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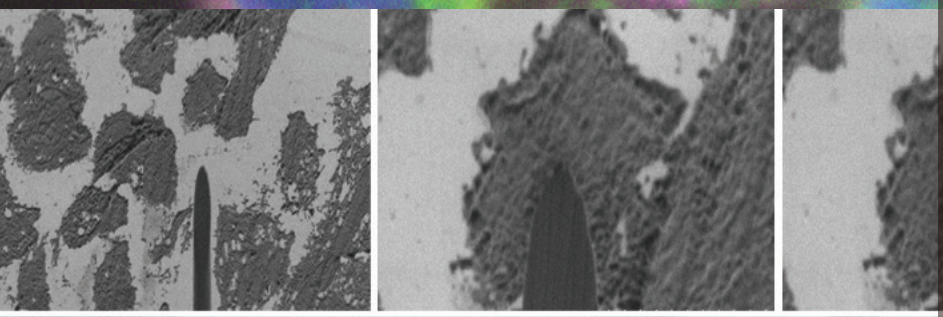

Gene Organization

A technique for extracting DNA from a cell nucleus is reported by Y. Sun, D. P. Bazett-Jones, and co-workers. The nano-dissection tool is shown above the specimen in a scanning electron microscope image before and after DNA extraction. On page 3267, extracted DNA is then identified by sequencing. Regions of interest identified by fluorescence microscopy are determined by correlative imaging and are precisely manipulated.

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Nano-Dissection and Sequencing of DNA at Single Sub-Nuclear Structures
Y. Sun, D. P. Bazett-Jones, and co-workers

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Nano-Dissection and Sequencing of DNA at Single Sub-Nuclear Structures

Brandon K. Chen, David Anchel, Zheng Gong, Rachel Cotton, Ren Li, Yu Sun, and David P Bazett-Jones**

The relative positioning of gene loci within a mammalian nucleus is non-random and plays a role in gene regulation. Some sub-nuclear structures may represent "hubs" that bring specific genetic loci into close proximity where co-regulatory mechanisms can operate. The identification of loci in proximity to a shared sub-nuclear structure can provide insights into the function of the associated structure, and reveal relationships between the loci sharing a common association. A technique is introduced based on the nano-dissection of DNA from thin sections of cells by high-precision nano-tools operated inside a scanning electron microscope. The ability to dissect and identify gene loci occupying a shared site at a single sub-nuclear structure is demonstrated here for the first time. The technique is applied to the nano-dissection of DNA in vicinity of a single promyelocytic leukemia nuclear body (PML NB), and reveals novel loci from several chromosomes that are confirmed to associate at PML NBs with statistical significance in a cell population. Furthermore, it is demonstrated that pairs of loci from different chromosomes congregate at the same nuclear body. It is proposed that this technique is the first that allows the de novo determination of gene loci associations with single nuclear sub-structures.

1. Introduction

In recent years, it is accepted that spatial positioning of chromosomes and genes within the cell nucleus is critically important for accurate gene regulation and integrity of the genome. However, it is unclear how multiple loci, separated across large regions of the same chromosome, or across multiple chromosomes, are co-regulated and give rise to stable, genome-wide transcription profiles that characterize a cell type. The location of a gene within the cell nucleus, both with respect to other genes on the same chromosome (in *cis*) or on separate chromosomes (in *trans*), and with respect to multi-protein nuclear sub-structures (i.e., nuclear bodies or NBs), is an actively regulated process that underlie a mechanism of genetic control.[1–4] Examples of such locus associations are chromosome loci associating with nuclear foci containing high concentrations of their regulatory factors,[5] the shared regulation of certain gene pairs requiring their inter-chromosomal convergence,[6] and NBs serving as regulatory centers for the multiple convergent loci.[3,7] This has led to the idea of the NB as a "gene hub",[8–10] whereby the co-regulation of multiple loci, separated across large regions of the same chromosome or across multiple chromosomes, emerges by virtue of their shared affinity with a *single* NB.

Testing the gene hub model necessitates the identification of genomic regions that preferentially associate at a single NB. The identification of these gene locus sequences will also provide insights into the function of the associated body, and reveal novel co-regulatory relationships that may not be detectable using conventional genome-wide transcription

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profiling approaches.[11] Whereas fluorescence *in situ* hybridization (FISH) is effective at identifying interactions between specific loci or between specific loci and sub-nuclear structures (immuno-FISH), these methods require *a priori* information for selection of loci to probe. Hence, immuno-FISH is not appropriate for use in a naïve or unbiased context.

Although there are techniques available to detect protein/DNA interactions, including ChIP (Chromatin Immuno Precipitaion),[12] the 3C technique and its variants,[13,14] e4C,[7] and ImmunoTrap,[15] these approaches detect the associations of gene loci in cell populations, and are therefore not suited for the *de novo* detection of pairs of loci that are convergent at a single nuclear body. Moreover, there still remain NBs, by virtue of their transience,[16,17] limited number (e.g. embryos,[18] or structural heterogeneity within single cells,[17,19,20] that cannot be assayed using the existing population-based approaches. Particularly when these "aberrant" or rare NBs occur in cell lines with concomitant genome-wide disregulation,[21] they are particularly attractive models for studying NBs as regulatory centers for multiple convergent loci. The identification of convergent sequences at these otherwise inscrutable NBs requires an alternative approach, one capable of determining the genomic neighbourhood of a single NB in a single cell.

Physical extraction of sub-cellular structures under optical microscopes using laser based microdissection[22] and glass capillary needles[23] have been reported; however, the minimum extractable area is limited to the micrometer scale. Atomic force microscopy (AFM) has been used for imaging, cutting, and extracting sub-micrometer-sized regions of an isolated chromosome.[24–26] The use of a single cantilever tip for both imaging and manipulation, however, leads to concerns of contamination. Furthermore, material extraction using AFM relies solely on adhesion to the sharp cantilever tip, making the process poor in reproducibility.

We report a nano-dissection technique for determining DNA in the immediate vicinity of a sub-nuclear structure (e.g., NB) in a single cell. The technique involves using a nano-manipulation system and custom made nano spatulas for DNA extraction inside a scanning electron microscope (SEM). The nano-manipulation system controls the motion of nano spatulas, under real-time SEM imaging, to accurately extract a minute amount of DNA that is then amplified and sequenced. As a proof-of-principle, we demonstrated the technique's feasibility in the dissection of minute volumes in the vicinity of a single histone locus body (HLB). HLB is an NB that has previously been shown to associate with histone gene loci on both chromosomes 1 and 6.[27] From nano-dissection of single HLBs in HT1080 cells, we obtained sequences that are enriched for chromosomes 1 and 6, including sequences within 500 kilobases (kb) and 30 kb of their histone gene clusters, respectively.

We also applied the technique to the dissection of single promyelocytic leukemia (PML) bodies in Jurkat cells. These NBs have been shown to specifically associate with particular gene loci in a cell population,[28,29] although it has yet to be demonstrated that there are specific pairs of trans loci that preferentially associate at a shared PML NB. Because there are multiple PML bodies per nucleus in a mammalian cell (typically between 10 and 20), our single body dissection approach is ideally suited to find these convergent trans associated loci, as loci obtained via a population-based approach would not necessarily originate at a shared body. Importantly, the results confirm our capability to recover multiple loci in *trans* from the dissection of a single PML NB.

2. Results

DNA nano-dissection. Targeted regions within a single cell nucleus are removed via nano-dissection inside an SEM, followed by amplifying and sequencing of the extracted DNA. Cell sample preparation (described in Experimental Section) involves fixation, immuno-labelling, post-fixation, cryoptrotection and freezing, before cryo-sectioning fixed cells into 300 nm-thick sections. The cell sections are first imaged by fluorescence microscopy to locate structures of interest before finding the same structures by correlative methods in the SEM, prior to nano-dissection. Sections are supported on a smooth and clean working surface of a doped silicon substrate that is electrically conductive to reduce charging effects caused by electron-solid interactions under SEM imaging. This also mitigates the complex electrostatic force interactions during nano-manipulation. To control for contaminating DNA that may be introduced to the sample prior to PCR amplification, the cells are pre-ligated with fluorescent double-stranded DNA linkers that are complimentary to primers used for the PCR (see Experimental Section).

The extraction tool, termed nano spatula, is made from solid glass rods heated and pulled into a gradually tapered needle, which is then precision grinded to produce a bevelled-shaped spatula with the tip end narrowed down to <100 nm (**Figure 1**a, Experimental Section). The nano spatula is then carbon coated to make it electrically conductive.

The cell sample is mounted on a piezoelectric nano-manipulator (Figure 1b), which provides nanometer motion resolution along X, Y, and Z axes.[30] The assembly is then transferred into the high vacuum chamber of SEM. The first step of the extraction process is to locate the cell of interest by correlating the SEM and fluorescence microscope images (Figure 1c,d). The target sub-nuclear region is then mechanically extracted using the nano spatula (Figure 1e-h). To ensure the extracted biomaterial stays attached to the spatula tip during SEM chamber venting and during manual handling, a fixation step called electron beam induced deposition (EBID) is conducted to deposit a line of hydrocarbon across the biomaterial and nano spatula to enhance adhesion (Figure 1i,j). The SEM is then vented, and the nano spatula with adhered chromatin fragments are carefully immersed into the buffer solution inside a test tube. This process is repeated until all desired sub-nuclear regions are dissected. The collected samples are then PCR amplified and sequenced.

2.1. SEM Imaging, DNA Integrity, and Nano-Manipulation

Exposure to electrons of just tens of eVs is sufficient to alter the organic chemical and biochemical integrity of

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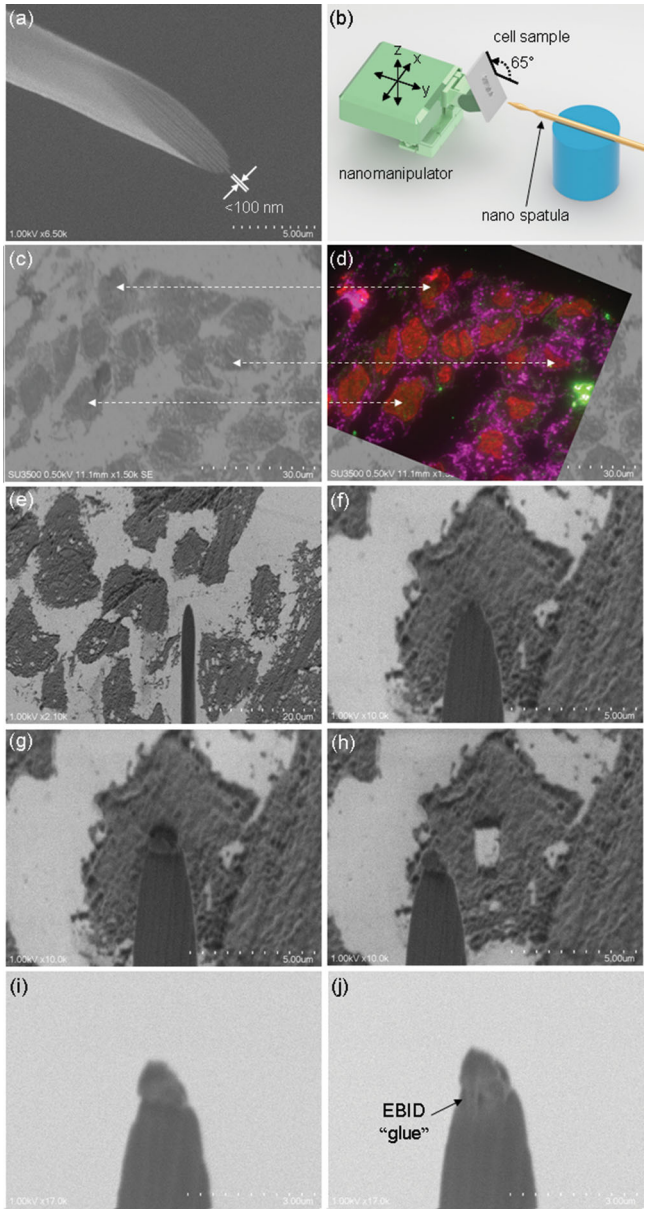

Figure 1. Chromatin extraction setup and experimental process. a) SEM image of the nano spatula with tip size less than 100 nm in width. It is made from heated glass rod, mechanically grinded to produce a beveled surface. b) DNA extraction setup within the SEM. The cell sample is mounted on an XYZ nano-manipulator, facing the stationary nano spatula. c-d) Image correlation between SEM image and fluorescence image. The arrows point to examples of matching features between the two images. e) Locating the cell of interest inside SEM, guided by correlated fluorescence image (not shown). f) Landing the nano spatula tip onto the target of interest. g) Pressing the nano spatula against the sample causes cell fragments to slide onto the bevel surface. h) Lifting up the nano spatula along with the extracted cell fragment. i,j) To ensure the cell fragment does not get detached due to vibration or airflow during SEM chamber venting, electron beam induced deposition (EBID) is used to 'glue' it in place. The nano spatula is then removed from the SEM and stored in buffer solution.

DNA. Under electron beam irradiation inside the SEM, DNA within the volume penetrated by the electron beam is damaged and cannot be sequenced. Thus, minimizing the electron penetration depth is critical, which can be controlled by reducing the accelerating voltage at the cost of the image signal-to-noise ratio. The signal-to-noise ratio can be improved by increasing the number of irradiating electrons (e.g., by increasing current, spot size, and aperture size), with the trade-off of reduced imaging resolution. Increasing the sample tilting angle also reduces electron penetration depth, but requires image processing to compensate for the tilt-induced image distortions. In summary, a balance between preserving DNA's biochemical integrity under electron irradiation and the ability to observe the extraction process must be achieved.

In experiments, we systematically varied the SEM imaging parameters (accelerating voltage, emission current, spot size, aperture size, and sample tilting angle) and conducted chromatin extraction under each set of parameters. These parameters were correlated to the success rate of DNA sequencing. We found that the optimal parameters for nano-dissection are: 0.4 kV accelerating voltage, 2 to 5 μA emission current, 50% spot size, 3 aperture size (50 μm diameter), and sample tilt of 65 degrees. No accumulation of negative charge on the sample surface was observed due to the low accelerating voltage used.

Despite the strong surface adhesion forces present at micro and nanometer scales,[31] vibration induced by the nano-manipulators and manual handling of the nano spatula, and turbulence of the air flow from venting the SEM vacuum chamber often detached the extracted materials from the nano spatula. To enhance the tool-sample adhesion, the nano spatulas were constructed using different materials (tungsten, glass, silicon, silicon dioxide, silicon nitride), combined with chemical coating (poly-L-Lysine), physical coating (carbon and gold film), oxygen plasma treatment, and an applied voltage to induce electrostatic forces. However, none of these combinations provided a consistent adhesion in the vacuum environment. Thus, we used the EBID procedure to fix the extracted cell fragments onto the nano spatula. This worked effectively regardless of the material and surface properties of the nano spatula or the substrate. Prolonged exposure of the sample to electron beam during EBID did not lower the success rate of obtaining sequenceable DNA, likely because the depth of electron interaction volume does not increase significantly with longer exposure time.

To extract DNA successfully, nano-manipulation must be properly performed. The thickness of the nano spatula tip and its angle relative to the sample substrate dictate how the fragment slides onto the bevelled surface of the nano spatula tip. If the cell fragment curls up or flips over during extraction, both sides of the cell fragment would be exposed to the electron beam thereby risking beam-induced specimen damage. Having the collected cell fragment break away cleanly from the rest of the cell when the nano spatula is lifted is also critical. A high success rate was achieved when the cell fragment was first pushed to an area outside of the cell, free of any surrounding tethering, before being lifted. To extract a target in the center of the cell, unwanted material is first scraped away, after which the target material is extracted.

2.2. Enrichment of Expected Sub-Chromosomal Regions from a Single Nano-Dissected Nuclear Body

To confirm the feasibility of extracting nano-volumes of chromatin from a specific targeted region, a nuclear structure that is known to associate with a particular gene locus is needed. We chose the histone locus body (HLB) as a model subnuclear target. These 0.5–1 μm diameter structures closely associate in a large percentage of cells with the histone gene clusters on both chromosomes 1 and 6.[27] The protein component of the body is called NPAT, and immuno-labelling against this protein was used to identify it in sections imaged by fluorescence microscopy. Structures of interest were identified in the SEM by correlative imaging, and DNA was extracted by nano-dissection of individual HLBs from 300 nm cryosections of HT1080 cells (see Experimental Section).

In five separate experiments, we obtained 11 sequences, 7 of which are from chromosome 6, indicating a significant enrichment for this chromosome ($p < 5.3 \times 10^{-7}$, **Figure 2** and Figure S1). In one dissection of a single HLB, a sequence within 500 kb of the histone cluster on chromosome 1 was obtained (Figure 2a). More remarkably, a sequence mapping to within 30 kilobases (kb) upstream of the histone cluster *HIST1H2BJ* from chromosome 6 was identified (Figure 2b). The dissections thus yielded a significant enrichment of sequences in the vicinity of the histone gene clusters on chromosome 1 and 6 ($p < 9.3 \times 10^{-5}$; see Experimental Section). Taken together, these results, for the first time, demonstrate the feasibility of amplification and identification of DNA dissected from targeted nano-scale volumes of single NBs.

2.3. Nano-Dissection of Single PML NBs Identifies Novel Loci-Body Associations

The nano-dissection technique was developed to investigate whether a well-defined, limited set of genes cluster at a single PML NB. We applied the technique to isolate DNA from nano volumes (500 nm × 500 nm × 300 nm, **Figure 3**a) around and including PML NBs in 300 nm cryosections of Jurkat T-cell leukemia cells. Several sequences were identified. Scraping of a single PML NB yielded sequences from multiple chromosomes. Moreover, owing to the small nuclear volumes extracted, clusters of genes were frequently observed (Figure 3b; chromosome 17 and 18 sequences). In fact, the single red bar on Chromosome 17 represents two sequences that mapped to within 30 kb of each other. We then used FISH to determine which of these sequences by themselves associated with a PML NB in a population of Jurkat cells.

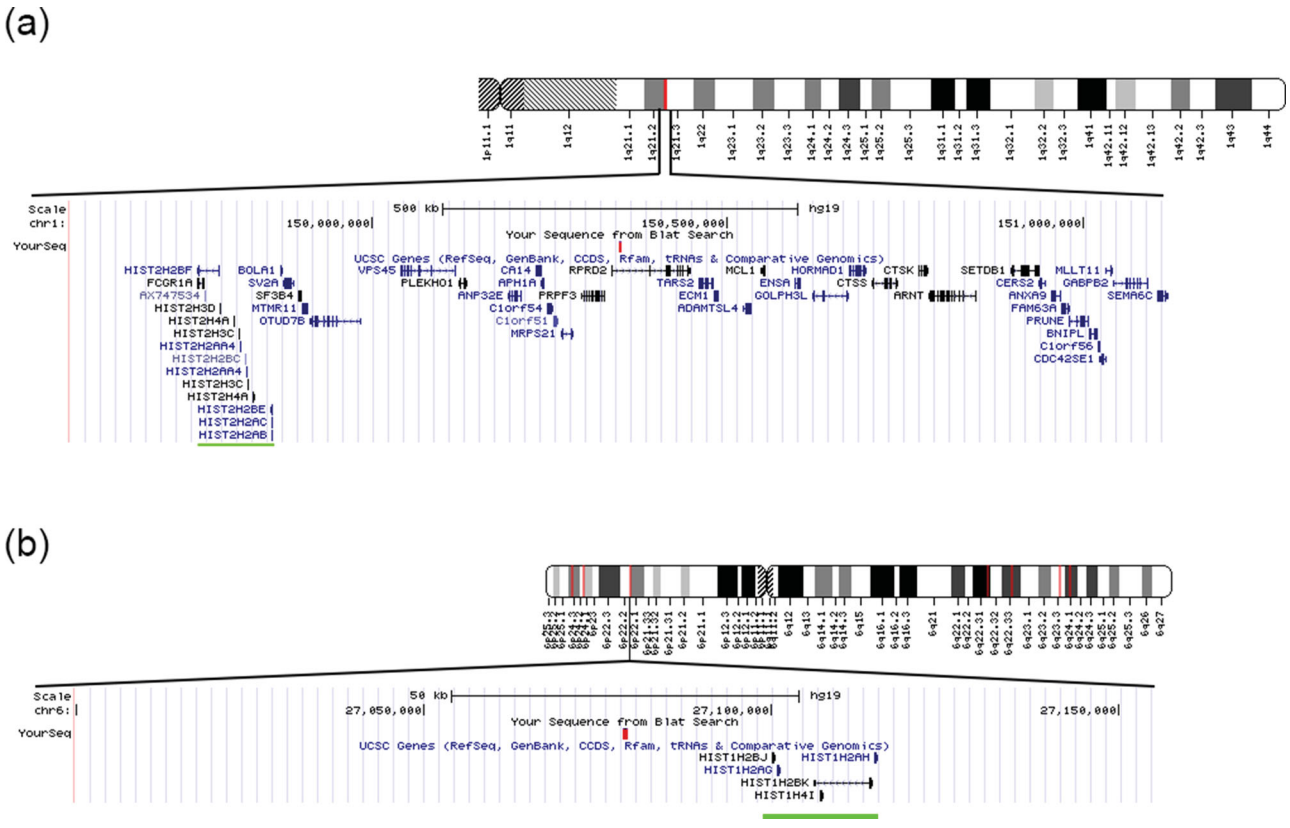

Figure 2. Five independent dissections of single HLBs yielded 11 bona fide DNA sequences (i.e., that are flanked on at least one end with probe that contains "signature" sequence; see 5. Experimental Section). The sequences obtained were enriched for those that mapped to chromosome 6 (7/11; $p < 5.3 \times 10^{-7}$). Red bars indicate the chromosomal location of sequences obtained that map to a) chromosome 1, or b) chromosome 6. The histone locus clusters found on chromosome 6 and 1 are indicated by green bars. Furthermore, the obtained sequences were significantly enriched for those proximal (within a 2 Mb window) of a histone gene cluster, with two sequences mapping to within 30 kb and 500 kb of the histone clusters on chromosome 6 and 1, respectively ($p < 9.3 \times 10^{-5}$).

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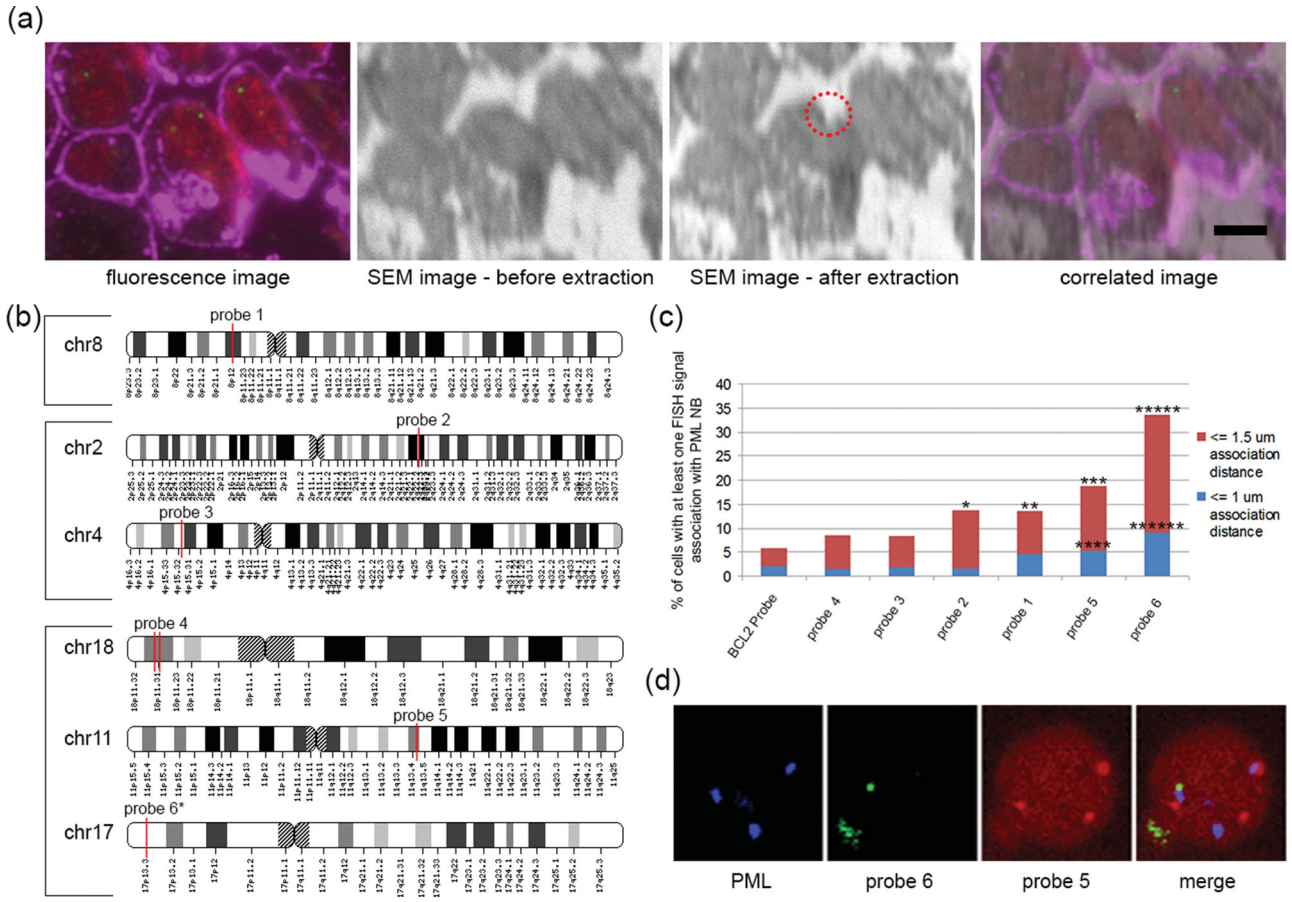

Figure 3. Nano-dissection of single PML NBs. a) Typical extraction volume is 500 nm × 500 nm × 300 nm on a 300 nm thick Jurkat cell sample (indicated by red circle). The scale bar represents 2μm. b) Sequences yielded from three nano volume dissections of single PML bodies from 300 nm Jurkat cryosections. Mapped sequences contained in the same bracket were amplified from the same dissection. Interestingly, we obtained sequences across multiple chromosomes from two of these single PML NB dissections. Their mapped chromosomal location is indicated with a red bar (*with the exception of the mapped sequences within 30kb of each other on chromosome 17, which are represented with a single bar). Sequences were found clustered in the genome (chromsome 17 and 18 sequences), as would be expected from dissection of a limited nuclear volume. c) Center-to-center distances of 3D FISH signals of indicated BACs to PML bodies were measured in whole Jurkat nuclei. For each FISH experiment, at least 100 nuclei were analyzed. Significantly higher percentages of FISH signals within 1 μm and 1.5 μm of PML bodies were obtained compared with a BAC overlapping with the *BCL2* gene. (* $p < .0086$; ** $p < .0014$; *** $p < 6 \times 10{-}7$; **** $p < .03$; ***** $p < 5 \times 10{-}15$; ****** $p < .0006$). d) 3D double FISH was performed using probe 5 and 6, the two BACs that showed significant PML association, and that corresponded to sequences obtained from the same dissection. Shown is an optical section of a Jurkat cell nucleus in which both FISH signals were within 1.5 μm of a shared PML NB.

Using BACs that contained the dissected loci DNA, we identified four genomic loci that were significantly associated with PML bodies in a population of Jurkat cells (Figure 3c). A BAC containing the *BCL2* locus served as a negative control.[28] Of these four loci, two loci (mapping to p15.4 and q12 of chromosome 11 and 17 respectively; see Figure 3b, probes 5 and 6) originated from the dissection of a single PML NB. Using immunoFISH on a cell population, we observed alleles containing these two loci together at a single PML NB in some cells (see Figure 3d), indicating that this co-association at a PML NB was not a rare occurrence. Hence, we demonstrate that our nano-dissection and identification strategy could be used to test whether pairs or sets of gene loci frequently cluster together at a single PML NB in a cell population. Such a clustering could correlate with the unique biochemical composition of that particular PML NB, such as the relative concentrations of certain transcription regulatory factors. Alternatively, that particular PML NB may be non-randomly positioned in the nucleus, such as next to the nucleolus or at the nuclear envelope. Such spatially confined PML NBs may then associate with particular subsets of loci. The nano-dissection technique is presently applied to testing these ideas.

3. Discussion

The spatial arrangements of specific gene loci relative to each other and to specific sub-nuclear neighbourhoods represent a level of gene organization that has regulatory potential.[1] Hence, the ability to identify specific gene loci at particular sub-nuclear landmarks in single cells provides a powerful step in generating hypotheses on the mechanisms of regulating nuclear events. The work described herein provides proof-of-principle demonstrations of a transformative chromatin extraction and identification technique.

We demonstrate the feasibility of isolating nano volumes of chromatin followed by amplification and identification of the extracted DNA sequences. We first showed that the dissection is accurate enough to target a single HLB, and enrich for sequences previously shown to associate with it. We then applied the technique to dissect chromatin from single PML NBs. Combined with FISH, we were able to identify novel loci that specifically associate with PML NBs in a cell population. In its current state, the nano-dissection technique is useful as a hypothesis generator, uncovering novel NB-gene associations.

The nano-dissection technique is particularly suited for asking whether pairs or sets of gene come together at specific sub-nuclear structures. In these first nano-dissection experiments of single PML NBs, we identified a pair of loci that were together at a PML NB. When probing these two loci in a cell population with immuno-FISH, we observed co-association at single PML NBs, although this occurrence was less than each locus being found at a different PML NB. We thus concluded that these two loci, although spatially associated with PML NBs, do not have to come together at the same body.

At present, the technique has a success rate of ~16% (Figure S2a), which represents the frequency of dissections yielding *bona fide* DNA sequences (i.e. those sequence that originated from the dissected region by virtue of the presence of a "signature" sequence; see Experimental Section) that can be unambiguously mapped to a genomic locus. The present accuracy of the scrape, based on the accuracy of correlative imaging, is +/– 350 nm. Further optimization of the technique is expected to improve the extraction accuracy and success rate.

Density and porosity of the cell nucleus plays a key role in the depth of electron interaction volume. To minimize the electron beam induced damage on DNA in future studies, cell samples can be "encapsulated" to fill the pores, or coated with a thin conformal material layer deposited over the sample to act as the electron shield. Encapsulation with Tween-20 detergent for example, has recently shown promise as a means to protect biological specimens from the ionizing damage of the SEM beam.[32]

In this study, we used correlative microscopy (i.e., correlating SEM and fluorescence microscopy images) to find structures of interest to nano-dissect. Imperfections in image alignments can lead to reduction of spatial resolution in dissecting the material of interest. A possible improvement is to immuno-label the target sub-nuclear structure with gold nanoparticles, which can be directly visualized within the SEM and hence, eliminate the need for correlative imaging.

It is possible that we successfully amplified only a portion of DNA that was dissected onto the tip of nano spatulas. Independent of electron-induced DNA damage, we suspect two other factors that could have limited the amplification efficiency. First, our approach used to reduce DNA contamination may have limited the sensitivity of the PCR amplification (See Experimental Section). DNA contained on the tip that is otherwise amplifiable (i.e., not damaged by the SEM beam) but not flanked by ligated probe, may be insufficiently amplified due to a lack of sites complimentary to the PCR primer, or discounted after sequencing by a lack of probe "signature" sequence (See Experimental Section). Thus, the sensitivity of PCR amplification is dependent on the proportion of DNA that is flanked by ligated probe. The proportion of DNA ligated with flanking probe may be improved by employing a staggered-ended probe instead. Second, because single molecule amplification requires a high number of PCR cycles for sufficient yields for cloning or direct sequencing, the final detectable PCR amplification products may only represent those dominant species that emerge over successive PCR cycles. As an alternative approach, a more representative population of template can be obtained by reducing the number of PCR cycles to yield sufficient amounts of DNA enough for either microarray analysis or next generation sequencing.

Our technique complements the scope of the present tools available to interrogate spatial relationships of gene loci (i.e. ChIP, 3C). It offers unprecedented sensitivity (e.g., the identification of genomic sequences converging on a single NB) and captures chromatin loci associations that may occur at distances that preclude direct or indirect chemical cross-linking to each other or a protein constituent of the nuclear substructure, as required by 3C and ChIP, respectively. This allows for the interrogation of chromatin in an arbitrary vicinity of a nuclear structure, which may reveal otherwise undetectable transient or indirect chromatin associations. This work thus far serves as a proof-of-principle and is intended as a basis for further investigation. In addition to being particularly suited to the detection of pairs or limited sets of loci that converge at single NBs, our approach can be used to find novel chromatin/nuclear body associations that cannot be detected by the present population-based methods, either because of limiting starting material (embryos and tissue), or because the NB in question cannot be biochemically isolated. PML NBs, for example, show differences in the ratios of particular PML protein isoforms from body to body,[19] as well as other factors, and these compositional differences may reflect the preferential association of genomic loci to a particular variant of a PML NB among the several found within a single cell. Such associations would not necessarily be detected by ChIP because it relies on the immunoprecipitation of a pooled population of cells.

The question of specific locus associations with PML NBs originally motivated the development of the nano-dissection technique. However, we expect that this technique has wide applicability and can be useful as a novel assay for general questions of interactions of chromatin with sub-nuclear domains that are so far intractable.

4. Conclusion

We report a novel technique that allows for the targeted dissection and sequencing of DNA obtained from a nanoscale region of a single cell nucleus using high-precision nanotools operated inside a scanning electron microscope. We demonstrated, for the first time, the ability to identify loci occupying a shared site at a single sub-nuclear structure. As a proof-of-principle, we nano-dissected the vicinity of single nuclear sub-structure known to colocalize with the histone gene clusters on chromosomes 1 and 6 (histone locus bodies)

and obtained a significant enrichment of DNA that mapped to within 500 kb and 30 kb of these clusters respectively. We applied the technique to a PML NB in Jurkat cells and yielded four novel loci that were found to have a significant frequency of association with PML NBs in a population of cells. Furthermore, we showed that a pair of these loci from different chromosomes can converge at the same PML NB. Because our approach allows for the dissection and identification of DNA in the vicinity of a *single* nuclear sub-structure, we propose that it is uniquely suited for identifying specific sets of loci that converge non-randomly at a shared NB. We expect that if the "gene hub" model is a general feature of nuclear organization, then our technique, applied in this manner to other NBs, will reveal specific sets of convergent loci that may reflect novel gene co-regulatory relationships.

5. Experimental Section

Sample Preparation: Cells are fixed in 2% formaldehyde for five minutes and washed in PBS. Cell is pelleted and prepared for cryosectioning as previously described.[15] Cryosections (300 nm thick) are then washed with PBS, and restriction digested with Sau96I and MSE1 enzymes. The resultant double strand breaks are then blunted with Klenow fragment for 1 hour at room temperature, washed 3 × 5 minutes in PBS and then ligated overnight at room temperature with the following fluorescently tagged double stranded oligo: Link1 5′ (Cy3)AGT GGG ATT CTT GCT GTC AGT TA*G CTG* 3′, Link2 5′ *CAG C*TA ACT GAC AG(ddC) 3′ (ddC: dideoxy C). Note that this oligo Linker contains the priming sites for the PCR amplification. The section is then washed 3 × 5 minutes with 0.1% Tween 20/2XSSC solution at 42 °C, and 3 × 5 minutes at 60 °C with 0.5XSSC solution. The efficiency of the ligation is confirmed by the Cy3 signal under fluorescence microscopy. Now the genomic DNA is flanked by priming sites specific to those primers used for the PCR amplification. Contaminating DNA introduced during downstream processing steps will not be recognized by the linker specific primers and thus not be amplified. By performing this preligation step prior to the nuclei scraping we were able to reduce the background amplification problem. Invariably, contaminants do get amplified by mispriming events; however, the presence of a 4bp "signature sequence" in the ligated linker (denoted by the italicized subsequences of Link1 and Link2), allowed us to distinguish bona fide amplicons that originated from DNA in the sample from those that arose from a mispriming event (see Figure S2b).

Nano Spatula Fabrication: We used a micropipette puller (P-97 Sutter Instrument) to process 1 mm diameter solid glass rod (GR100–4 from World Precision Instruments) for creating nano spatula tools. Fabrication parameters are summarized in Table S1. With the first recipe, a neck structure is created. With the second recipe, the sharp tip is created (Figure S3a). The sharp tip is then mechanically grinded using a beveler (BV-10 Sutter Instrument) to create the beveled surface. The nano spatula is mounted on the nanomanipulation system (see Figure S3b) for DNA extraction.

DNA Extraction and PCR Amplification: Samples are incubated overnight at 42 °C in 4.5 µL of DNA extraction buffer as used in Langer et al. 2005[33]: 0.5 µl of 10 One-Phor-All-Buffer-Plus (Amersham Pharmacia Biotech), 0.13 µl 10% Tween 20 (Sigma, Germany), 0.13 µl 10% Igepal CA-630 (Sigma), 0.13 µl Proteinase K (10 mg ml^{-1}, Sigma). Proteinase K is then inactivated with an 80 °C incubation for ten minutes. PCR is performed in 50 µl total volume using the Titanium Taq polymerase kit (Clonetech) under the following conditions: 72 °C 1 minute, 68 °C 3 minutes, then 14 cycles of 94 °C 40 seconds, 57 °C 30 seconds, 68 °C 1 minute 30 seconds increasing by one second each cycle. Then 8 cycles of 94 °C 40 seconds, 57 °C 30 seconds, 68 °C 1 minute 45 seconds increasing by one second each cycle. Then 22 cycles of 94 °C 40 seconds, 65 °C 30 seconds, 68 °C 1 minute 53 seconds increasing by one second each cycle. Then 68 °C for 3 minutes 40 seconds. Primer sequence is as follows: 5′ AGTGGGATTCTTGCTGTCAGTTA 3′. Then 10 µl of the amplification product is analyzed on a 2% agarose gel, and 2 µl is cloned for sequencing using the CloneJET PCR cloning kit (Thermo Scientific).

3D FISH and Imaging: For Jurkat cells immuno flouresence and FISH was preformed as previously described.[15] BACs corresponding with the mapped location of Jurkat sequences (Figure 3b) are described as follows: *BCL2* Probe- rp11–299P2, Probe 1- rp11–164H24, Probe 2- rp11–881M16, Probe 3- rp11–366H2, Probe 4– rp11–66P2, Probe 5- rp11–15D18, Probe 6- rp11–510P20. Confocal stacks with a Z increment of 0.1 µm were taken with an Olympus IX81 microscope.

3D FISH Measurements and Statistics: The percentage of cells with at least one BAC signal associating with a PML NB (using thresholds of 1 and 1.5 µm) was determined by measuring the Euclidian center to center distances between FISH signals and PML with ImageJ. Significant differences ($p < 0.05$) in association frequencies were determined by taking the two-tailed p value under an exact Fisher test. To determine if the shared association at a PML NB of two BACs was not due to their independent association, the frequency of their association (using a 1.5 µm threshold) was compared to a model in which the loci associations are independent and equally likely between any PML NB within a cell. This gives a probability of association of two BACs at the same PML NB in a cell as: (frequency of BAC A)X(frequency of BAC B)/(Avg. # of PML bodies per cell). We use this as the probability of success in a binomial distribution test to determine the p value for the frequency of paired associations at a single PML NB. To calculate lower estimate for the p value associated with finding two sequences within 500 kb of the two histone clusters on chromosome 6 and 1 ($P_{insidewindow}$), we considered it as the complement probability of a sequence *not* being within either of 2 Mb windows centered on these two histone clusters ($P_{outsidewindow}$). This gives us: $P_{insidewindow} = 1 - P_{outsidewindow} = 1 - [((\text{number of base pairs in human haploid genome}) - (2 \times 2\ \text{Mb}))/(\text{number of base pairs in human haploid genome})] \approx 0.0013$. We then used this value as the probability of success in a binomial test. To determine a p value for the enrichment of chromosome 6 sequences obtained from the HLB dissections, we considered an upper estimate of the chance of getting a sequence from chromosome 6 as: (size in base pairs of chromosome 6)/(size of haploid genome) $\approx (171 \times 10^6)/(3 \times 10^9) = 5.7 \times 10^{-2}$. We then used this value as the probability of success in a binomial test.

Supporting Information

Supporting Information is available from the Wiley Online Library or from the author.

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