Safety Precautions for Practical Laboratories Involved in the Use of Genetically Modified Cellular Slime Mould *Dictyostelium discoideum* Cells.

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Abstract

Genetic composition of living organisms can be modified with a technique called genetic modification (GM) in order to give special characteristics for human benefit. Living modified organisms (LMO) have significant potential to improve our lives. GM is an important part of revolutionary medical treatment associated with induced pluripotent stem (iPS) cells. When regular applications of iPS cells are available, a large number of technicians trained in handling cultured cells and in GM techniques will be required. However, the science literacy of Japanese high school students is rapidly degrading under the current educational system. In order to adminster a proper education in GM and LMO, practical laboratories involving GM and animal cell culture should ideally be included. However, as the school budget for scientific education is limited in general, costly practical laboratories are not desirable. The morphology of social amoebae, cellular slime mould (CSM) *Dictyostelium discoideum* cells, resembles cultured animal cells and the cost for culturing them is lower. GM methods for CSM cells is well established. They are one of the first species whose genome has been fully sequenced. Therefore, they can be an ideal model organism in high school education. We propose the use of CSM in high school practical laboratories involved with GM and discuss how to be compliant with Japanese domestic law, which is based on the international agreement of the Cartagena Protocol on Biosafety.

1. Introduction

Genetic modification (GM) has been developed to modify living organisms in order to add favourable characteristics for humans, such as capabilities to produce valuable substances or tolerances to certain kinds of environmental pressures. Large numbers of living modified organisms (LMO) have been produced and used for many different kinds of applications. Although LMO have significant potential to improve human lives, an adverse effect on the global environment or on human health has been recognized. Following media reports regarding the hazardous health effect of imported GM maize in Japan, food items containing LMO have been firmly refused by Japanese consumers. Although there are several scientific reports describing the safety of certain kinds of LMO, the attitude of Japanese consumers does not seem to improve. A potential reason for such a public mood is the lack of scientific knowledge about GM technology and LMO. In developing countries, GM crops with high nutrient values have an undoubtedly large potential to improve the nutritional condition of the people. GM crops containing medicinal substances may give hope to patients who cannot afford proper medical treatment.

With recent developments in producing induced pluripotent stem (iPS) cells, a totally new way of medical treatment using iPS cells will be possible in the future. When regular applications of iPS cells become available, a large number of medical technicians trained in handling cultured cells and in GM techniques will be required. However, the science literacy of Japanese high school students is rapidly decreasing, as the attached survey indicates[1-2]. A decrease in staff quality in

industrial and scientific research is pointed out in certain technological/scientific areas by the survey of experts in science and industries[3]. Concerning GM technologies, the survey for high school teachers indicates that the safety of LMO is not fully appreciated among them, and many teachers admit that they do not have enough information to properly teach students about LMO [4]. They also recognize that the time allowed them to teach the complex subject of GM technology is not sufficient[4]. Under existing circumstances, it is difficult to arouse the real interest of students in life sciences involving GM technology and LMO.

In order to arouse the interest of high school students in science, ideally they should be able to experience practical laboratories on a regular basis. However, the lack of resources in scientific education is another problem. For practical laboratories, high school teachers are allowed to spend only 510 (yen /student/year) on average under the current educational budget[2].

In order to administer a proper education in GM and LMO, practical laboratories with hands-on experience of GM should be included. In addition, the course should include cell culture. However, the course has to be administered at a nominal cost under the school budget. For this purpose, we have been developing a way to apply cellular slime mould (CSM) *Dictyostelium discoideum* cells in high school education. Cellular slime mould has been used in a wide variety of biological research fields, including cell motility, cell differentiation, development, and morphogenesis. The shape of the cells is amoeboid and resembles cultured animal cells. The GM methods used on this organism are highly similar to the methods used on cultured animal cells. The cultured CSM cells can also be manipulated by methods similar to those applied to the cultured animal cells. Laboratory techniques have been established and widely shared in public through the internet site DictyBase, together with the whole genome information identified by an international consortium[5, 6].

Under the Convention on Biological Diversity, international regulations associated with LMO were discussed, and the Cartagena Protocol on Biosafety has been in force since September 2003. As one of the countries that ratified the protocol, the use of LMO in Japan is strictly regulated by domestic law. Among many different kinds of usage of LMO, laboratory experiments using non-pathogenic microorganisms belonging to Fungi or Protista are categorized in the lowest risk class, termed as Class 1 in Japanese domestic law, and, depending on the design of the experiments, the containment level can be classified as P1, which is also in the lowest containment level. However, as the law has relatively recently come into force, the use of even the lowest class LMO organisms has not been established in the high school laboratory environment yet. In this article, we discuss the following two points in order to find how to use CSM in GM associated practical laboratories: (i)Safety precautions that should be taken in practical laboratory uses of cellular slime mould cells, (ii)Examples of practical laboratories.

2. Safety precautions required in practical laboratory works

2.1 Legal requirements for GM laboratory safety

The use of CSM cells in practical laboratories has to meet the legal requirements of Type 2 Use of LMO for research and development. CSM is a microorganism that has no known pathogenic properties. The experiments using CSM are classified as Class 1. The vector used for GM has to be a certified non-pathogenic plasmid such as the pBR322-derived plasmid. Necessary vectors have to be provided by the universities or research laboratories involved in CSM research. In some cases,

another organism such as bacterial cells may be required. In such cases, all of the organisms have to belong to Class 1. For example, non-pathogenic certified *Escherichia coli* must be used. The containment levels of the GM CSM cells and certified *E. coli* cells are classified as P1 level. P1 level organisms should be handled in laboratory conditions that satisfy the following legal requirements [7].

A. With regard to facilities, the laboratory shall have the structure and equipment as a laboratory for ordinary organisms.

B. In carrying out genetic recombination experiments, the matters stipulated in the following shall be observed.

(1) Before disposing waste products (including effluent. Hereinafter the same applies) containing living modified organisms, a measure for inactivating the living modified organisms shall be taken.

(2) Before disposing or reusing (or washing when it is washed beforehand; hereinafter "the disposal.") an equipment, an apparatus or an appliance stuck with living modified organisms, a measure for inactivating the living modified organisms shall be taken.

(3) A testing bench shall be subjected to a measure for inactivating living modified organisms on each day on which an experiment is carried out upon end of the day's experiment and immediately when a living modified organism sticks to it.

(4) The door to the laboratory shall be kept closed (except when one gets in and out of it).

(5) The windows of the laboratory shall be kept closed and other necessary measures shall be taken to prevent insects from entering.

(6) In all operations, the production of aerosol shall be minimized.

(7) When a living modified organism is taken out of the laboratory in process of an experiment, including the case when one intends to inactivate a living modified organism in any other place than the laboratory, the living modified organism shall be put in a container of the structure that prevents it from leaking or other dispersion.

(8)To prevent a living modified organism from sticking to or infecting a person who handles it, necessary measures including hand washing after handling it shall be taken.

(9)A measure shall be taken to prevent anyone having no knowledge of the contents of experiment from entering the laboratory without permission.

In order to implement the legal requirements shown above and others, we propose the following measures be taken for practical laboratories using genetically modified CSM cells.

(1)Before any use of genetically modified organisms, individual schools should be under the supervision of organizations that have sufficient experience of GM techniques, such as universities or governmental research laboratories. The head of the school should contact one such organization before planning practical laboratories.

(2)The supervisor of practical laboratories should have experience of GM techniques. For this purpose, local universities or governmental bodies should organize training courses for the teachers.

(3) The windows of the laboratory should be kept closed at all times until cleaning of benches, floors and equipment has been completed, and genetically modified cells are either properly stored in a sealed container or inactivated (i.e. completely terminated).

(4) The door of the laboratory should be kept closed and unnecessary entrance/exit should be avoided where possible.

(5) Students should be well briefed before starting experiments and they should be aware of the potential hazards. They should also be aware of the principles and process of the experiments.
(6) The laboratory should be equipped with washbasins in order to de-contaminate CSM cells and other experimentally used LMO, such as Class 1 certified non-pathogenic *E. coli* cells, which may remain on the experimenter's hands.
(7) For cleaning benches, floors and any other surfaces, a 70% ethanol solution is effective. Therefore, sprayers containing a 70% ethanol solution should be prepared, and whenever a solution containing LMO is suspected to have been spilled, the surface should immediately be sprayed with 70% ethanol and cleaned with tissues/paper towels for decontamination.
(8) Genetically modified CSM cells and recipient organisms, such as Class 1 certified non-pathogenic *E. coli*, must be inactivated whenever required in order to contain them in the laboratory environment. For this purpose, autoclaves are normally used in research laboratories. However, autoclaves are relatively expensive for high school budgets and not all schools may have them. In order to satisfy legal requirements, we tested to use diluted household bleach as an alternative method for inactivation. We tested several brands, and found that any household bleach containing sodium hypochloride would effectively inactivate CSM and *E. coli* cells. With our test, incubating the culture medium containing CSM or *E. coli* in a solution containing household bleach in 100-fold dilution (approximated concentration of sodium hypochloride is more than 200 ppm) and household wash-up liquid (the product should be at neutral pH) in 100-fold dilution for 1 hour was enough to inactivate them. However, for safety precautions, we recommend a mixture of freshly diluted bleach in 20-fold dilution (more than 1000 ppm in final concentration) and household wash-up liquid in 50-fold dilution. As the quantity of sodium hypochloride and detergent should be far exceed the necessary level, all cells should be effectively inactivated. As sodium hypochloride is corrosive and can cause irritation, students should be well briefed before handling household bleach.

As another method, pressure cookers could be used as an alternative to autoclaves. Pressure cookers are sometimes used for sterilizing culture media and laboratory equipment in an emergency. However, we found that the temperature in the pressure cookers can be variable depending on the manufacturer. Therefore, inactivation with pressure cookers may not be sufficient, particularly for inactivating *E. coli* cells, as they tend to tolerate high temperatures compared to CSM cells. Therefore, we believe that further evaluation is necessary in the use of pressure cookers for inactivation of the organisms.
(9) At the end of each day, bench tops should be sprayed with 70% ethanol and wiped with tissues/paper towels. When any spill on the floor is suspected, the area should also be cleaned in the same way as cleaning bench tops. Genetically modified CSM or E. coli cells should be stored in sealed containers.
(10) As LMO should never be taken out of the classroom, supervisors should take extra care in containing LMO in the room at all times.

2.2 Verification of containment of LMO

Although the methods of verification of containment of Class 1 organisms in Type 2 use for research and development is not specified, precautions should be taken in order to guarantee laboratory safety and to fully observe legal requirements. In order to verify containment of LMO in the

Name of the experimenter: Date of experiment:
Time for starting: Time for Finishing:

1. Did all the windows and doors of the room remain closed during experiment until cleaning of the benches, floors and equipments was completed?
☐Yes ☐No

If not, please answer following questions in writing.
Which window/door was opened? _____
When and how long were they opened? _____

2. Were the used genetically modified cells inactivated (=totally killed)?
☐Yes ☐No
If the answer is No, please inactivate cells immediately, following the supervisor' s instructions. If there is anything uncertain, ask the supervisor.

3. Are any of genetically modified cells still alive?
☐Yes ☐No

If yes, please answer following questions.
Which type of cells is alive?
☐cellular slime mould ☐bacteria

4. Are the live cells stored in sealed containers?
☐Yes ☐No
If the answer is No, please store the cells in sealed container immediately, and specify the time when live cells are stored: _____(AM / PM).

5. Did you clean the bench tops with 70% ethanol?
☐Yes ☐No
Did you clean the area of the floor around/under your bench with 70% ethanol?
☐Yes ☐No
Did you clean any of equipment which directly touched the genetically modified cells with 70% ethanol?
☐Yes ☐No
Did you ask the supervisor for inspection?
☐Yes ☐No

6. Did you wash your hands carefully?
☐Yes ☐No

Hand this check list to the supervisor when completed.

Figure 1: An example of the safety check list

laboratory environment, supervisors should prepare check-up lists for the decontamination and inactivation process, and the lists should be provided for each student. An example of a check-up list is shown in Figure 1. Students should check whether inactivation, cleaning and decontamination processes are properly performed, and mark it on the list. At the end of each day, the lists should be returned to the supervisors for verification and should be filed and stored for a certain period. A record of planning and execution of practical laboratories should also be stored in the

Name of type of living modified organism		
Place intended for Type 2 Use		Name
		Location
Purpose and outline of Type 2 Use		
Properties of Living modified organism	Recipient organism or species to which the recipient organism belongs	Taxonomical position and state of distribution in natural environment
		History and present State of use
		Mode of reproduction or proliferation
		Pathogenicity
		Other information
	Donor nucleic Acid	Composition, and origins of component elements
		Functions of component elements
	Vector	Name and origin
		Properties
	Modified micro-organism	Method for Preparation
		State of existence of nucleic acid transferred in cell and stability of expression
		Difference from recipient organism or the species to which the recipient organism belongs
Contain- ment measures	Category of use	
	Position of working area	
	Equipments	Arrangement
		Structure
		Production Process
Name of the supervisor		
Qualification and experience of handling of genetically modified organisms		

Figure 2: An example of the record form of the planning of practical laboratories involved with GM and LMO. This example is based on the form of an application stipulated in Article 13 Paragraph 2 of the Act on the Conservation and Sustainable Use of Biological Diversity through Regulations on the Use of Living Modified Organisms (Act No. 97 of 2003).

individual schools. The record should indicate the necessary information shown in Figures 2 and 3.

3. Examples

With the co-operation of the students and supervising teacher of the Biology Club in Sendai Nika High School, a practical laboratory trial was carried out in Ishinomaki Senshu University under the supervision of the author of this article. In order to fully satisfy safety requirements, we chose to perform trial in the university, as it has internal regulations in accordance with the law, and the university facility provides a higher standard of laboratory safety.

Four students participated in the course, which lasted 4 days. The total hours for the course work was 12 hours. The course was designed to identify a mutated gene in a REMI-mutant strain (8), and classes were designed as follows: (Day1) Part 1. Lecture on principles and general methods for identifying the mutated gene, Part 2. Briefing of the safety precautions for handling GM CSM cells (Day 2) (Part 1) Observation of REMI-mutant CSM cells under the microscope (handling of LMO was involved). (Part 2) Collecting cells and extracting genomic DNA (handling of

Name of type of living modified organism		
Place for Type 2 Use		Name
		Location
Purpose and outline of Type 2 Use		
Properties of Living modified organism	Recipient organism or species to which the recipient organism belongs	Taxonomical position and state of distribution in natural environment
		History and present State of use
		Mode of reproduction or proliferation
		Pathogenicity
		Other information
	Donor nucleic Acid	Composition, and origins of component elements
		Functions of component elements
	Vector	Name and origin
		Properties
	Modified micro-organism	Method for Preparation
		State of existence of nucleic acid transferred in cell and stability of expression
		Difference from recipient organism or the species to which the recipient organism belongs
Contain- ment measures during practical lab.	Category of use	
	Position of working area	
	Equipments used	Arrangement
		Structure
		Production Process
Inactivation of all the modified micro-organisms		(Yes or No)
Methods of inactivation of modified micro-organisms		
Name of the supervisor		
Date of completion		

Figure 3: An example of the record form of the completion of practical laboratories involving GM and LMO.

LMO was involved). (Day 3) Digesting genomic DNA and ligating the fragments in order to produce circular DNA fragments. (Day 4) (Part 1) Cleaning up the circular DNA fragments and setting up for inverse-PCR (Part 2) Lecture on the principle of PCR (Part 3) Electrophoresis of amplicons of inverse-PCR (Part 4) Extraction of amplicons, cloning into cloning vectors and transforming bacterial cells (a GM technique was involved)

Using genetically modified CSM cells was a new experience for the students, and they showed strong interest in the organism, as the cells show dynamic movement under the microscope, and shape of the multicellular structure also changes dramatically. At the moment of preparation of this article, students are preparing for presentations in several local scientific meetings. Proper evaluation of the educational effect has not yet been carried out and further trials will be necessary.

(Disclaimer)

This article proposes the precautions for the use of genetically modified CSM cells in high school practical laboratories. Although legal research has been done with extreme care, the proposal itself is still under trial. Therefore, the use of genetically modified CSM cells following the methods shown in this article is at the individual reader' s risk. As the use of genetically modified eukaryotic cells in high school environment is still new to schools, the process needs to be done with extreme care.

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