The *Allium* Test— A Simple, Eukaryote Genotoxicity Assay

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To demonstrate genotoxicity, laboratory exercises in courses such as General Biology or Genetics usually focus on the induction of mutation in a procaryote test system or a simple eukaryote system, such as yeast. For example, often studied exercises include the induction of colorless mutants of *Saccharomyces cerevisiae* (Mayo & Friedrichsen 1993) or *Serratia marcescens* by ultraviolet light or of nutritional mutants of *Salmonella typhimurium* by chemical agents (e.g. Cappuccino & Sherman 1996). An exercise that visually demonstrates clastogenicity, that is, the ability of a test agent to cause chromosomal aberrations, is, for the most part, nonexistent in most laboratory manuals. Perhaps this is because many of these eukaryotic chromosomal assays utilize animal test systems, which are either too complex, time consuming, or costly for laboratory instruction in high schools or undergraduate colleges. Here, we describe a short-term clastogenicity assay that has been standardized using chromosomes of meristematic root cells of the common yellow onion, *Allium cepa* (2n = 16) (Fiskesjo 1985, 1988, 1994; Fiskesjo & Levan 1993). Among other plant test systems, *Allium cepa* has been listed as an example of a plant species useful in the screening of chemicals that damage chromosomes (Nilan 1978; Grant 1982).

The *Allium* test is effective, yet simple. Onions are inexpensive, easy to store and handle, and their root tip cells constitute a convenient system for microscopic evaluation of genetic chromosomal damage. In fact, the excellent cytological conditions in the cells in the root tip of the yellow onion have made this species the classical material for classroom demonstration and study of normal mitosis in plants. We have utilized the *Allium* test as a laboratory exercise in the course, Introduction to Genetics, and it has generated interesting and exciting learning experiences for the undergraduates. Apart from the use of a brightfield compound microscope, the protocol does not require sophisticated scientific equipment (Table 1) and, thus, the *Allium* test can also be used in most high schools. An overall summary of the *Allium* test is as follows. Roots are excised from onion bulblets grown in aqueous solutions of a test agent, root tips are then isolated and stained with aceto-orcein, and chromosomal aberrations are microscopically observed.

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Procedure

A. Growth of the Bulblets

Onion bulblets are preferable to larger onions because they fit into 50-ml test tubes. Bulblets can be obtained locally or purchased through a supply house. Conical-bottom plastic

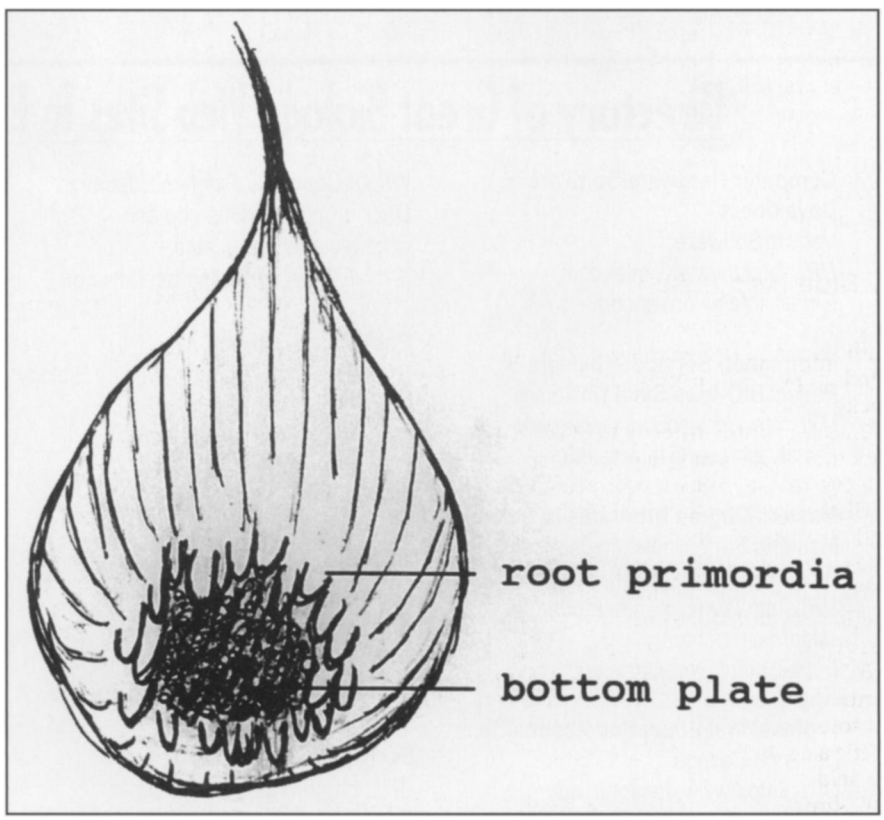

Figure 1. Underside of a dormant, nongrowing onion bulblet.

Table 1. Materials needed for the *Allium* test

Material	*Company*	*Catalog #*
Onion bulblets	Carolina Biological Supply Co. (800) 334-5551	K3-17-1140
Irradiated onion root tip (microscope slide)		B 555C
Orcein	Sigma Chemical Co. (800) 325-3010	0 7380
50 ml polystyrene tubes	Fisher Scientific Co. (800) 766-7000	0 5-539-11

50-ml centrifuge tubes are available in disposable racks that can be used to store the tubes during the experiment.

1. Using pointed forceps, peel off the yellow-brown outer scales and scrape off some of the brownish bottom plate, carefully leaving intact the surrounding ring of root primordia (Figure 1).
2. Place individual bulblets into test tubes containing either water (control) or aqueous solutions of the test agent. Orient the onion with the root primordia immersed into the water. If needed, toothpicks can be inserted into the onion bulblet to allow it to gently rest in the solution (Figure 2). Tubes are maintained at room temperature in the dark for two days, a time period that yields the maximum number of mitotic cells. Evaporation after 24 hours will require additional water to be added to prevent the growing roots from drying.
3. Before excising the roots to assess the genotoxic effects, measure and compare relative lengths of the roots in the control tube (between 2 and 4 cm) and in the tubes containing progressively increasing concentrations of the test agent.

B. Slide Preparation

1. Place the growing onion bulblet with its white roots on a piece of darkened paper to provide a contrasting background.
2. Use pointed forceps to clip off the root and, with a single-edge razor, cut the top of the excised root at an angle to distinguish the terminal from the cut end. If desired, the root tips may be placed in a preservative consisting of 3 parts ethanol to 1 part concentrated acetic acid and stored in a freezer until needed.
3. Place the root tips into test tubes containing 3 ml of a fixation/hydrolysis solution consisting of 9 parts of 45% acetic acid and 1 part of 1 N hydrochloric acid.
4. Immerse the tubes in a 48–52° C water bath for 6 minutes. The heated acid helps to dissolve the "cement" that binds the cells to each other, thereby facilitating their spread on the slide. After this interval in the fixation/hydrolysis solution, the root tips are very soft and should be carefully removed with pointed forceps.
5. Place the root tips on the darkened background paper and remove excess fixative with a paper towel.
6. Using a single-edge razor blade, cut off the terminal 2 mm of the root tip and place it on a microscope slide; the remainder of the root is discarded.
7. Add two drops of aceto-orcein stain and let stay for 2 minutes.
8. Using a flat-end spatula, squash and mix the softened root tip in the stain. Allow 2 minutes for the cells to absorb sufficient stain.
9. Add a coverslip and cover the entire slide with a paper towel. Gently press the coverslip to further squash and spread the cells and to blot the excess stain. Examine microscopically for chromosomal aberrations. The oil immersion objective is not needed.

The aceto-orcein stain, which is specific for DNA, is used to visualize the chromosomes. The stain is prepared as follows: 5 g of orcein is added to 150 ml of acetic acid already heated on a stirring hotplate. A foam will appear. The solution is heated (not boiled) again. Retain the orcein stain for 2–3 days in a dark bottle (e.g. a vessel covered with aluminum foil); shake several times a day. Thereafter, add 150 ml of distilled water, filter, and store in a dark bottle. This stain is stable for several weeks. Should excess precipitate be evident, centrifuge the amount of stain needed for that day's exercise.

Before examining root tips from onions grown in the presence of test agents, it is important to have a clear understanding of the morphology of normal mitotic chromosomes. Figure 3 shows a typical microscopic field of view of these cells, including some in various stages of mitosis. After examining normal mitotic chromosomes, proceed to study cells from roots treated with a test agent.

A vast variety of unusual chromosomal morphologies may be encountered, the most common types of chromosomal aberrations include:

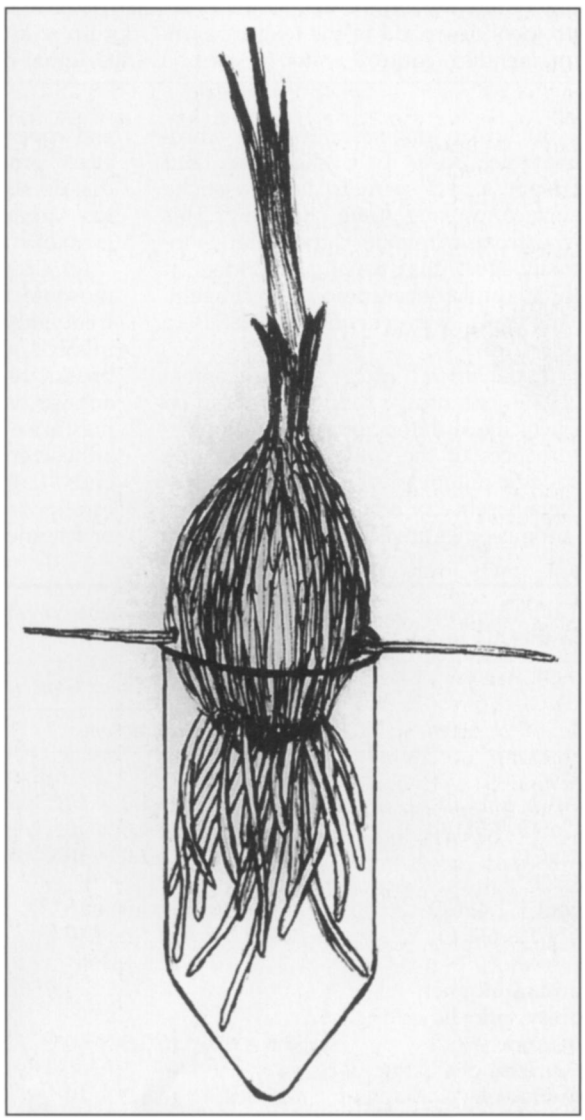

Figure 2. Growing onion bulblet supported in a test tube by toothpicks inserted into the bulb portion.

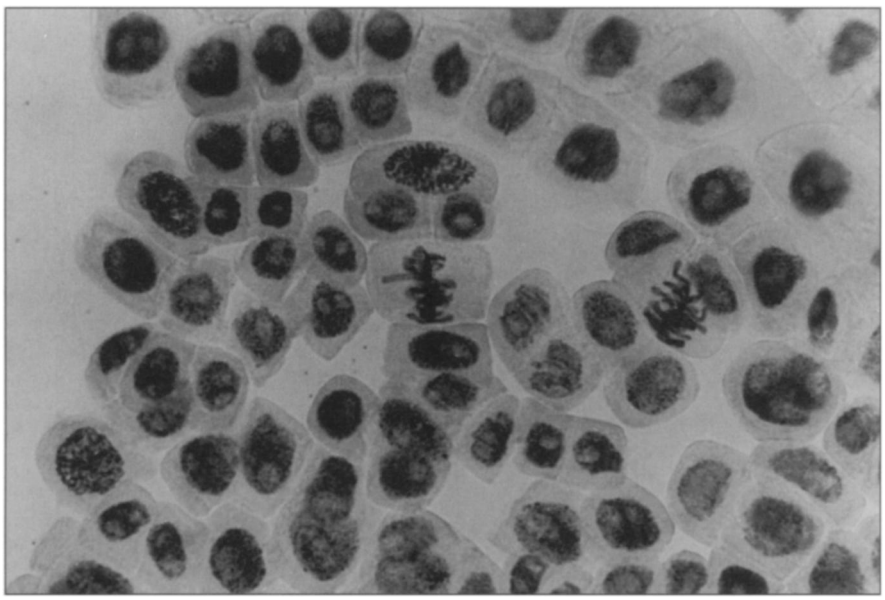

Figure 3. Microscopic field of view of cells, prepared and stained according to the protocol described in the text, from the root tip of an onion bulblet maintained in unamended (control) water for two days. (Original magnification: 400×)

(a) *Sticky chromosomes*—The chromosomes lose their sharpness and crispness and seem to have a sticky surface, causing some clumping. This is a strong cytotoxic, rather than genotoxic, effect, that usually is evident at the highest concentration of test agent. This effect is irreversible and leads to cell death.

(b) *C-mitotic effects*—Chromosomal aberrations similar to those induced by colchicine, a mitotic poison causing disturbances of the spindle apparatus. A strong c-mitotic effect may result in complete absence of a spindle; here, all the chromosomes are arrested in metaphase and appear contracted. A weak c-mitotic effect produces vagrant chromosomes that do not attach to the spindle apparatus. Vagrant chromosomes may lead to genotoxic effects, such as aneuploidy.

(c) *Clastogenic effects*—Refer to chromosome damaging effects. The most frequently observed clastogenic effects involve chromosome and chromatid breaks, resulting in fragments and anaphase bridges. This latter aberration may arise by translocation or simply by adhesiveness of sticky chromosome ends. Lagging chromatin, another aberration, includes whole chromosomes or fragments observed between poles.

Table 2. Range of concentrations of test agents causing chromosomal aberrations in the *Allium* root tip test.

	Molar Concentrations Causing	
Test Agent	*Sticky Chromosomes*	*C-Mitosis/Clastogenicity*
Inorganic[a]		
$AlCl_3 \cdot 6H_2O$	3.3×10^{-4} to 1.0×10^{-3}	3.3×10^{-5} to 3.3×10^{-4}
$CuSO_4 \cdot 5H_2O$	2.5×10^{-6} to 1.0×10^{-5}	1.0×10^{-6} to 5.0×10^{-6}
$HgCl_2$	3.3×10^{-6} to 1.0×10^{-4}	1.0×10^{-3} to 3.3×10^{-6}
LiCl	1.0×10^{-1}	1.0×10^{-3} to 3.3×10^{-2}
$MnCl_2 \cdot 4H_2O$	1.0×10^{-2} to 1.0×10^{-1}	1.0×10^{-3} to 1.0×10^{-3}
$NiCl_2 \cdot 6H_2O$	1.0×10^{-3} to 1.0×10^{-2}	3.3×10^{-5} to 1.0×10^{-3}
Organic[b]		
acetaminophen	—	1.3×10^{-4} to 6.6×10^{-4}
acetylsalicylic acid	—	1.1×10^{-4} to 2.7×10^{-4}
ethanol	8.6×10^{-2} to 3.5×10^{-1}	8.6×10^{-3} to 3.5×10^{-1}
ethylene glycol	—	1.7×10^{-1} to 8.9×10^{-1}
methanol	2.5×10^{-2} to 1.2×10^{0}	2.5×10^{-2} to 1.2×10^{0}

[a]Fiskesjo (1988)
[b]Fiskesjo and Levan (1993)

Genotoxic effects, such as the presence of micronuclei (resulting from vagrant chromosomes or chromosomal fragments that form their own small nuclei) or giant nuclei (resulting from polyploidy), may be noted in cells in interphase. Examples of these aberrations are noted in Figure 4.

Every chromosomal aberration shown in Figure 4 is not induced by each test agent. Thus, it is suggested that several different test agents be studied simultaneously; enough roots are produced per onion for students to exchange samples. Table 2 is a listing of suggested test agents. However, interested individuals are encouraged to evaluate other test agents and applications. For example, an interesting application of the *Allium* test is to assess the quality of natural aquatic systems and of drinking waters, e.g. tap water versus bottled water (Fiskesjo 1985, 1993). Students can be encouraged to test aqueous samples obtained from polluted sites, such as ponds or streams near gasoline stations or landfills. These studies can be further developed into mini-research projects for courses in ecology, botany or genetics.

This experiment has been performed by our undergraduates, and much enthusiasm and excitement was generated when the students identified chromosomal aberrations in their own preparations. Especially exciting were the aberrations seen in plants treated with acetaminophen and other over-the-counter drugs. Although there are differences in metabolism between plants and animals, there are also basic similarities. Plants, as animals, have mixed the function oxidase systems needed for the activation of promutagens to genotoxic chemicals (Higashi 1988). Results reported in the *Allium* test are in good agreement with those from mammalian cell test systems (Grant 1978, 1982). Thus, there is strong support for extrapolating data from the *Allium* test to potential health risk to humans. A test agent that induces a clastogenic effect in the *Allium* test may also be genotoxic to human beings.

The topic of environmentally-induced chromosomal damage can be extended to physical clastogens, such as ionizing radiations. A commercially-available microscope slide of radiation-induced chromosomal damage in the root tip of *Allium cepa* can be used to supplement this laboratory exercise. Many of the same chromosomal aberrations (e.g. anaphase bridges; fragments) induced by chemical clastogens (Figure 4) are also noted in this prepared slide. Interesting discussions on radiation-induced genetic damage can be developed, such as the increased

risk of specific cancers in people, children in particular, exposed to radiation from the Chernobyl incident in the former Soviet Union.

In summary, the easy handling, low costs, and excellent chromosomal constitution of the onion, make the *Allium* test an excellent hands-on teaching tool to study chemically-induced genotoxicity in eukaryotic systems. The materials for and the protocol of the *Allium* test allow it to be conducted in laboratories in most high schools and undergraduate colleges. The uniqueness of this exercise is that it demonstrates genotoxicity at the level of chromosome damage.

Acknowledgments

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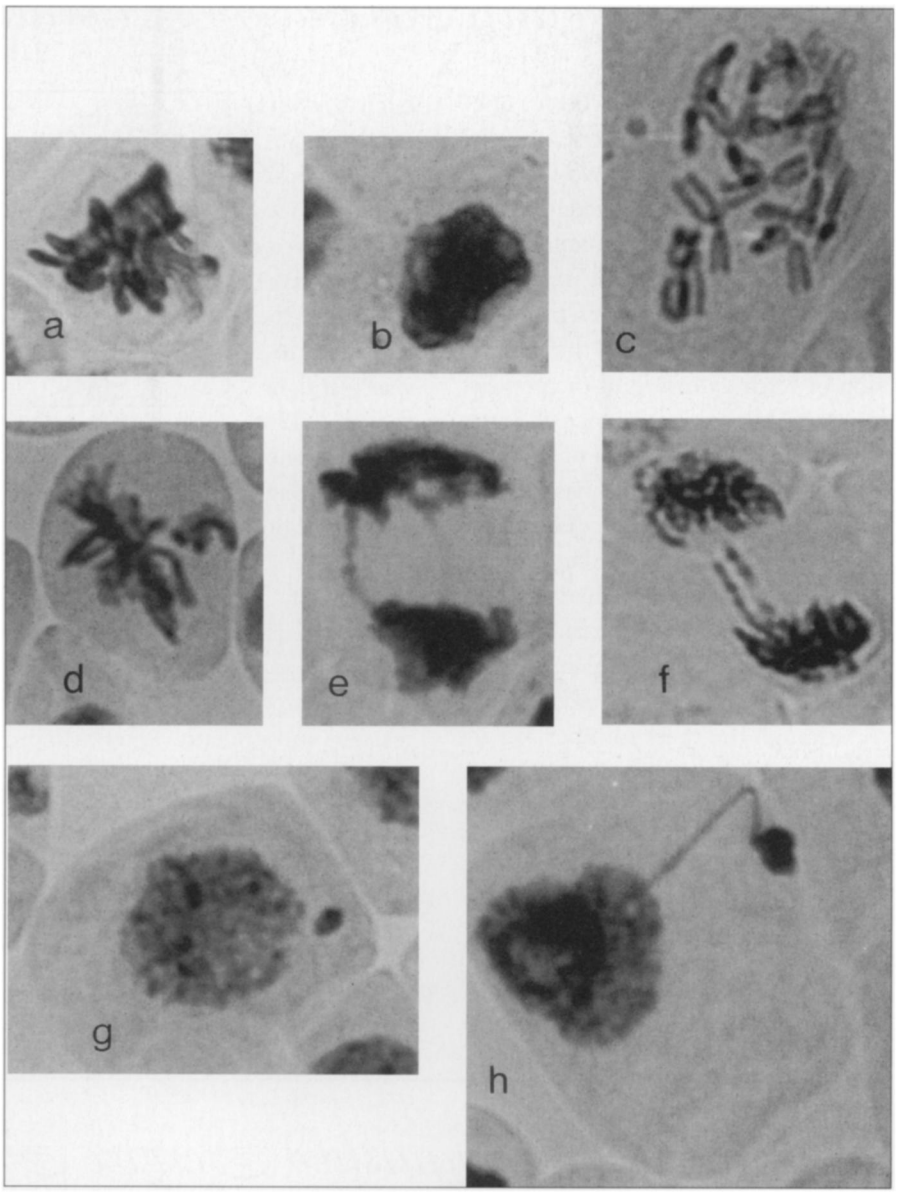

Figure 4. Chromosomal and nuclear aberrations in cells from onion bulblets exposed for two days to various test agents.
(a) sticky chromosomes; 1 × 10^{-2} M $MnCl_2 \cdot 4H_2O$
(b) sticky, rather than granular, nuclear chromatin; 1 × 10^{-5} M $CuSO_4 \cdot 5H_2O$
(c) c-mitosis; 1 × 10^{-4} M $NiCl_2 \cdot 6H_2O$
(d) vagrant chromosomes; 1 M methanol
(e) anaphase bridge; 1 × 10^{-4} M $NiCl_2 \cdot 6H_2O$
(f) lagging chromosomes; 1 × 10^{-4} M $NiCl_2 \cdot 6H_2O$
(g) micronucleus; 1 M methanol
(h) nuclear filament with terminal expansion; 1 × 10^{-3} M acetaminophen. [We noted this aberration only with acetaminophen].
(Original magnification: 400×)