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# Biotechnical and Systematic Preparation of Artificial Cells (DNA Crown Cells)

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*Received: 10 December 2016 Accepted: 4 January 2017 Published: 15 January 2017*

## Abstract

The first cell biology studies were conducted half a century ago and it has long been known that cells consist of a membrane made of lipid-polymer complexes comprising proteins and carbohydrates complexed with lipids. Many cell biology studies have been based on this understanding of the structure of the cell membrane, yet there have been no reports of a cell membrane associated with DNA.I have demonstrated that cyto-cells and cyto-particles covered with DNA are formed when cultured cells are mixed with sphingosine-DNA (Sph-DNA). Recently, I have been studying the preparation of artificial cells and have demonstrated the formation of cells (named DNA crown cells) which are surrounded by a membrane comprising lipid-DNA. Moreover, I have synthesized DNA crown cells using a known lipid (monolaurin).

***Index terms***— artificial cells, biotechnology, biomedical engineering, DNA crown cells, sphingosine-DNA.

## 1 Introduction

here has been significant progress in the generation of artificial cells since the first studies in the 1960s (Zhang, Ruder, and Leduc, 2008, Lin, Hansen, Marques and Kiyoshi, 2013, Uruma, Stano, Ueda and Luisi, 2009), yet no artificial cells that can replicate autonomously have been reported to date. Recent work on artificial cells has focused on cell division or replication (Noireaux, Maeda and Libehaber, 2011). I have studied approaches for generating fully operational (self-replicating) artificial cells (Inooka, 2012) and have established a method **??**Inooka, 2016, Ref. 1) by which artificial cells can be cultivated and produce protein in egg white by combining adenosine with sphingosine (Sph) and DNA. I have studied the mechanism underlying the formation of artificial cells and have demonstrated that artificial cells are generated from Sph-DNA aggregates formed using components in egg white **??**Inooka, 2016, Ref. 4).

The surface of artificial cells generated from Sph-DNA consists of DNA and these cells are called DNA crown cells. Experiments **??**Inooka, 2016, Ref. 4) Author: s3inooka@aol.com suggest that DNA crown cells may be proto-cells of artificial cells generated within egg white. I previously reported that cyto-cells and cyto-particles are generated when cultured cells are lysed with Sph-DNA (Inooka, 2000). These cells are also DNA crown cells because their surface consists of DNA. DNA crown cells contain large loops or circles of DNA, similar to the general structure of plasmids. All prepared DNA crown cells can replicate, making it very important to clarify whether the loop structure of DNA is associated with self-replication and thus potentially uncover a new phenomenon applicable to various fields of the life sciences.

Here, I describe three methods for the preparation of DNA crown cells.

## 2 II.

## 3 Methods Summarized and Results

## 4 a) Method to prepare DNA crown cells using animal cell materials

Sph (Sigma, USA); DNA (extracted from quail blood lymphocytes); murine fibro-sarcoma cell line L929/LM (L-M cells)

Step 1 Preparation of assembled sphingosine-DNA (Sph-DNA) and particles 2) Sph-DNA assemblies were formed, as shown in Fig. **??**A. Russert light was observed from the assembly (Fig. **??**C). Russert light was not observed in the sample which not contained Sph (Fig. **??**B) 3) To prepare Sph-DNA particles, Sph (30 µl) and DNA (100 µl) were added to 300 µl distilled water. After mixing, the solution was heated and boiled for several minutes, then concentrated to approximately 100 µl. To detect DNA, ethidium bromide solution was added and the sample was observed under a fluorescence microscope. Sph-DNA particles were formed and fluorescent particles and aggregates were observed (Fig. **??**D). Sph-DNA fibrous assemblies were prepared from a mixture of Sph and DNA and particles were prepared by heating the assembly. Sph-DNA fibers or particles were

Step 2 Cell lysis with Sph-DNA aggregates and particles (Fig. **??**)

2) Sph-DNA particles (0.3-0.05 ml) were added to the confluent cultures of L-M cells. Cell growth was examined under a phase contrast microscope. 3) Sph-DNA aggregates (arrow) were observed on the L-M cells, as shown in Fig. **??**A. 4) L-M cells surrounding the Sph-DNA aggregates were lysed (Fig. **??**B; arrow). 5) 24 hours after the addition, L-M cells surrounding the Sph-DNA aggregates formed plaques with the cell lysis (Fig. **??**C; arrow). 6) 24 hours after the addition, cell particles (arrow)

were observed among the lysed L-M cells (Fig. **??**D).

1) L-M cells were cultured as described in Step 2.

A drop of cells on the slide was observed under a fluorescence microscope.

Sph-DNA particles are cytotoxic and thus can kill neighboring cells, resulting in the generation of DNA crown cyto-cells and DNA crown cyto-particles.

Step 4 Structural integrity of DNA crown cyto-cells Light microscopic observation showed ovalshaped cyto-cells (Fig. **??**A). Fluorescence microscopic observation of L-M cells treated with ethidium bromidelabeled Sph-DNA particles (Fig. **??**B~G) showed fluorescence on the surface of cyto-cells, indicating that Sph-DNA particles were adsorbed on the surface of cells (Fig. **??**B), shrunken cells (Fig. **??**C), rod-shaped cells (Fig. **??**D and E), and cocci or rod-shaped cells (Fig. **??**F). The cocci-shaped cells were <3.0 µm in size (Fig. **??**G).

Step 5 Structural integrity of DNA crown cyto-particles Light microscopic observation of L-M cells after 3 hours of Sph-DNA treatment (Fig. **??**H~J) showed numerous small particles within the cyto-plasma (arrow) (Fig. **??**H), and individual or aggregates of particles (Fig. **??**IandJ) were also observed in the separated cyto-Figures **??**K and 4L show fluorescence microscopic images of DNA crown cyto-particles. Fluorescent particles were incorporated into the cytoplasma (Fig. **??**K) and cyto-particles (arrow) and fluorescent aggregates were also observed (Fig. **??**L), showing that aggregates of cyto-particles were released from cyto-plasma. DNA crown cells were generated from cultured cells lysed due to the cytotoxicity of Sph-DNA. Free Sph is a chelator and exhibits strong cytotoxicity, and thus the addition of Sph to cultured cells immediately lyses the cells. However, I found that some agents bind Sph so that Sph lyses cells slowly. Hence, Sph-DNA may exhibit unique cytotoxicity and generate DNA crown cells.

DNA crown cyto-cells of various sizes can be formed by simply adsorbing Sph-DNA particles on the surface of L-M cells (Fig. 5Aa and b). The formation of DNA crown cells may be based on changes in osmotic pressure caused by covering the cell surface with Sph-DNA, resulting in the cells shrinking and formation of rod-shaped or cocci-shaped cyto-cells (Fig. 5Ac and d). Cyto-cells are composed of Sph-DNA in the outmost layer, followed by a plasma membrane from L-M cells and nucleus from L-M cells.

DNA crown cyto-cells may contain an L-M cell or L-M cell components.

DNA crown cyto-cells have been generated using two kinds of DNA: quail DNA and DNA from L-M cells.

Here, L-M cells were used as cultured cells and quail DNA was used as DNA.

DNA crown cyto-cells can be prepared from cells other than L-M cells, such as monolayer cells, and from DNA other than quail DNA. Numerus DNA crown cells can be prepared with different combinations of cells and DNA.

# 5 DNA crown particles can be formed as follows:

Sph-DNA particles are adsorbed onto cell membranes(Fig. 5B a and b) and incorporated into the cells (Fig. 5B c and d).These cells are then lysed and used to generate DNA crown cyto-particles (Fig. 5B e and f). The surface of these particles therefore consists of the cytoplasm from L-M cells and Sph-DNA particles enclosed in the L-M cell membrane. Many kinds of DNA crown cyto-particles can be generated, similar to the case of DNA crown cyto-cells.

Here, I call DNA crown cells 'DNA crown cytocells' or 'DNA crown cyto-particles' to distinguish between DNA crown cells prepared from cell materials and these prepared from Sph-DNA, as described in the following section.

# 6 -

Step 3 Formation of DNA crown cyto-cells and DNA crown cyto-particles 1) L-M cells were cultured in Dulbecco's modified Eagles medium containing 10% fetal calf serum. L-M cells were seeded (1-2 × 10 4 /well) in 96-well plates (FALCON) and confluence cultures (6-7 × 10 4 /well) were used for the experiments.

2) Sph-DNA particles (0.3 ml) were added to confluent cultures of L-M cells (6-7 × 10 4 /well) and incubated for 3, and 24 hours. The cells were trypsinized, and then the cell pellets were fixed to a glass slide and stained with Giemsa's stain solution. 3) Sph-DNA particles were stained with ethidium bromide. The suspension of

trypsinized L-M cells (10 5 /well) was incubated with labeled Sph-DNA particles (0.2 ml/well) for 5-60 min in a 96-well plate.

iii. Comments on the possible formation and structure of DNA crown cyto-cells and cytoparticles iv. Comment on DNA crown cyto-cells

# 7 v. Comments on DNA crown cyto-particles b) Method to prepare DNA crown cells using egg white components

Sph (Sigma, USA); DNA (Escherichia coli strain B, Sigma); adenosine (Sigma); white Leghorn eggs purchased from a market.

Step 1 Preparation of the components (F-fraction and Dfraction)

Egg whites were injected with 0.5 ml of adenosine (0.1 M) and the eggs were incubated for 5 days at 37°C, after which the egg whites were collected and kept at 4°C until use. Components were prepared from egg white using a protocol similar to the protocol for extracting DNA. One case is described below.

Egg white (300 µl ) was incubated at 65°C for 30 minutes in a microfuge tube, then 400 µl of F-solution (DNA extraction kit, Rizo Inc. Japan) was added and the tube contents were mixed. Then, equal volumes (400 µl each) of phenol and chloroform were added. After mixing, the tube was centrifuged for 10 minutes at 6714 × g.

The aqueous phase was separated from the organic phase and an equal volume of isopropanol was mixed with the aqueous phase. This mixture was centrifuged for 10 min at 15107 × g and the upper layer was collected and kept at 4°C and used as the Ffraction. Then, 1 ml of 70% ethanol was added to the tube. The precipitated DNA fraction was dissolved in 50 µl of distilled water. The fraction was divided between 10 tubes (5 µl aliquots). This sample was used as the Dfraction. The F-fraction was dried and dissolved in 100 µg/ml of distilled water. Distilled water (50 µl) was added to each 5 µl D-fraction aliquot.

Step 2 Preparation of the compound forming by mixing F-fraction and adenosine (F-A solution) F-fraction (1.0 ml, 100 µg ) was added to 1.0 ml of adenosine solution (0.1 M). After mixing, 4.0 ml of ethanol was added, the precipitate (F-A compound) was dried, and then the dried precipitate was re-dissolved in 1 ml of distilled water to provide F-A solution

Step 3 Aggregation of Sph-DNA with D-fraction and F-A solution Aggregates of Sph-DNA could be formed using D-fraction or F-A solution. Sph (90 µl) was added to DNA solution (40 µl), then D-fraction (50 µl) was added. Aggregates of Sph-DNA were formed, as shown in Fig. **??**a.

Using D-fraction or F-A solution, two types of aggregates were formed: mucoid type (Fig. **??**a) and crystal type (Fig. **??**c). Staining the aggregates with ethidium bromide resulted in the observation of Russert light in mucoid type (Fig. **??**b) and crystal type (Fig. **??**d) aggregates, indicating that these aggregates contain DNA.

Figure **??**a shows mucoid type aggregates formed using D-fraction, whereas Fig. **??**c shows crystal type aggregates formed using F-A solution.

Crystal type aggregates were also formed using D-fraction and mucoid type aggregates were also formed using F-A solution. Therefore, the forces driving the aggregation of Sph-DNA were the same in D-fraction and F-A solution.

Step 4 Preparation of DNA crown cells DNA crown cells could be formed using either D-fraction or F-A solution.

Using D-fraction, Sph (90 µl) was added to the DNA solution (40 µl) and then D-fraction (50 µl) was added. After mixing, F-fraction (100 µl) was added. Using F-A solution, Sph (90 µl) was added to DNA solution (40 µl). After mixing, F-A solution (50 µl) was added and mixed, and then F-fraction (100 µl) was added, resulting in the preparation of DNA crown cells. A typical cell stained with ethidium bromide is shown in Fig. **??**.

I only show Fig. **??** to illustrate DNA crown cells because this paper has a page limitation. A detailed description has been published **??**Inooka, 2016, Ref. 4). Comments on possible mechanisms of formation and the structure of DNA crown cells.

DNA crown cells are generated from aggregates of Sph-DNA.

# 8 Sph-DNA aggregates may be formed as follows:

When Sph is mixed with DNA, thread-like fibers are formed (Fig. **??**A).

These fibers may aggregate upon the addition of D-fraction or F-A solution, resulting in the formation of aggregates of Sph-DNA. The addition of F-fraction (which includes lipids) results in the formation of DNA crown cells.

Therefore, the membrane of the cells may consist of Sph, with DNA and Sph in the middle. The cells may be empty or may contain liquid such as distilled water.

D fraction contains adenosine and lipids and Ffraction contains lipids. The components have not been identified and the use of these complex components For F-A solution, Sph (90 µl) was added to DNA solution (40 µl). After mixing, F-A solution (50 µl) was added and mixed.

Aggregates of Sph-DNA were formed, as shown in Fig. **??**c. allows the preparation of various DNA crown cells using DNA rather than Escherichia coli cells. Step 2 Preparation of aggregates of Sph-DNA with A-M Sph (90 µl, 10 mM) was added to 40 µl of DNA (1.7 µg/µl). After heating the mixture, A-M solution (50 µl) was added.

To observe aggregates, one drop of ethidium bromide solution was added to one drop of the Sph-DNA-A-M mixture, then a drop of this mixture was placed on a glass slide and observed using phase contrast microscopy and fluorescence microscopy.

Two types of aggregates, mucoid type (Fig. 8a) and crystal type (Fig. 8b and c), were prepared. Fluorescence was observed on the surface of the crystal type (Fig. 8d), suggesting the presence of DNA.

Thus, aggregates of Sph-DNA could be prepared in a simple manner.

Step 3 Synthesis of DNA crown cells:

To synthesize DNA crown cells, Sph (90 µl, 10 mM) was added to 40 µl of DNA (1.7 µg/µl). After heating the mixture, A-M solution (50 µl) was added and monolaurin solution (50 µl, 0.1 M) was added to the Sph-DNA-A-M mixture. After mixing, the cells were observed as described above.

Cells of various sizes were formed (Fig. **??**) and the surfaces of all cells were covered with DNA (Fig. **??**1), indicating that all cells were DNA crown cells.

A typical cell as observed under a phase contrast microscope is shown in Fig. **??**0.

A layer-like ring or membrane is observed outside. A typical cell as observed under a fluorescence microscope is shown in Fig. 12. Russert light was observed in the outmost layer.

iii. Comments on the possible mechanism underlying structure formation of synthetic DNA crown cells fibers were formed. These branches may spontaneously seal, resulting in the formation of cells. Accordingly, the cell structure may comprise Sph outside, DNA in the middle, and Sph inside, which is the same as cells formed with D-fraction or F-A solution.

Many types of DNA crown cells can be generated using this method.

Artificial cells were generated using Sph-DNA and nucleosides, including uridine **??**Inooka, 2016, Ref. 3), showing that compounds prepared using combinations of nucleosides and monolaurin may aggregate Sph-DNA. Moreover, compounds prepared using combinations of nucleosides and lipids related to monolaurin can also form aggregates of Sph-DNA. Here, I used Escherichia coli DNA, but DNA crown cells can also be prepared using DNA from other sources.

Thus, various DNA crown cells consisting of different components can be prepared by combining nucleosides, lipids and DNA.

iv. Summary of Comments Here, three basic methods were described for preparing DNA crown cells.

Interestingly, these cells can self-replicate (Inooka, 2017).

All DNA artificial cells contain Sph-DNA fibers in the membrane of the cells, whereas the contents of the cells can differ.

DNA crown cyto-cells contain two types of DNA and whole L-M cells may be encapsulated within DNA crown cyto-cells.

Therefore, L-M cells can grow. Enzymes in L-M cells may stimulate Sph-DNA in DNA crown cyto-cells, resulting in the multiplication of DNA.

DNA crown cyto-particles are enclosed by a cell membrane. The DNA in DNA crown cyto-particles may stimulate enzymes in the membrane of the cells, resulting in division of the DNA in the artificial cells.

In contrast, DNA crown cells formed using adenosine-lipids may be empty or contain water. It is unclear how these DNA crown cells could self-replicate. Some components to stimulate division of DNA may be contained in egg white. Thus, DNA crown cells (cytocells and cyto-particles) prepared from cell materials differ from those prepared using egg white components or A-M compound.

The current method for preparing DNA crown cells is performed easily.

Cells whose membranes consist of DNA-lipid have not previously been reported.

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# 10 C

DNA crown cells can be prepared using the purified reagents sphingosine, DNA, adenosine, and monolaurin.

During DNA crown cell formation, Sph-DNA aggregated with A-M solution and branches of Sph-DNA new findings in a wide number of fields in the life sciences DNA crown cells are artificial cells containing a large loop of DNA, similar in structure to general plasmids. Studies using DNA crown cells may provide A-M was prepared by adding 0.4 ml (0.1 M) monolaurin to 0.4 ml (0.1 M) of adenosine solution. After mixing, 0.15 ml of ethanol was added and the precipitate was collected and dried. The A-M precipitate was dissolved in 1.0 ml distilled water and used.

iii.

# 11 Conclusion

Here, I described two biotechnological methods using cell materials and egg components, and a systematic method using A-M compound to prepare artificial cells (DNA crown cells: cells whose membrane consists of DNA). These DNA crown cells were formed using Sph-DNA. Mixing Sph with DNA resulted in the formation of fibrous sphingosine-DNA.

DNA crown cells prepared using cell materials resulted in Sph-DNA fibers covering the surface of the target cell or being enclosed by the cell membrane, resulting in the formation of DNA crown cells.

DNA crown cells prepared using the components of eggs or A-M compound resulted in fibrous sphingosine-DNA spreading with the components or A-M compound, and these sphingosine-DNA bilayers may spontaneously seal, resulting in the formation of DNA crown cells.

A cell membrane associated with DNA has not previously been reported.

Therefore, studies using DNA crown cells may provide new findings in a wide range of fields in cell biology and the life sciences in general. iv. [1] [2] [3]

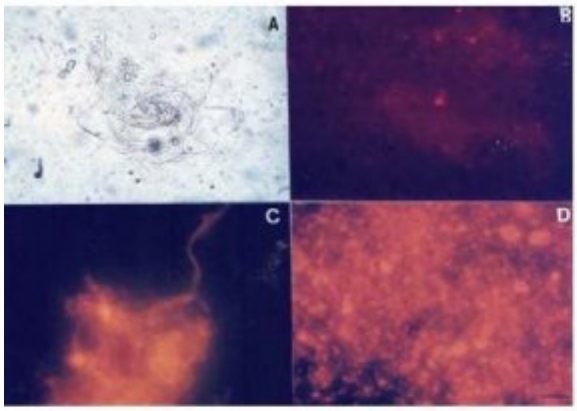


Figure 1: C

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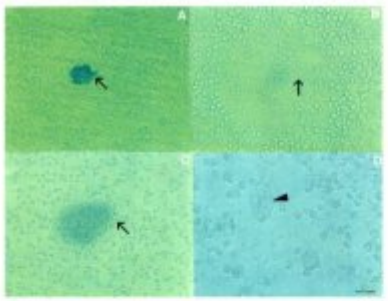

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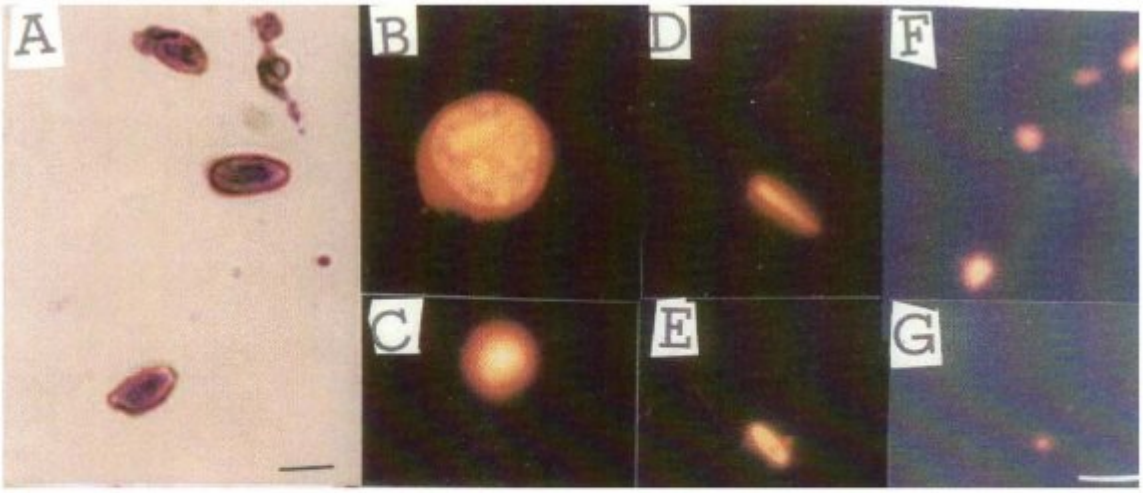


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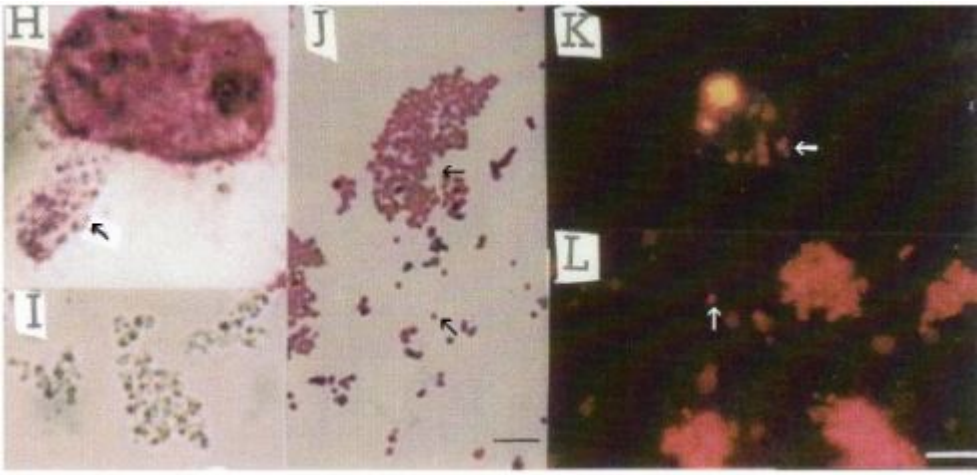


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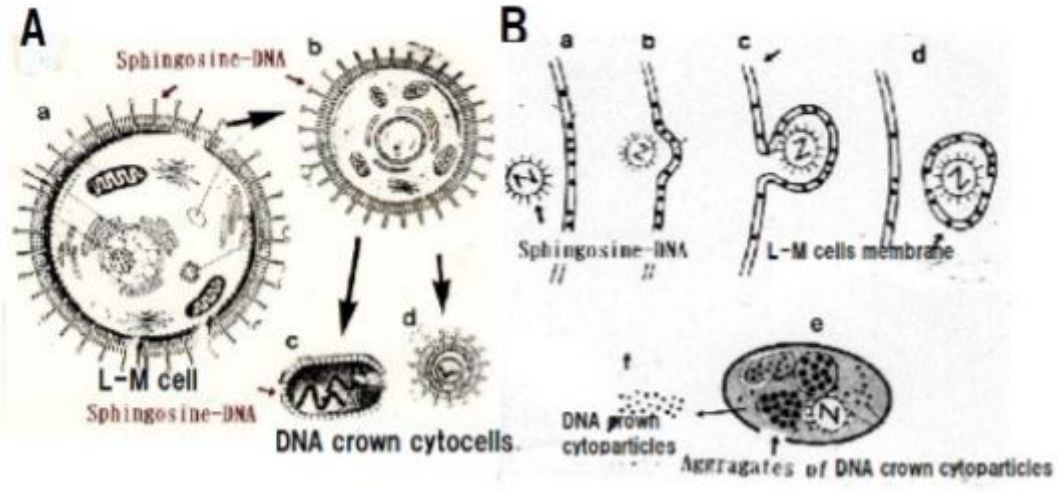
Figure 4: Figure 2 :Fig. 4 :

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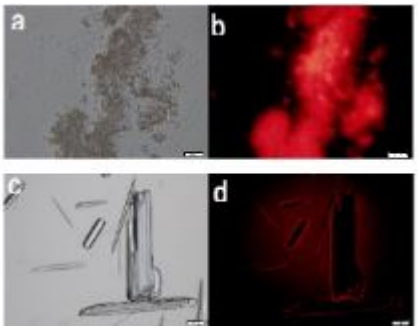


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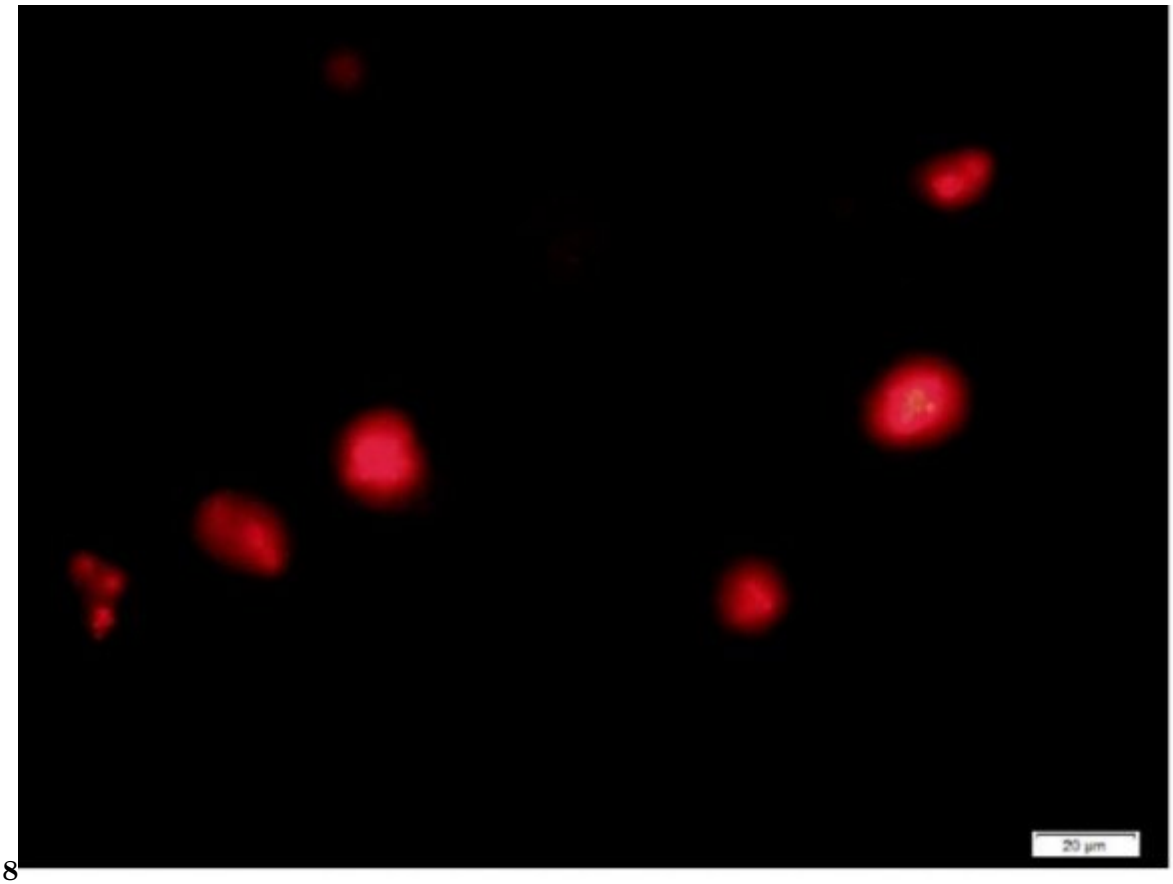
Figure 6: Fig. 6 :Fig. 7 :C

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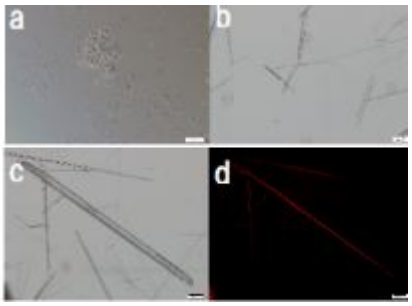


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Figure 8: Figure. 9 :Figure 11 :

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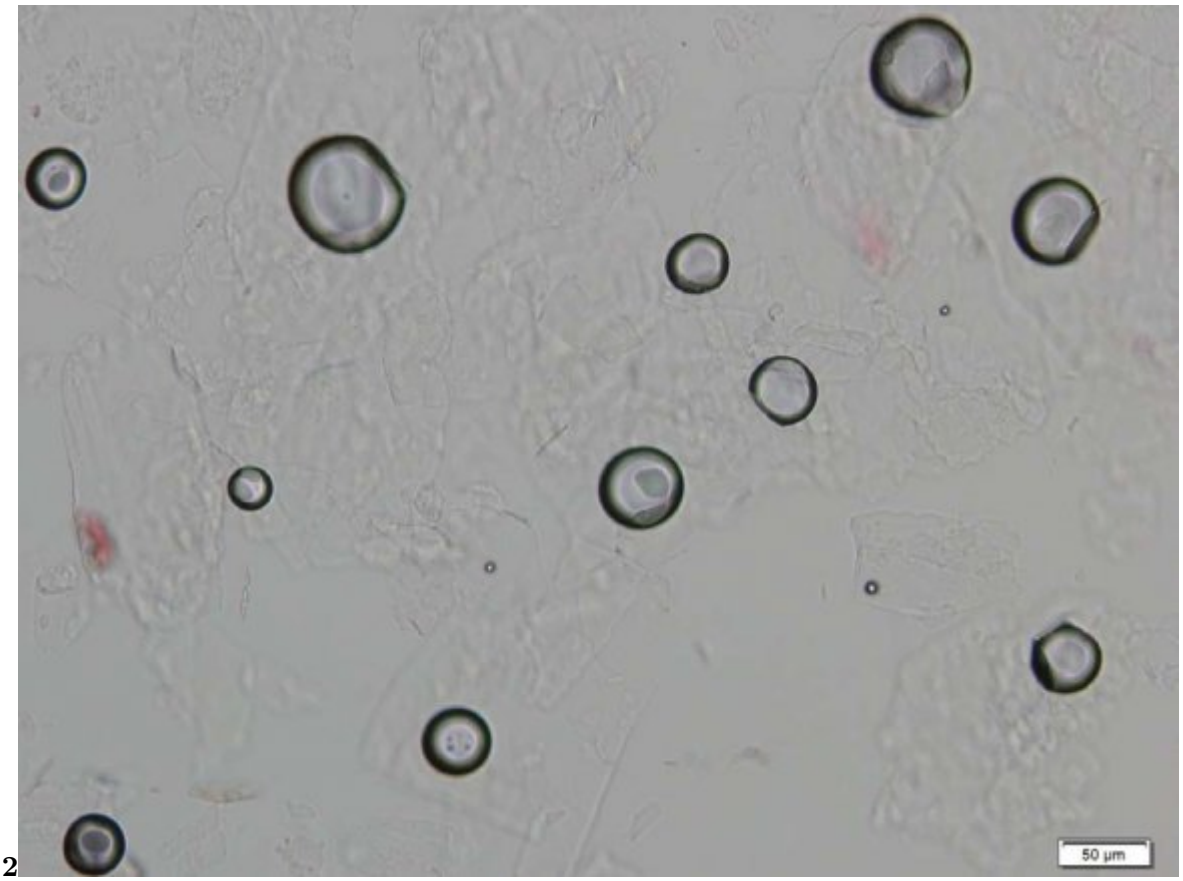


Figure 9: Figure 12 :

# .1 Acknowledgements

I would like to thank Professor Sen-itirou Hakomori (University of Washington, Seattle, USA) for guidance on sphingosine research.

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