Chromosomal analysis and identification based on optical tweezers and Raman spectroscopy

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Abstract: The ability to identify specific chromosomes with certainty has been established by the development of several cytogenetic techniques based on staining. Here, we report the use of a new optical technique, laser tweezers and Raman spectroscopy (LTRS), to capture and manipulate chromosomes in order to obtain their spectral patterns for molecular analysis without the need for staining. The purpose of this study was to obtain Raman spectroscopy patterns for chromosomes number 1, 2, and 3 and to test if the Raman spectroscopy pattern could be used to distinguish these three chromosomes. In our experiment, optical tweezers were used to capture the individual chromosomes and the Raman spectral patterns were collected for the trapped chromosomes. Then, the captured chromosome was manipulated with the optical tweezers and moved to another chamber through a micro - channel, in which the chromosomes were G–banded for positive identification as chromosome number 1, 2, or 3. Generalized discriminate analysis (GDA) was used to compare the Raman signatures. This analysis revealed that chromosomes 1, 2, and 3 could be distinguished and identified based on their Raman spectra. Development of this approach will lead to more rapid automatic methods for chromosome analysis and identification without the use of prior staining. Moreover, the Raman spectral patterns may lend themselves to more detailed analysis of chromosomal structure than is currently available with standard staining protocols. Such analysis may some day be useful for rapid, automated screening and diagnosis for certain cancers.

OCIS codes: (170.5660) Raman spectroscopy; (170.1530) Cell analysis

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1. Introduction

Chromosomes are made up of a complex combination of DNA and proteins organized into a structure known as chromatin [1, 2]. Chromatin structure acts to compact DNA in the cell nucleus and can participate in transcriptional regulation. In general, gene transcription requires an open chromatin confirmation, whereas gene repression is associated with compacted chromatin. Studies over the past few years have revealed that histone acetylation can regulate the formation of higher ordered chromatin structure and thus gene transcription. Histone acetylation is associated with active gene transcription. When histone is deacetylated, it can act as a transcriptional repressor [3].

Altered chromosome structure is associated with several malignancies [4, 5]. This is perhaps best documented for leukemia, in which specific chromosomal aberrations, deletions, or translocations correlate well with specific leukemic disease subtypes [6]. These leukemia specific chromosomal abnormalities often have prognostic value in predicting therapeutic outcome.

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Cytogenetic analyses of chromosomes is almost always based on examination of chromosomes fixed during mitotic metaphase [1]. During that phase of the cell cycle, DNA has already been replicated and the chromatin is highly condensed, making the chromosome visible for normal light microscopy. The ability to identify specific chromosomes with certainty has been established by the development of several different cytogenetic techniques that stain specific regions of the chromosome [1]. Because each chromosome is composed of slightly different DNA/protein compositions and has distinct morphological characteristics during metaphase, each chromosome can be independently distinguished. The most widely used technique to classify chromosomes is G-banding. G-banding, however, has several limitations. The technique is time consuming and laborious and the identification of G-banded chromosomes requires considerable training and expertise. Moreover, some disease - associated chromosomal aberrations are not detected during G-banding. In addition, there are 24 different chromosomes in human cells and genome sequences of each chromosome have been determined. However, the Raman spectra of these individual human chromosomes have not yet been reported.

Here, we report a new optical technique of combining the laser tweezers and Raman spectroscopy (LTRS) to capture and manipulate chromosomes in order to obtain their spectral patterns for a real time analysis of chromatin without the need for staining. In a LTRS system, a near-infrared laser beam is tightly focused to form an optical trap and the same laser beam is used as the excitation source for Raman spectroscopy, which contains information about species, structure, and molecular conformation of the trapped particles [7, 8]. LTRS has been applied to identifying and characterizing single optically - trapped red blood cells [7, 9], yeast cells [10], bacterial cells and spores [11-13], liposomal membranes [14], and activation of T cells [15]. The LTRS has also been used to sort Raman-identified microorganisms [16]. The major advantage of using LTRS to study chromatin is that it can be done on a macromolecular level without invasive staining procedures. Using Raman spectroscopy, the whole chromosome can be studied without the physical destruction of the chromatin, which permits a more accurate macromolecular analysis of chromosome structure. In addition, this platform is amenable to the subsequent development of automatic high throughput chromosomal analysis. The use of the laser tweezers to trap the chromosomes in this study was essential for several reasons. First, human chromosomes at their largest are only approximately 3 microns in size, which means that they will naturally have a low signal. Trapping them above and away from the slide not only ensures a much higher signal by averaging the reading for the entire chromosome that is enveloped by the laser tweezers, but also greatly reduces the background that a slide would produce in close contact with the chromosome. Also, a chromosome that is attached to the slide will be flatter and therefore larger than a chromosome that is in suspension. The smaller the chromosome, the more likely it is to be read in its entirety by the Raman spectroscope. Lastly, by obtaining the average spectrum for the chromosome in suspension, there is no apparent bias for the telomere, centromere, or any other isolated piece of the chromosome. The laser tweezers allow for a more complete spectrum to be obtained on any given chromosome.

For the present study, human chromosomes 1, 2 and 3 were separated using optical tweezers and subjected to Raman spectral analysis. Following Raman analysis, the optical tweezers were used to deposit the chromosome onto a fresh slide for cytogenetic analysis via G-banding. This permits the generation of Raman spectra for each chromosome number 1, 2 and 3 (22 chromosomes were obtained for each number) whose identity was confirmed. The subsequent GDA analysis of the collected Raman data set [11] was capable of segregating chromosomes 1, 2 and 3 based on their respective spectra. Thus, this validates the use of optical tweezer manipulation and Raman spectroscopy for the identification of human chromosomes without the prior chromatin staining.

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2. Experimental procedure

Healthy peripheral blood cells were obtained from an antecubital blood draw. The white blood cells (buffy coat) were separated by centrifugation on Accu-Paque Lymphocytes at a density of 1.086 g/ml (Accurate Chemical and Scientific, Westbury, NY). The buffy coat was removed carefully with a small glass pipette and resuspended into 10 mls of Amniomax Blood Media, consisting of 75 ml of AmnioMax Basal (Invitrogen, Carlsbad, CA), 12.5 mls of AmnioMax Supplement (Invitrogen), 87.5 ml of Minimal Essential Media (Invitrogen), 20 ml of Fetal Bovine Serum (Invitrogen), 2 mls of L-Glutamine (Invitrogen), and 2 ml of Penicillin/ Streptomycin (Invitrogen). The flask was then incubated for 72 hours, which is the highest growth peak for a harvest. At this time, colcemid (10 µg/ml) (Invitrogen) was added to the flask to arrest the cells in metaphase of mitosis. For peripheral blood cells, 45 minutes was found to be appropriate for optimal chromosome length to both avoid entanglement and assure quality G-banding. Next, the cell pellet was carefully resuspended in a prewarmed chromosome isolation buffer solution consisting of 20 mM NaCl, 8 mM $MgCl_2$, and 20m M TrisHCl pH 7.5 [17]. The cells were then incubated at 36.5 ºC for 10 minutes, centrifuged, and 0.8% Triton X-100 (prewarmed) was added in a dropwise fashion. The suspension was then incubated at 36.5 ºC for 5 minutes. The cells were passed through a glass pipette for lysis. In order to check for chromosomes, a slide was made and a chromosome stain was prepared by mixing Wright's stain (Invitrogen) with Gurr buffer (pH 6.8) (Invitrogen) in a 1:4 ratio and generously applied to the top of the slide. After one minute, the excess stain was rinsed off and the presence of chromosomes could be verified by oil immersion microscopy.

The wavelength, Raman shift, and spectral response of the LTRS system was calibrated with polystyrene beads according to standard routine calibration practices [7]. Then, a special slide for the Raman was constructed, utilizing a three-hole design, with one hole larger than the other two (Fig. 1). A glass coverslip was secured to one side, the slide was turned upside down, and chromosome isolation buffer was added to both of the small wells. In the first and largest well, an aliquot was added from the top of the chromosome solution after it had been allowed to settle (1xG force) for 20 minutes to facilitate large debris and cells settling to the bottom. A glass coverslip was added, and surface tension allowed the slide to be turned upside down again and immersion oil to be added to the bottom of the slide for the microscope objective.

After allowing the channels to clear, chromosomes 1, 2, or 3 were screened based on size and centromere location. After a chromosome was located, it was trapped in the optical tweezers and suspended distal to the coverslip to avoid high background Raman signal. The Raman laser was then used at 50 mW for the average spectra between two 30-second readings. The resulting spectra were recorded with a symphony CCD detector. Then the chromosome, still trapped in the optical tweezers, was maneuvered into the first canal. As long as there was little current, the laser power could be lowered to 10mW in order to navigate the wells and canals without attracting unwanted debris. The chromosome was moved to the third well, where there was little or no debris. The chromosome was then attached to the bottom of the slide by the combined use of the laser tweezers and focus knob to raise the slide up to the level of the laser-trapped chromosome. Once the chromosome was attached to the bottom coverslip and its position recorded, another chromosome could be captured and the process repeated. After several chromosomes were collected in this manner, they were fixed to the slide. The top coverslip was removed and the excess buffer was removed. A fixative, methanol – acetic acid solution (3:1), was then quickly added, causing the chromosomes to spread flat on the slide in the traditional X shape. The fixative was removed and the slide allowed to dry as photographs were taken of the chromosomes.

The slide was set aside in a dark, dry location overnight to allow the chromosomes to become dry and brittle, facilitating the denaturation of the proteins for clear G–banding. The slide was immersed in a solution containing 5 ml trypsin (Invitrogen) in 45 ml 8.9 g/L NaCl

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for 35 seconds. The slide was then promptly removed and immersed in two separate containers each containing 1 ml FBS (Invitrogen) in 49 ml 8.9 g/L NaCl for a couple of seconds each for consecutive rinses. Next the slide was stained with a solution containing Wright's stain and Gurr buffer (1:4) for 35 seconds, and submersed in water to rinse off excess stain. The slide was air dried and placed on a microscope. Pictures of the G-banded chromosomes were taken and examined to positively identify each chromosome.

3. Results and discussion

The technique of sorting the chromosomes using the LTRS system allowed for individual chromosomes to be chosen based on visual recognition. Figure 1 shows the basic idea and experimental scheme of sorting the different chromosomes using the Raman tweezers. The target chromosome was selected with optical tweezers in the sample chamber and moved to an area (the middle chamber) with no debris so that the spectrum was obtained without the risk of contamination. Due to the microflow between the sample chamber and the middle chamber, there was some debris in the middle chamber. In order to perform G-banding, the isolated chromosome was moved to the third chamber and deposited on the slide. The deposited chromosome was then far away from the debris. Principally, different chromosomes could be discriminated according to the accumulated Raman spectra. In order to confirm their identity, it was necessary to G–band the chromosomes. Thus, Raman spectral patterns could be assigned to specific known chromosomes.

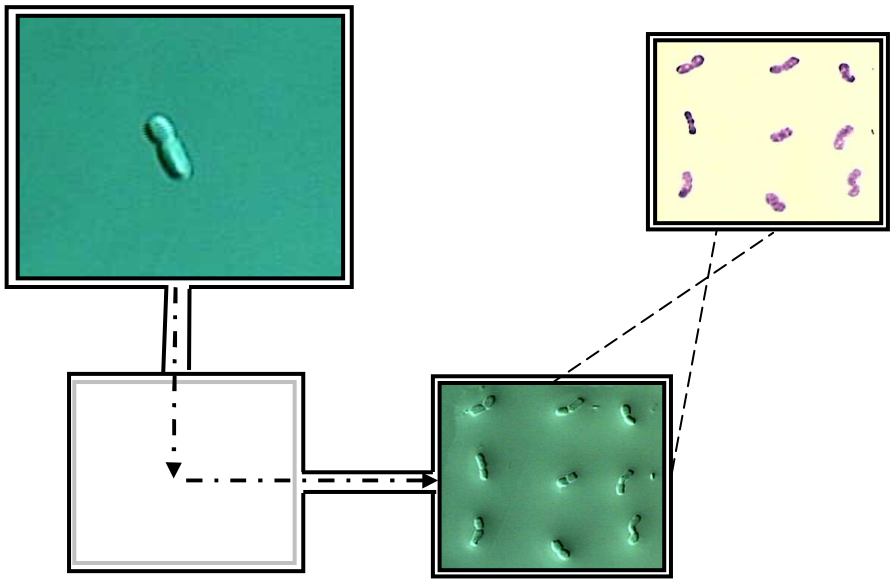

Fig. 1. Illustration of the Raman sorter for this study. The slide has three wells, one large and two smaller. The chromosomes start out in the large well and are transported using the optical tweezers to the third well, where they are fixed to the slide and then G-banded. A cytogeneticist then identified the chromosome number when possible. The chromosomes here were identified as follows: from left to right: Top row - #2, nv, #2; Middle row – nv, #2, #3; Bottom row – nv, #2, #3 (nv - not verifiable).

The ability to flow sort chromosomes is limited within certain groups of chromosomes such as 1 – 2 and 9-12, however the Raman tweezers have proven capable of physically sorting each chromosome regardless of its size or shape. While it was necessary to G- band the chromosome for positive identification in this study, our data indicate that it will be

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possible to rely on the Raman spectral peaks for positive identification of each chromosome. Determination of the experimental conditions that would best distinguish each chromosome will help to provide a basis for studying the variation within each chromosome type.

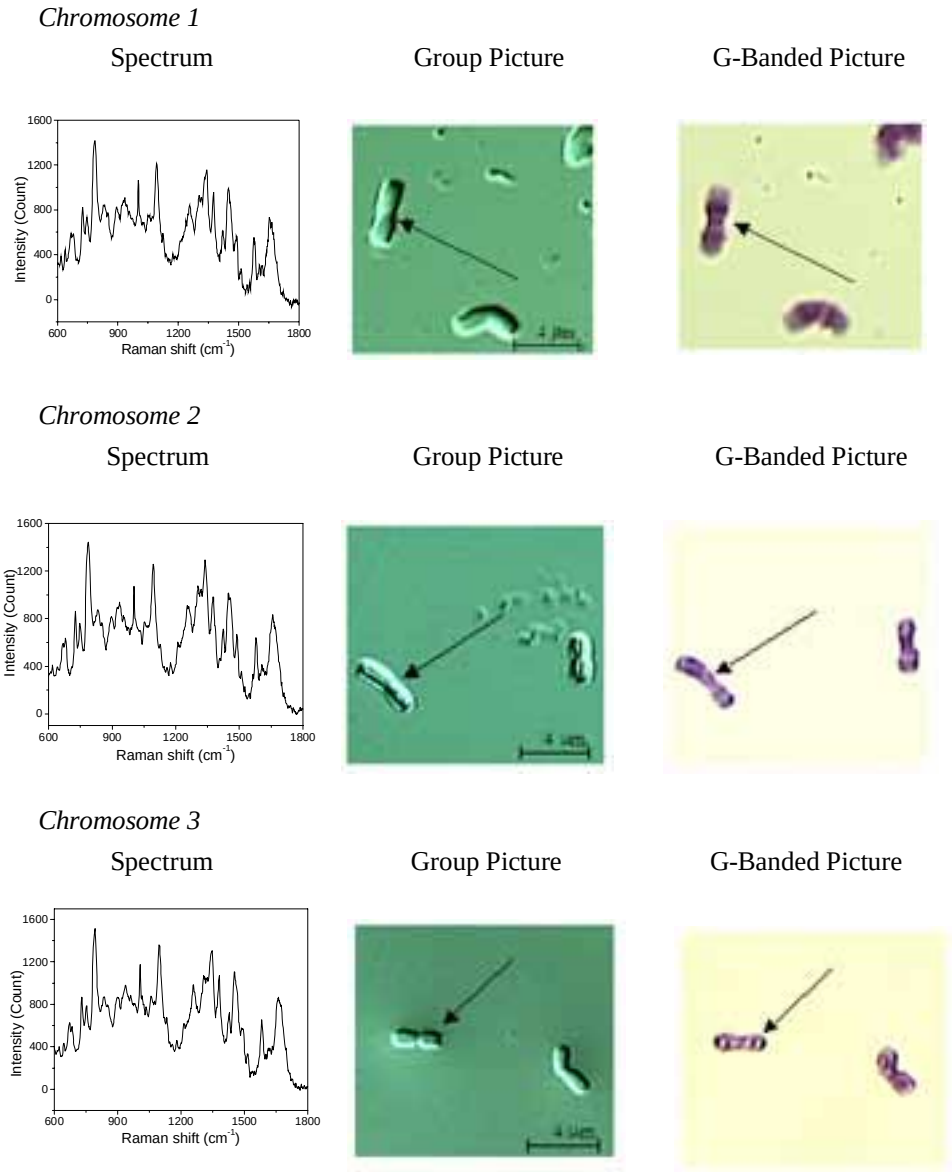

Fig. 2. Typical Raman spectra and G-banding images of individual chromosomes. The purpose of this Fig. is to show what the Raman spectrum of an individual chromosome looks like and how it is related to the positive identification with G-banding.

In our scheme, different group chromosomes were discriminated based on the collected Raman patterns and confirmed with the G-banded results. For every group of chromosomes (#1, #2 and #3), the typical Raman spectra and G-banding images of individual chromosomes

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were given in Fig. 2. All of the chromosomes were composed of the same bases (A, T, C, G) and the associated proteins, their Raman spectra contain the similar characteristic Raman peaks, such as those located at 785, 1004, 1094, 1340, 1450, and 1660 cm^{-1}. However, the order and ratio of the bases for different chromosomes are different; the specific associated proteins are also different. Therefore, the Raman pattern should be different. The variation among those three groups of chromosomes are more obviously from the G-banded patterns. As can be seen from the Fig. 2. G-banded picture, the band position, thickness, and shape are different,which highly suggests an ability to discriminate different groups of chromosomes.

The Raman patterns of different single chromosomes are quite similar, which defers us to discriminate different groups of chromosomes conclusively. However, the different spectra are due to the difference among the chromosomes, not due to the instrumentation. The resolution of our system is about 6 cm-1 (the HWFM of polystyrene at Raman position of 1001.4 cm-1) and the Raman sift of the instrument was calibrated daily to within 1 cm-1 (using the polystyrene Raman pattern as a criterion) [7]. Figure 3 depicts the averaged Raman spectra of three groups of chromosomes and the different spectra among different groups. The different spectra are multiplied by 3 for ease of visualization. The molecular compositions of the Raman peaks associated with chromosomes are known [21,22,23]. Comparing the averaged Raman spectra of the three chromosomes, there are several subtle differences. Of particular interest when looking at these different spectra are those located at 725 785, 1004, 1094, 1340, 1450, and 1660 cm^{-1} (see Fig. 3(a), the different spectra of d, e and f). For example, the different spectrum (curve d) between the group #1 and #2 might demonstrate that the molecular weight of the DNA and proteins are dissimilar. For group #1, the ratio of DNA to protein molecules is higher than that of group #2. The height of any given peak is based on the total intensity of the signal, which can vary from moment to moment. In order to get a definite discrimination, every chromosome's Raman spectrum was normalized to the band intensity at 1004 cm^{-1}. A comparison of the ratios among the three groups were shown in Fig. 3(b). From Fig. 3(b), it can be seen that the ratio of three groups are different. Group #3 is quite unlike the other two groups. For group #1 and #2, the variances overlap too much. It's difficult to discriminate these two groups from the comparison of the ratio of other Raman band intensities to that at 1004 cm^{-1}.

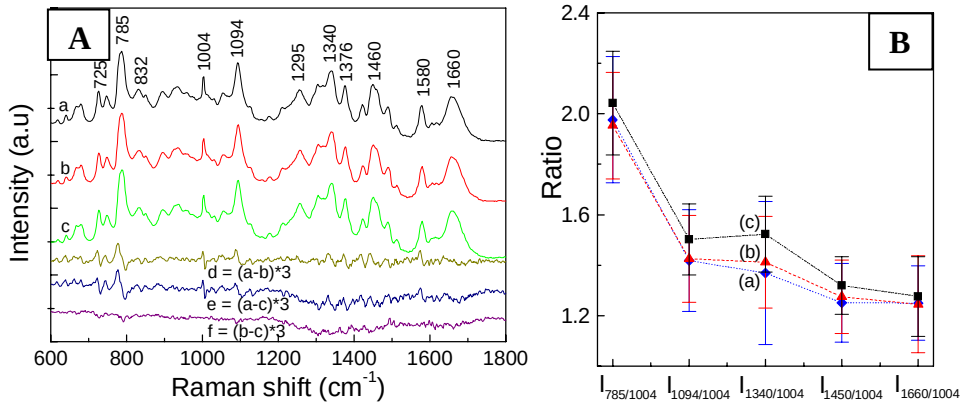

Fig. 3. (a) The averaged Raman spectra of three groups and the differences among them. The different spectra are multiplied by 3 for visualization. a: #1; b: #2, and c: #3. For d,e, and f, each peak is a difference between chromosomes 1 and 2, 1 and 3, and 2 and 3, respectively. (b) The variance among the ratios of Rama peak intensity of main peaks to that at 1004 cm^{-1} of the three chromosomes. The variance was calculated over 22 chromosomes per group.

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A Raman spectrum could be counted as a multidimensional vector. The averaged Raman spectra, the different spectra and the ratio of other Raman band intensities to that at 1004 cm^{-1} were just focused on a few dimensions. Other dimensions might also contain useful information which can help us to discriminate the three groups of chromosomes. This prompted a more thorough analysis using generalized discriminate analysis (GDA). The three average spectra were subjected to the multivariable analytical method of (GDA) [11]. GDA produces the kernel data projection which increases class separability of the projected training data [24]. One point in a GDA plot represents one chromosome. The distribution of the supervised points represents the similarity among different chromosomes. To eliminate commonalities, the spectra used in the first GDA plot was normalized at 783 cm^{-1} (see Fig. 4(a)). Also, we isolated the data from one chromosome donor only and the GDA shows less variance within each chromosome group (see Fig. 4(b)).

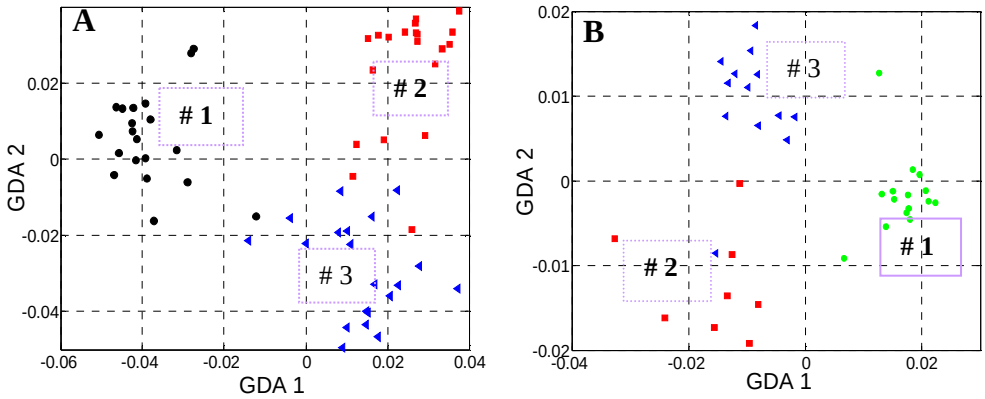

Fig. 4. Generalized discriminate analysis (GDA) plots of all three chromosome numbers. GDA plot using all data collected from all three chromosomes and normalizing the peaks by a chosen standard of 783cm-1. Chromosome 1 is represented as black circles, chromosome 2 as red squares, and chromosome 3 as blue triangles. (a) are the Raman spectra of chromosomes isolated from 6 donors over 12 different days. (b) are Raman spectra from the chromosomes of a single individual over 6 different days.

In these data there is variance within each chromosome set (see Table 1). This may be due to different activation states for each chromosome or perhaps trapping each chromosome in the laser at a slightly different angle due to minor inconsistencies in morphology. The different activations states could be addressed by taking the Raman spectra of whole cells, sorting out the ones that have the most similiar spectra, and then harvesting them. The variability due to the possibility of trapping each chromosome at slightly different angles was addressed by obtaining twenty two chromosomes for each number. Each spectrum that was taken was an average reading of most if not all of each chromosome, and after taking twenty two different readings of different chromosomes, a characteristic spectrum representing that chromosome number could be made. The variance was not a result of instrument differences, since the LTRS system was calibrated daily using polystyrene beads. The sample preparation itself was as consistent as possible, with the use of the same stocks and quantities each time and following the same protocol. In fact, in Fig. 4, both sample populations were over multiple days: (a) was over 12 separate days and (b) was over 6 separate days. They both involved multiple sample preparations that were only used on the day they were made in order to avoid possible degradation over time. The major observable difference between the two graphs is that there is less variance for one donor than there is for four separate people. This would suggest that the variance is biological and a product of the chromosomes themselves, whether it involves their different levels of expression due to the different activation states of

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the cells or whether it may be due to the discreet differences among the people themselves, the data infers that the chromosomes themselves are the source of the variance.

Table 1. (a). The statistical results of the three groups of chromosomes at the main Raman peak positions. (b) The statistical results of the ratio of the main Raman peak positions to that at 1004 cm^{-1} for three groups of chromosomes. (AV, average value; SD, standard deviation)

(a)	Chromosome 1		Chromosome 2		Chromosome 3	
Raman band position (cm^{-1})	AV (Counts)	SD	AV (Counts)	SD	AV (Counts)	SD
785	702.8	117.5	692.0	94.3	695.3	104.6
1004	359.6	71.94	356.0	47.5	342.4	51.4
1094	508.8	109.0	505.3	75.3	511.9	76.7
1340	494.6	139.3	502.1	84.7	515.4	49.0
1450	450.1	99.82	452.5	69.6	449.3	62.9
1660	452.7	114.2	443.4	90.4	438.1	89.5

(b)	Chromosome 1		Chromosome 2		Chromosome 3	
Raman band position (cm^{-1})	AV (Counts)	SD	AV (Counts)	SD	AV (Counts)	SD
785	1.97	0.25	1.95	0.21	2.04	0.20
1094	1.41	0.20	1.42	0.17	1.50	0.14
1340	1.36	0.28	1.41	0.18	1.52	0.15
1450	1.25	0.15	1.27	0.14	1.31	0.11
1660	1.25	0.14	1.24	0.19	1.27	0.15

In summary, while others have obtained nonhuman chromosomal readings using only the Raman spectroscope [20], this study has taken that utility to a much higher level by combining the laser tweezers with the use of Raman spectroscopy on human chromosomes, which are much smaller and more challenging to work with. The trapping is important for measuring human chromosomes since it greatly increases the signal of such a small macromolecule. Also, the use of the trapping for chromosomal sorting and verification is new and holds great potential. This novel technique has opened up several research possibilities for the future. It is possible that this LTRS system may be able to detect the minute differences between one person's chromosomes and another's. Based on the data collected from this study, it may be possible to study CpG I methylation and histone acetylation as epigenetic causes of cellular differentiation and organismal development. This LTRS system will help us to gain a fundamental understanding of the regulation of gene activity that defines cellular differentiation. In doing so, it may be possible to gain a better understanding of all different types of cancer, including leukemia, as well as any heritable diseases that are based on the improper regulation of cellular gene expression.

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