Image Processing Methods for Quantitative Analysis of Mitochondrial DNA Dynamics

Toshiki Matsuo, Kyohei Nakayama, Kazuki Nishimoto and Yasutomo Nomura*

Department of Systems Life Engineering, Graduate School of Engineering, Maebashi Institute of Technology, Japan

Abstract
Mitochondria and mitochondrial DNA are known to be highly motile. Molecular analysis revealed that several proteins are bound between mitochondrial DNA and mitochondrial inner membrane. However, nucleoid formed by these proteins is involved in the replication and duplication of mitochondrial DNA and it remains unclear whether mitochondrial DNA keeps binding the mitochondrial inner membrane or not. When nucleoid is necessary during cell cycle, it would be formed probably. When nucleoid is formed in the manner dependent on mitochondrial DNA sequence, the changes in the sequence would result in the equilibrium shifted to dissociation. This implies that the important information on changes in mtDNA accompanied with various symptoms is obtained by quantitative analysis of mtDNA dynamics. In this review, theoretical background of two typical methods, i.e., particle trajectory and image correlation spectroscopy, and several results of the pilot experiment are introduced.

Keywords: Mitochondrial DNA; Particle trajectory; Image correlation spectroscopy; Diffusion constant; Mean squared displacement

Introduction
Mitochondrion is one of major organelles within a cell. Mitochondria generate ATP when electron is transferred from respiratory substrates to oxygen by a series of redox reaction in which respiratory enzymes pump protons across the mitochondrial inner membrane from the matrix space [1]. In the isolated mitochondria as well as the intact cells, respiration frequently produces reactive oxygen species (ROS). Especially, ROS increases if respiration is perturbed, e.g., ischemia-reperfusion injury [2]. ROS can attack almost all biomolecules unspecifically. In case of DNA, ROS causes single and double-strand breaks, and base damage [3].

Mitochondrial DNA (mtDNA) that mitochondria contain independently of nucleus is particularly vulnerable because mtDNA repair system is weaker than that of nucleus [4]. As shown in Figure 1A, since mtDNA with ~17kbp codes a part of respiratory enzymes pump protons across the mitochondrial inner membrane from the matrix space [1]. In the isolated mitochondria as well as the intact cells, respiration frequently produces reactive oxygen species (ROS). Especially, ROS increases if respiration is perturbed, e.g., ischemia-reperfusion injury [2]. ROS can attack almost all biomolecules unspecifically. In case of DNA, ROS causes single and double-strand breaks, and base damage [3].

Mitochondrial DNA (mtDNA) that mitochondria contain independently of nucleus is particularly vulnerable because mtDNA repair system is weaker than that of nucleus [4]. As shown in Figure 1A, since mtDNA with ~17kbp codes a part of respiratory enzymes pump protons across the mitochondrial inner membrane from the matrix space [1]. In the isolated mitochondria as well as the intact cells, respiration frequently produces reactive oxygen species (ROS). Especially, ROS increases if respiration is perturbed, e.g., ischemia-reperfusion injury [2]. ROS can attack almost all biomolecules unspecifically. In case of DNA, ROS causes single and double-strand breaks, and base damage [3].

Mitochondrial DNA (mtDNA) that mitochondria contain independently of nucleus is particularly vulnerable because mtDNA repair system is weaker than that of nucleus [4]. As shown in Figure 1A, since mtDNA with ~17kbp codes a part of respiratory enzymes pump protons across the mitochondrial inner membrane from the matrix space [1]. In the isolated mitochondria as well as the intact cells, respiration frequently produces reactive oxygen species (ROS). Especially, ROS increases if respiration is perturbed, e.g., ischemia-reperfusion injury [2]. ROS can attack almost all biomolecules unspecifically. In case of DNA, ROS causes single and double-strand breaks, and base damage [3].

When mtDNA dynamics has been analyzed, the standard
fluorescence imaging systems (e.g., laser scanning microscope, LSM) are adopted [22]. As shown in Figure 2A, the high quality image data were obtained with FluoView FV1000 Olympus and were used in this paper. HEK293 cells were incubated in 300x diluted PicoGreen reagent (Molecular Probes) under culture conditions for 1 hr. Since DNA intercalater PicoGreen was used, mtDNA in cytoplasm and nuclear DNA were observed [23]. Ashley et al. [23] pointed out that PicoGreen staining of mtDNA was dependent on a mitochondrial membrane potential. This contributes to the fluorescence of mtDNA stronger than that of nuclear DNA within a living cell in contrast to DNA solution. In order to analyze mtDNA dynamics, an area of cytoplasm was selected (Figure 2B) and the time image series was recorded. Each image series (256 x 256 pixels x 30 frames in the case of particle trajectory, 100 frames in image correlation spectroscopy) with 10 s between sequential frames was collected from single cells.

Diffusion Equation

If mtDNA assumes to diffuse freely, the first step for the analysis is to derive diffusion equation. The equation is usually given as follows [24]:

$$\frac{\partial c}{\partial t} = D \frac{\partial^2 c}{\partial x^2}$$  \hspace{1cm} (1)

Where $c$ indicates the concentration at the point $x$ and time $t$. $D$ represents the diffusion constant. When the linear partial differential equation of second order is solved analytically by using Dirac delta function as initial conditions, the following equation is obtained:

$$c(x,t) = \frac{1}{\sqrt{4\pi Dt}} e^{-\frac{x^2}{4Dt}}$$  \hspace{1cm} (2)

Using Einstein-Stokes equation, $D$ is as follows:

$$D = \frac{RT}{6\pi \eta N_A}$$  \hspace{1cm} (3)

where $R$, $T$, $\eta$, and $N_A$ are the gas constant, the absolute temperature, viscosity and Avogadro number, respectively. Because $\eta$ is pointed out to be spatially heterogeneous within a cell, the value should be considered carefully [25,26]. In this paper, two methods to analyze dynamics of mtDNA which diffuse freely within mitochondria are outlined, (i) trajectory method of fluorescent particles selected in temporally serial images [27-31] and (ii) image correlation spectroscopy using the entire images [32,33].

Particle Trajectory Method

In the particle trajectory method of fluorescent mtDNA, the image is quiet frequently processed by a public domain Java image processing program, e.g., ImageJ. In this paper, first of all, particles were extracted from background in the time image series through the setting proper threshold. Five particles used in this study are numbered from 1 to 5. D: Trajectories of center points of particles obtained by fitting ellipses. Analyzed particles are the same as C. E: Relationship between mean squared displacement and time. Particle #1 in C denotes closed triangle and the linear regression shows thin solid line, #2: open circle and thick solid line, #3: open square and chain line, #4: closed circle and solid line, #5: closed diamond and dotted line.
Diffusion constant is calculated from tracking data of particles. If the particles move from position 0 at time 0 to new position \( x \) after time \( t \) due to the simple diffusion, mean squared displacement (MSD) is given by integral using probability distribution of Equation (2) [24]:

\[
\langle x^2 \rangle = \int_{-\infty}^{\infty} x^2 \exp \left(-\frac{x^2}{4Dt}\right) dx = 2Dt.
\]

(4)

MSD is \( 4Dt \) in the case of two dimensions (2D) and \( 6Dt \) in three dimensions (3D). Equation (4) indicates that MSD increases with time linearly. Indeed, Figure 2E indicates linear relationship between time and MSD calculated from Figure 2D. Each slope of Figure 2D shows diffusion constant of five mtDNAs, and data of 4 cells are summarized in Table 1. Although diffusion constant of 20 mtDNA particles from 4 cells was 9.8 + 5.5 \( \mu m^2/s \) (mean + SD), careful interpretations for the data were required. When the actual 3D dynamics of mtDNA within a cell was observed by using LSM, 2D serial images with \( x \) axis and \( y \) axis were recorded with time but contained no information about \( z \) axis. Therefore, since a true diffusion constant in 3D space was not calculated, the apparent value was comparable with two thirds of the diffusion constant. Although the calculation of temporal series of 3D reconstructions from 2D image stacks is not always possible, the reconstruction is the computationally intense task and it would be not employed practically. Therefore, at the single living cell was two digits smaller than that of them in vitro [34,35] careful considerations about the spatially heterogeneous viscosity within a cell would be required.

**General Expression of Image Correlation Spectroscopy (ICS)**

In correlation analysis, the cross-correlation of a signal with itself is calculated and the similarity between signals observed as a function of time or space is obtained [32]. A generalized spatiotemporal correlation function is defined as following equation:

\[
r(\xi, \eta, \tau) = \frac{\delta I(x,y,t) \cdot \delta I(x+\xi, y+\eta, t+\tau)}{I(x,y,t)^2}.
\]

(5)

Where a fluctuation in fluorescence, \( \delta I(x, y, t) \), is given as follows:

\[
\delta I(x,y,t) = I(x,y,t) - \bar{I}(x,y,t).
\]

(6)

Where \( I(x,y,t) \) is the intensity at pixel \( (x, y) \) in the image recorded at time \( t \) in the image series as shown in Figure 3A. \( \bar{I}(x,y,t) \) is the average intensity of that image at time \( t \). Every image obtained by LSM is a convolution of the microscope PSF (point spread function) with the point source emission from the fluorescent particle due to diffraction [36]. This convolution causes the signal from a point-emitter to be spread over a number of pixels. Dynamics of the fluorescent particle can be calculated by correlating fluorescence fluctuation signals arising from the particles entering and exiting observation area defined by the microscope PSF.

**Spatial ICS**

To obtain the microscope PSF, fluorescence fluctuations over plane of each image are correlated from Equation (5):

\[
r(\xi, \eta, 0) = \frac{\delta I(x,y,t) \cdot \delta I(x+\xi, y+\eta, t)}{I(x,y,t)^2}.
\]

(7)

The correlation function is then fitted to a 2D Gaussian using a nonlinear least squares algorithm:

\[
r(\xi, \eta, 0) = g(0,0,0) \exp \left(-\frac{\xi^2 + \eta^2}{\omega_0^2}\right) + g_o.
\]

(8)

Where \( g(0,0,0) \) is the zero-lags amplitude and is inversely proportional to the number of fluorescent particles, and \( g_o \) is the long-spatial lag offset to account for an incomplete decay of the correlation function. In Equation (8), a Gaussian function is used because the laser beam has a Gaussian intensity profile. A typical result is shown in Figure 3B. The beam radius of the microscope PSF (\( \omega_0 \)) can be confirmed by using methods such as imaging of fluorescent microspheres with diameter less than diffraction limit.

**Temporal ICS**

Because the observation area is defined as \( \omega_1 \) in the preceding section, dynamics of fluorescent particles is calculated from temporal fluctuation of fluorescence intensity in the area (Figure 3C). As shown in Figure 3D, temporal autocorrelation function of an image series as a function of time lag \( \tau \) is obtained from Equation (5) when \( \xi = \eta = 0 \):

\[
r(0,0,\tau) = \frac{\delta I(0,0, t) \cdot \delta I(0,0, t+\tau)}{I(0,0,t)^2}.
\]

(9)

Where the angular brackets denote spatial and temporal averaging. Experimentally, \( \tau \) values are determined by the time between subsequent images in the image series. Depending on the microscope system used, sampling time of image acquisition is usually between...
of fluorescent particles, and with results obtained by the particle trajectory method, but 3D simple larger than that of trajectory method. This difference may be resulted was comparable with that of the particle trajectory method, SD was

<table>
<thead>
<tr>
<th>Table 1: Diffusion constant of mtDNA evaluated by two analysis methods.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Diffusion constant</td>
</tr>
<tr>
<td>---------------------</td>
</tr>
<tr>
<td>(x 10-3µm²/s)</td>
</tr>
<tr>
<td>9.8±5.5</td>
</tr>
</tbody>
</table>

0.03 and 10 s was10 s in this paper. Here, the correlation function \( r(0,0,r) \) is fitted to a simple one component 2D model which can be derived from Equation (2):

\[
r(0,0,r) = \frac{g(0,0,0)}{1 + \frac{r}{\tau_d}} + g_c. \tag{10}
\]

Where \( g(0,0,0) \) is the zero-lags amplitude dependent on number of fluorescent particles, and \( g_c \) is the long-time offset. For confocal excitation, the characteristic diffusion time, \( \tau_d \) is related to the diffusion coefficient, \( D \) by:

\[
D = \frac{(e \omega_s)^2}{4 \tau_d}. \tag{11}
\]

Where \( \omega_s \) is \( e^2 \) radius of the focused beam of the microscope

As shown in Table 1, diffusion constant of mtDNA particles from 25 cells was 9.4 ± 10.2 µm²/s (mean ± SD). Although this average value was comparable with that of the particle trajectory method, SD was larger than that of trajectory method. This difference may be resulted from exclusion of extraordinary movement during particle selection. In Equation (8), a 2D simple diffusion model is adopted for comparison with results obtained by the particle trajectory method, but 3D simple diffusion model should be used in the next step.

Concluding Remarks and Future Prospects

In this paper, typical two methods to analyze mtDNA dynamics were explained concisely. At present, it is difficult to compare between the particle trajectory method and the image correlation spectroscopy quantitatively because of the different number of cells analyzed. However, we try to apply particle trajectory method to more cells. The reason of the standard deviation less than results of image correlation spectroscopy would attribute to the selection of the several particles which were observable easily. If we select all particles in the time image series in the case of particle trajectory method as same as image correlation spectroscopy, the standard deviation would be comparable to that of image correlation spectroscopy. The merit of image correlation spectroscopy is that one can analyze the average diffusion constant for all particles without the complicated procedures of the selection of particles. Thus, because the method would be effective in an automated diagnosis for mitochondrial diseases, this method is expected to be applied to various sample containing patients suspected of having these diseases. At present, we plan to determine the effect of mtDNA damage on the diffusion constant in detail. Furthermore, because mitochondria within a cell move through the interaction with cytoskeleton, the effects of the cytoskeleton inhibitors on mtDNA dynamics may be quite interesting.

Acknowledgments

This study was financially supported in part by Grant-in-Aid for Scientific Research (C) (23500523) and Knowledge Cluster Initiative (Hokkaido Marine Bio-Industrial Cluster) from the Ministry of Education, Science, Sports and Culture of Japan.

References


