromatin organization in driving transcription, enhancer-promoter communication, and response mechanisms to external signaling cues. I co-authored a review article on novel techniques for studying chromatin organization in single cells and am currently co-editing a forthcoming Methods in Molecular Biology volume detailing experimental, theoretical, and computational approaches to the study of Phase-Separated Biomolecular Condensates. My group contributed a chapter on single molecule and super-resolution imaging approaches in this context[d]. Closely related to this proposal, I will chair two sessions during the March Meeting of the American Physical Society on “Biomolecular Condensates” and “Genome Organization and Subnuclear Phenomena”.
The combination of these previous works lays the foundation for in depth studies of functional structures of chromatin scaffolds and biomolecular condensates in biological processes.

a. Cho, W.K.* & Spille, J.-H.*, Hecht, M., Lee, C., Li, C., Grube, V., Cisse, I. **Mediator and RNA Polymerase II clusters associate in transcription-dependent condensates.** Science 361 (6400), 412-415 (2018). *contributed equally PMCID: PMC6543815
b. Guo, E., Manteiga, J., Henninger, J., Sabari, B., Dall’Agnese, A., Hannett, N., Spille, J.-H., Afeyan, L., Zamudio, A., Shrinivas, K., Abraham, B., Boija, A., Decker, T.-M., Rimel, J., Fant, C., Lee, T.I., Cisse, I., Sharp, P.A., Taatjes, D.J., Young, R.A. **Pol II phosphorylation regulates a switch between transcriptional and splicing condensates.** Nature 572 (7770), 543-8 (2019). PMCID: PMC6706314
c. Zamudio, A., Dall’Agnese, A., Henninger, J., Manteiga, J., Afeyan, L., Hannett, N., Coffey, E., Li, C., Ozgur, O., Sabari, B., Boija, A., Klein, I., Hawken, S., Spille, J.-H., Decker, T.-M., Cisse, I., Abraham, B., Lee, T.I., Taatjes, D.J., Schuijers, J., Young, R.A. **Mediator condensates localize signaling factors to key cell identity genes.** Molecular Cell 76 (5), 753-766 (2019). PMCID: PMC6898777
d. Pandey, G, Budhathoki, A., Spille, J.-H. **Characterizing properties of biomolecular condensates below the diffraction limit in vivo.** In: Methods in Mol. Bio.: Phase-Separated Biomolecular Condensates. (2022) PMID: 36227487.

A complete list of publications is available at:
https://www.ncbi.nlm.nih.gov/myncbi/jan-hendrik.spille.1/bibliography/public/

PHS OTHER SUPPORT
For All Application Types – DO NOT SUBMIT UNLESS REQUESTED

There is no "form page" for reporting Other Support. Information on Other Support should be provided in the format shown below.

*Name of Individual: Jan-Hendrik Spille
Commons ID: JSPILLE

Other Support – Project/Proposal

*Title: Ig locus function in immunosenescent mice

*Major Goals: The goal of this study is to investigate if altered lineage specific transcription factor expression profiles lead to chromatin topological changes in aged vs young mice, and if those changes underlie diminished immune-repertoire formation and immuno-senescence.

*Status of Support: active

Project Number: 1R21AI159626-01A1

Name of PD/PI: Kenter

*Source of Support: NIH NIAID

*Primary Place of Performance: University of Illinois at Chicago

Project/Proposal Start and End Date: (MM/YYYY) (if available): 07/2021 – 06/2023

* Total Award Amount (including Indirect Costs): $429,041

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2021	1.00 (summer)
2. 2022	1.00 (summer)
3. 2023	0.00

*Title: The Butterfly Effect in Cellular Phase Separation: from Molecular Interactions to Emergent Behavior

*Major Goals: The goal of this collaborative grant is to investigate the effect of single amino acid mutations in intrinsically disordered protein domains on phase separation propensity at the cellular level.

*Status of Support: active

Project Number: SCIALOG: CMC #28407

Name of PD/PI: Spille

*Source of Support: Research Foundation for the Advancement of Science

*Primary Place of Performance: University of Illinois at Chicago

Project/Proposal Start and End Date: (MM/YYYY) (if available): 01/2022 – 12/2022

* Total Award Amount (including Indirect Costs): $55,000

Name of Individual:
Commons ID:

* Person Months (Calendar/Academic/Summer) per budget period.

None/not allowed by sponsor.

*Title: Visualizing Inheritance through the Lens of Phase Separation

*Major Goals: The goal of this collaborative grant is to identify proteins that gain dominant function through phase separation in human disease and map orthogonal condensate structures.

*Status of Support: active

Project Number: SCIALOG: CMC #28419

Name of PD/PI: Spille

*Source of Support: Research Foundation for the Advancement of Science

*Primary Place of Performance: University of Illinois at Chicago

Project/Proposal Start and End Date: (MM/YYYY) (if available): 01/2022 – 12/2022

* Total Award Amount (including Indirect Costs): $55,000

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2022	0.50 (summer)

*Title: Mapping protein signatures to single allele chromatin topologies at genomic resolution

*Major Goals: We propose to develop a novel super-resolution microscopy assay that combines chromatin tracing and protein mapping to enable characterization of combinatorial protein signatures at chromatin elements with genomic to reveal drivers of cell-to-cell variation in chromatin topology.

*Status of Support: pending

Project Number:

Name of PD/PI: Spille

*Source of Support: NIH NIGMS

*Primary Place of Performance: University of Illinois at Chicago

Project/Proposal Start and End Date: (MM/YYYY) (if available): 04/2023 – 03/2025

* Total Award Amount (including Indirect Costs): $422,354

* Person Months (Calendar/Academic/Summer) per budget period.

None/not allowed by sponsor.

Name of Individual:
Commons ID:

*Title: Enhancer-enhancer interactome and Igh locus function

*Major Goals: We propose to characterize the chromosomal infrastructure that spatially orients V gene segments and makes them available for use during VDJ recombination in immune cell biogenesis. We hypothesize that a network of enhancer interactions orchestrates locus topology. Our studies address basic questions of how antibody genes are recruited for recombination during immune responses.

*Status of Support: pending

Project Number:

Name of PD/PI: Kenter

*Source of Support: NIH NIAID

*Primary Place of Performance: University of Illinois at Chicago

Project/Proposal Start and End Date: (MM/YYYY) (if available): 07/2023 – 06/2028

* Total Award Amount (including Indirect Costs): $3,720,877

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2023	1.00 (summer)
2. 2024	1.00 (summer)
3. 2025	1.00 (summer)
4. 2026	1.00 (summer)
5. 2027	1.00 (summer)

*Title: Mapping protein signatures to single allele chromatin topologies at genomic resolution

*Major Goals: We propose to develop a novel super-resolution microscopy assay that combines chromatin tracing and protein mapping to enable characterization of combinatorial protein signatures at chromatin elements with genomic to reveal drivers of cell-to-cell variation in chromatin topology.

*Status of Support: pending

Project Number:

Name of PD/PI: Spille

*Source of Support: NIH NCI 1 R21 CA282715-01

*Primary Place of Performance: University of Illinois at Chicago

Project/Proposal Start and End Date: (MM/YYYY) (if available): 04/2023 – 03/2025

* Total Award Amount (including Indirect Costs): $422,534

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2023	1.00 (summer)
2. 2024	1.00 (summer)

Name of Individual:
Commons ID:

*Title: Structural and functional determinants of biomolecular condensates in transcription organization

*Major Goals: We propose to investigate if transcription condensates possess an internal structure related to their function, in particular if transcription activity is localized to the condensate surface where we find an enrichment of active chromatin. We further propose to use multiplexed chromatin and protein imaging to determine if condensate formation is facilitated by specific chromatin topologies. Finally, we will use single particle tracking to elucidate how individual proteins move through the condensate face and find cognate binding sites.

*Status of Support: pending

Project Number:

Name of PD/PI: Spille

*Source of Support: NIH NIGMS 1 R35 GM150560-01

*Primary Place of Performance: University of Illinois at Chicago

Project/Proposal Start and End Date: (MM/YYYY) (if available): 07/2023 – 06/2027

* Total Award Amount (including Indirect Costs): $1,893,276

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2023	1.50 (summer)
2. 2024	1.50 (summer)
3. 2025	1.50 (summer)
4. 2026	1.50 (summer)
5. 2027	1.50 (summer)

*Title: Collaborative Research: Tolls 4 Cells: Developing Next Generation Methods for Studying Cytoskeletal Factors in the Cell Nucleus

*Major Goals: To develop novel tools for rapid and specific perturbation of nuclear actin, and employ these tools to investigate the role of nuclear actin in orchestrating fine-scale chromatin architecture and RNA Pol II spatial organization.

*Status of Support: pending

Project Number:

Name of PD/PI: Spille

*Source of Support: NSF

*Primary Place of Performance: University of Illinois at Chicago

Project/Proposal Start and End Date: (MM/YYYY) (if available): 04/2023 – 03/2026

* Total Award Amount (including Indirect Costs): $630,937

* Person Months (Calendar/Academic/Summer) per budget period.

Name of Individual:
Commons ID:

Year (YYYY)	Person Months (##.##)
1. 2023	1.00 (summer)
2. 2024	1.00 (summer)
3. 2025	1.00 (summer)

*Title: Causality in epigenetic imprinting and chromatin remodeling during developmental gene activation

*Major Goals: To decipher the causal sequence of events in HoxB decondensation, translocation, and epigenetic imprinting during gene activation using newly developed internally calibrated micro-C and ChIP along with chromatin and protein multiplexed super-resolution microscopy.

*Status of Support: pending (THIS APPLICATION)

Project Number:

Name of PD/PI: Spille/Ruthenburg

*Source of Support: Chicago Biomedical Consortium Catalyst Award

*Primary Place of Performance: University of Illinois at Chicago

Project/Proposal Start and End Date: (MM/YYYY) (if available): 06/2023 – 05/2025

* Total Award Amount (including Indirect Costs): $125,000

* Person Months (Calendar/Academic/Summer) per budget period.

Year (YYYY)	Person Months (##.##)
1. 2023	0.18 (summer)
2. 2024	0.18 (summer)

Name of Individual:
Commons ID:

IN-KIND

*Summary of In-Kind Contribution:

*Status of Support:

*Primary Place of Performance:

Project/Proposal Start and End Date (MM/YYYY) (if available):

*Person Months (Calendar/Academic/Summer) per budget period

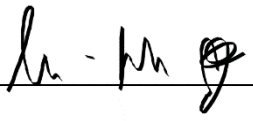
Year (YYYY)	Person Months (##.##)
1. [enter year 1]	
2. [enter year 2]	
3. [enter year 3]	
4. [enter year 4]	
5. [enter year 5]	

*Estimated Dollar Value of In-Kind Information:

***Overlap** (summarized for each individual):

There is no overlap of this proposal with the active or pending projects and activities, other positions, affiliations, and resources and this application in terms of the science, budget, or committed effort.

I, PD/PI or other senior/key personnel, certify that the statements herein are true, complete and accurate to the best of my knowledge, and accept the obligation to comply with Public Health Services terms and conditions if a grant is awarded as a result of this application. I am aware that any false, fictitious, or fraudulent statements or claims may subject me to criminal, civil, or administrative penalties.

*Signature: 

Date: 11/18/2022

BIOGRAPHICAL SKETCH

NAME: Ruthenburg, Alexander J., PhD.

eRA COMMONS USER NAME: RUTHENBURG

POSITION TITLE: Associate Professor

EDUCATION/TRAINING

INSTITUTION AND LOCATION	DEGREE	Completion Date MM/YYYY	FIELD OF STUDY
Carleton College, Northfield, MN	BA	06/1999	Chemistry
Harvard University, Cambridge, MA	Ph.D.	09/2005	Chemistry and Structural Biology
Rockefeller University, New York City, NY	Postdoctoral Fellow	07/2010	Chromatin Biology

A. Personal Statement

My research program spans a host of traditional disciplines (discovery biochemistry, chemical biology, biophysics, technology development, quantitative genomics and cell biology) with the goal of developing fundamental mechanistic understanding of epigenetic information systems through three main avenues. 1.) Accurate measurement of the individual chromatin marks, patterns and structures is a crucial prerequisite to deciphering how epigenetic systems work. To address this challenge, we developed internally calibrated chromatin immunoprecipitation (ICeChIP), which uses barcoded semisynthetic nucleosomal calibrants to enable absolute quantification of histone marks and variants and access finer scale chromatin features in cells, revealing many new insights and overturning existing dogma. 2.) A second major effort in the lab is the biochemical discovery of new epigenetic pathways that regulate gene expression, centered on orphaned histone marks and DNA-modifications that have resisted elucidation. 3.) My lab has discovered a new class of noncoding RNA molecules, which display potent enhancer activity and are defined by tight attachment at their site of transcription.

I am intensely committed to direct in-lab mentoring and the holistic development of my trainees into independent researcher-scholars. The 13 graduate students I have mentored at the University of Chicago have derived from five different programs: ranging from Cell and Molecular Biology to Chemistry. Each of my graduate trainees has published at least one first author paper, many have garnered fellowships and awards including NSF and AHA predoctoral fellowships. In terms of postgraduate outcomes, all of my students went to their first choice of labs for postdoctoral research; my first graduate student recently started a tenure track position at the University of Utah; two are scientists in small biotech companies, and the remaining are still postdoctoral researchers, one of whom has been awarded ACS/NRSA postdoctoral fellowships. I am deeply invested in broadening diversity in the next generations of scientists. In my own lab, my and 12 University of Chicago undergraduates, 13 graduate trainees and 4 postdoctoral fellows have included 13 women, 6 students from underrepresented backgrounds in the sciences, 10 immigrants, and 4 LGBTQ+ students.

At the institutional level, I am a leader in graduate education and graduate-student centered service. In my over ten years at the University of Chicago I have served on 25 doctoral preliminary exams and 44 graduate thesis committees, and serve on the DEI-ship Committee for the Biological Sciences. I have been on the Steering Committee for the GRTG NIGMS T32, and co-authored and co-direct our recently awarded Genetic Mechanisms and Evolution NIGMS T32 grant. I have been active in admissions and recruitment of graduate students in the programs that I am affiliated with (Cell and Molecular Biology [CMB] Program) and have served on 6 faculty search committees for three different departments.

In terms of recent extramural service, I have been an ad hoc reviewer for NIH R01/R21 (MGA, MGB), SBIR (NHGRI) and P01/U19 (NIAID) grants, served for several years as a permanent member of the American Cancer Society DMC study section (2x per year), am a permanent member of MCB→ MG study section at the NIH, and frequently review manuscripts.

1. Grzybowski AT, Chen Z, **Ruthenburg AJ**. Calibrating ChIP-Seq with Nucleosomal Internal Standards to Measure Histone Modification Density Genome Wide. *Mol Cell*. 2015 Jun 4;58(5):886-99. PubMed PubMed Central PMCID: PMC4458216.
2. Shah RS, Grzybowski AT, Cornett EM, Johnstone EM, Dickson BM, Boone BM, Cheek MA, Cowles MW, Maryanski D, Meiners MJ, Tiedemann RL, Vaughn RM, Arora N, Sun Z-W, Rothbart SB, Rothbart SB, Keogh M-C, **Ruthenburg AJ**. *Mol Cell*. 2018 Oct 4;72(1):162-177. PMC6173622. (Funded by R01 GM115945 to AJR).
3. Richter WF, Shah RS, **Ruthenburg AJ**. Non-canonical H3K79me2-dependent pathways promote the survival of MLL-rearranged leukemia. eLife. 2021 Jul 15;10:e64960. PMC8315800 (Funded by R01 GM115945 to AJR).
4. Grzybowski AT, Shah RS, Richter WF, **Ruthenburg AJ**. Native Internally Calibrated Chromatin Immunoprecipitation for Quantitative Studies of Histone Post-Translational Modifications. *Nat Protoc*. 2019 Dec;14(12):3275-3302. PMC7158589. (Funded by R01 GM115945 to AJR).

B. Positions, Scientific Appointments and Honors

Positions and Scientific Appointments

2021 –	Co-Director NIGMS T32 training grant (Genetic Mechanisms and Evolution)
2020 –	Permanent member of MGB/MG study section at the NIH
2017 – 2021	Reviewer for American Cancer Society (DMC study section, 2x per year)
2014 –	Ad hoc reviewer for NIH R01/R21 (MGA, MGB), SBIR (NHGRI) and P01/U19 (NIAID) grants
2011 – 2016	Reviewer for Catalyst grants, Chicago Biomedical Consortium
2017 -	Associate Professor, University of Chicago, Departments of Molecular Genetics and Cell Biology, Biochemistry and Molecular Biology
2011 – 2017	Assistant Professor, University of Chicago, Department of Biochemistry and Molecular Biology
2010 – 2017	Assistant Professor, University of Chicago, Department of Molecular Genetics and Cell Biology

Honors

2017	Research Scholar, American Cancer Society
2013	New Scholar in Aging, Ellison Medical Foundation
2011	Kavli Fellow, National Academy of Sciences
2010	Neubauer Family Foundation Fellow, University of Chicago
2010	Junior Investigator, Chicago Biomedical Consortium
2007	Irvington Institute Postdoctoral Fellow, Cancer Research Institute
2000	Graduate Research Fellow, National Science Foundation
1999	Distinction in Thesis/Major, magna cum laude, and Franz Exner Award, Carleton College

C. Contributions to Science

1. Two sources of histone mark data have led to a “Heisenberg Uncertainty Principle of Epigenetics”—one can measure either global absolute levels of a histone modification by MS without any information about where in the genome the mark resides; or, one can measure relative (and potentially problematic) amounts of a histone modification genome wide by ChIP-seq, without any information about how much, in an absolute sense, of a given mark exists at any arbitrary location. Yet knowing both of these parameters simultaneously is critical as histone modifications are thought to act locally in a density-dependent manner. With a method that my lab has developed, we can now measure both where and how much of a modification exists on a biologically meaningful scale genome wide with bp precision. Specifically, we have markedly improved Chromatin Immunoprecipitation (ChIP), the central technique in the field of chromatin biology, by using semisynthetic nucleosomes as internal standards so that: 1.) data is expressed on an accurate, reproducible and biologically meaningful scale of histone modification density, rather than an arbitrary and experimental condition-biased relative scale; 2.) this calibrated scale, when applied from experiment-to-experiment

enables legitimate and unbiased quantitative comparisons between experiments for the first time; 3.) Our method may be applied to assess within a given ChIP experiment how specific the enrichment step is, serving as a crucial *in situ* metric of whether the experiment actually worked and the strength of the conclusions that may be drawn from it. ICeChIP has revealed new quantitative relationships between the marks of enhancers and the transcriptional output of their target promoters, overturning longstanding dogma in the field that was established by less accurate methods. We have also used this method to quantitatively evaluate a number of existing histone modification paradigms based on conventional ChIP and found many to be a consequence of artifact. For example, our work has called into question every aspect of a very popular model of bivalency, the idea that paradoxical combinations of marks decorate developmentally poised genes—none of the model's predictions survive quantitative scrutiny. Although there are many highly-specific reagents available, to our horror we have found many of the most widely used antibodies lack sufficient ChIP specificity to draw robust conclusions, as prior antibody quality metrics are surprisingly uncorrelated to ChIP performance. Thus, our method also provides the only available approach to selecting antibodies that are specific in ChIP experiments.

a. Grzybowski AT, Chen Z, **Ruthenburg AJ**. Calibrating ChIP-Seq with Nucleosomal Internal Standards to Measure Histone Modification Density Genomewide. *Mol Cell*. 2015 Jun 4;58: 886-99. PMC4458216.

b. Shah RS, Grzybowski AT, Cornett EM, Johnstone EM, Dickson BM, Boone BM, Cheek MA, Cowles MW, Maryanski D, Meiners MJ, Tiedemann RL, Vaughn RM, Arora N, Sun Z-W, Rothbart SB, Rothbart SB, Keogh M-C, **Ruthenburg AJ.** *Mol Cell*. 2018 Oct 4;72(1):162-177. PMC6173622.

c. Grzybowski AT, Shah RS, Richter WF, **Ruthenburg AJ**. Native Internally Calibrated Chromatin Immunoprecipitation for Quantitative Studies of Histone Post-Translational Modifications. *Nat Protoc*. 2019 Dec;14(12):3275-3302. PMC7158589.

d. Shah RN, Grzybowski AT, Elias J, Chen Z, Hattori T, Lechner CC, Lewis PW, Koide S, Fierz B, **Ruthenburg AJ**. Re-evaluating the role of nucleosomal bivalency in early development. *BioRxiv* 2021.09.09.458948 [**Preprint**]. Sep 10, 2021.Available from: https://doi.org/10.1101/2021.09.09.458948

2. As a means to curate potential chromatin-based functions for a large set of long noncoding RNA (lncRNA), we performed stringent nuclear fractionation coupled to RNA sequencing. To our surprise, this approach also revealed several thousand novel *ch*romatin-*e*nriched RNAs (cheRNAs). Although chromatin-tethered by projection from paused or stalled polymerase II, cheRNAs resemble canonical lncRNA in several respects (and encompass several hundred annotated lncRNAs), but are clearly distinct molecularly, if not functionally from the recently described class of bi-directional enhancer RNA (eRNA). Remarkably, the proximity of cheRNAs is more highly correlated with neighboring gene expression than the presence of any other active enhancer annotation (chromatin signatures, eRNA production, etc.). We have studied a number of individual cheRNA-gene pairs in the context of human cancer lines and mouse cardiomyocytes and found a number of cases where it can be inferred that the cheRNA molecule itself plays a causal role in neighboring gene activation and local chromosome architecture. So far, we have defined this transcriptional activation by locally tethered cheRNAs as essential for the expression of several key calcium conductance genes and a chemokine in cardiomyocytes. To address an issue with existing toolkit to study RNA in the nucleus, we are developing new technology to specifically target RNA for destruction without concomitant transcriptional elongation effects and used these catalytic nucleic acid enzymes to demonstrate one of these cardiac cheRNA acts at the RNA level by tethering its promoter to the neighboring *Cxcl1* chemokine gene.

a. Werner MS, **Ruthenburg AJ**. Nuclear Fractionation Reveals Thousands of Chromatin-Tethered Noncoding RNAs Adjacent to Active Genes. *Cell Rep*. 2015 Aug 5; 12(7), 1089–1098. PMC5697714

b. Werner MS, Sullivan MA, Shah RN, Nadadur RD, Grzybowski AT, Galat V, Moskowitz IP, **Ruthenburg AJ.** Chromatin-enriched lncRNAs can act as cell-type specific activators of proximal gene transcription. *Nat. Struct. Mol. Bio.* 2017 Jul; 24(7):596-603. PMC5682930

c. Yang XH, Nadadur RD, Hilvering CR, Bianchi V, Werner M, Mazurek SR, Gadek M, Shen KM, Goldman JA, Tyan L, Bekeny J, Hall JM, Lee N, Perez-Cervantes C, Burnicka-Turek O, Poss KD, Weber CR, de

Laat W, **Ruthenburg AJ,** Moskowitz IP. Transcription-factor-dependent enhancer transcription defines a gene regulatory network for cardiac rhythm. *eLife*. 2017 Dec 27;6. pii: e31683. PMC5745077

d. Sun X, Wang Z, Hall JM, Perez-Cervantes C, **Ruthenburg AJ,** Moskowitz IP, Gribskov M, Yang XH. Chromatin-enriched RNAs mark active and repressive cis-regulation: An analysis of nuclear RNA-seq. *PLoS Comput Biol*. 2020 Feb;16(2):e1007119. PMC7034927

3. The key breakthrough and the centerpiece in deciphering the function of any epigenetic mark is the discovery of specific binding partners, yet many clearly important marks remain orphaned. My lab is in a unique position to make impact in this arena, as we have combined expertise in making semisynthetic nucleosomes, chromatin fractionation biochemistry, quantitative mass spectrometry and biophysical affinity measurements as well as a track record of functional genomics and cell line genetic engineering for critical follow-on functional studies. We have constructed a number of modified histone by scar-free chemical ligation for use in reconstituted nucleosome studies, including the only reported semisynthesis of the H3K79me1/2/3 methylation series, an important internal mark for our future studies. Indeed, we have discovered distinct pathways by which this mark functions to promote transcription and mediate alternative splicing. We have also become skilled in the dying art of fractionation biochemistry, and deployed it to discover proteins that bind internal nucleosomal marks, as well as newly appreciated oxidative adducts of 5-methylC in the context of duplex DNA. We have discovered that an hmC-specific binding protein, WDR76, acts as a transcriptional activator in mESCs, and demonstrated that the hmC-DNA binding interface is required for the development of MLL- rearranged leukemias.

   a. Richter WF, Shah RS, **Ruthenburg AJ**. Non-canonical H3K79me2-dependent pathways promote the survival of MLL-rearranged leukemia. *eLife*. 2021 Jul 15;10:e64960. PMC8315800

   a. Chen Z, Grzybowski AT**, Ruthenburg AJ.** Traceless semisynthesis of a set of histone 3 species bearing specific lysine methylation marks. *Chembiochem*. 2014 Sep 22;15(14): 2071-5. PMC4415702.

   b. Chen Z, Notti RQ, Ueberheide B, **Ruthenburg AJ**. Quantitative and Structural Assessment of Histone Methyllysine Analogue Engagement by Cog-nate Binding Proteins Reveals Affinity Decrements Relative to Those of Native Counterparts. *Biochemistry*. 2018 Jan 23;57(3):300-304. PMC5780203

   c. Malecek KE, Weng H, Sullivan MA, Kokontis CY, Werner MA, Chen J, **Ruthenburg AJ**. WDR76 promotes MLL-rearranged leukemia via selective recognition of 5-hydroxymethylcytosine in DNA. *BioRxiv* 511394 [**Preprint**]. January 3, 2019 Available from: https://doi.org/10.1101/511394.

4. My postdoctoral work in the Allis lab critically examined a central idea of the "histone code" hypothesis — that combinations of posttranslational modifications installed on histone proteins can locally collude in a functionally meaningful way. We developed the idea that the *multivalent nature* of cooperative-binding events explains the association energetics of chromatin binding complexes and contribute to composite specificity from a series of individually weak and modestly specific interactions in several influential reviews, then provided several concrete examples. Our work was the first to carefully examine multivalent binding properties integrating histone mark information at the nucleosome level in the form of rigorous biochemical, biophysical and genomic analysis —in effect providing proof of concepts for a three-dimensional histone code (beyond that of combinations of marks within a single tail).

   a. **Ruthenburg AJ**, Allis CD, Wysocka J. Methylation of lysine 4 on histone H3: intricacy of writing and reading a single epigenetic mark. *Mol Cell*. 2007 Jan 12;25(1):15-30. PMID: 17218268.

   b. **Ruthenburg AJ,** Li H, Patel DJ, Allis CD. Multivalent engagement of chromatin modifications by linked binding modules. *Nat Rev Mol Cell Biol.* 2007 Dec;8(12):983-94. PMID: 18037899.

   c. **Ruthenburg AJ**, Li H, Milne TA, Dewell S, McGinty RK, Yuen M, Ueberheide B, Dou Y, Muir TW, Patel DJ, Allis CD. Recognition of a mononucleosomal histone modification pattern by BPTF via multivalent interactions. *Cell*. 2011 May 27;145(5):692-706. PMC3135172

d. Milne TA, Kim J, Wang GG, Stadler SC, Basrur V, Whitcomb SJ, Wang Z, **Ruthenburg AJ**, Elenitoba-Johnson KS, Roeder RG, Allis CD. Multiple interactions recruit MLL1 and MLL1 fusion proteins to the HOXA9 locus in leukemogenesis. *Mol Cell*. 2010 Jun 25;38(6):853-63. PMC2902588

Complete List of Published Work in My Bibliography:
https://www.ncbi.nlm.nih.gov/myncbi/alexander.ruthenburg.1/bibliography/public/

OTHER SUPPORT

Alexander Ruthenburg
Commons ID: RUTHENBURG

ACTIVE

Title: Gene Regulatory Non-Coding RNAs in the Human Heart
Major Goals: The aims are to identify a novel class of lncRNAs in human cardiomyocytes and to subject these ncRNAs to functional and mechanistic studies to unveil their mode of action.
Status of Support: Active
Project Number: R01HL148719
PI: Multiple Principal Investigator - (Contact: Moskowitz)
Source of Support: NIH/NHLBI
Primary Place of Performance: University of Chicago
Start and End Date: 08/2019 – 07/2023
Total Award Amount (including Indirect Costs): $939,640 Total Award Amount to my Lab
Person Months per budget period: 1.80 calendar months

Year	Person Months
1. 2019	1.80
2. 2020	1.80
3. 2021	1.80
4. 2022	1.80

Overlap: None

Title: Genetic Mechanisms and Evolution
Major Goals: Students will receive rigorous pre-doctoral research training from world-leading scientists in quantitative, computational, and experimental approaches to answer fundamental questions in molecular, statistical, and evolutionary genetics.
Status of Support: Active
Project Number: 1T32GM139782-01
PI: Multiple Principal Investigator – (Contact PI: Joseph Thornton)
Source of Support: NIH/NIGMS
Primary Place of Performance: University of Chicago
Start and End Date: 07/2021 – 06/2026
Total Award Amount (including Indirect Costs): $9,765,385 Total Award – Effort only
Person Months per budget period: 1.80 calendar months

Year	Person Months
1. 2021	1.80
2. 2022	1.80
3. 2023	1.80
4. 2024	1.80
5. 2025	1.80

Overlap: None

Title: Defining the mechanisms of epigenetic information flow
Major Goals: The three main avenues of study: advances in quantitative interrogation of chromatin states drive new biologic insight, cheRNAs: a new class of long noncoding RNAs that act to promote neighboring gene transcription and biochemical discovery of epigenetic pathways.

Status of Support: Active
Project Number: 1R35GM145373
PI: Alex Ruthenburg
Source of Support: NIH/NIGMS
Primary Place of Performance: University of Chicago
Start and End Date: 08/2022 – 07/2027
Total Award Amount (including Indirect Costs): $2,398,015
Person Months per budget period: 6.12 calendar months

Year	Person Months
1. 2022	6.12
2. 2023	6.12
3. 2024	6.12
4. 2025	6.12
5. 2026	6.12

Overlap: None

Title: Developing Next Generation Methods for Studying Cytoskeletal Factors in the Cell Nucleus
Major Goals: This grant is centered on generating two new technologies for perturbing actin in the nucleus to study the chromosomal architectural consequences of actin filament formation. Although it is a collaborative project between the Spille and Ruthenburg labs, there is no overlap with this grant in any of the scientific objectives or methods used for readout.
Status of Support: Pending
Project Number: 2306187
PI: Multiple Principal Investigator (Spille, Jan-Hendrik, and Alex Ruthenburg)
Source of Support: NSF Tools 4 Cells
Primary Place of Performance: University of Chicago
Start and End Date: 04/01/2023- 03/31/26
Total Award Amount (including Indirect Costs): $630,939
Person Months per budget period: 1.6 calendar months

Year	Person Months
1. 2022	1.6
2. 2023	1.6
3. 2024	1.6
4. 2025	1.6
5. 2026	1.6

Overlap: None