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Fluorescent Focus Assay of Viruses on Cell Monolayers in Plastic Petri Plates. (32232)

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Fluorescence microscopy and fluorescent focus (FF) assay techniques have been used extensively in the study of viruses and the quantitation of viral infectivity(1-7). All of the methods reported to date, of which we are aware, employed glass cover-slips upon which cell monolayers were grown. Viruses were inoculated upon these cover-slips and each cover-slip put through a series of washings, a fixation, various treatments to reveal viral antigens, and a final mounting on a glass slide. We have found that these manipulations are tedious and usually involve significant risks in breaking the delicate cover-slips bearing infected cells. The present report describes a technique which permits cell growth, inoculation, fixation, staining and examination of fluorescing cells directly in plastic Petri plates.

Materials and methods. Plastic Petri dishes, 35 × 15 mm were obtained from Falcon Plastics Co., Los Angeles, Calif. Human amnion (HA, strain FL) cells were grown in 32-oz. bottles, dispersed by trypsinization, counted in a hemocytometer and mixed with Eagle's No. 2 plus 5% fetal bovine serum to give a concentration of 2×10^5 cells/ml. Two ml of this suspension were added to each plate. Plates were incubated undisturbed for 24 hours at 36°C under 5% CO_2. It was important to have an even distribution of cells in the plates.

Viruses. Adenoviruses 1, 2 and 12 and adeno-associated virus (AAV) type 1 were the same ones used in other studies(8,9). Reovirus type 1(10) and its specific antiserum (prepared in rabbits by injection of density gradient purified particles) were also used.

Inoculation of plates. Medium was removed and 0.50 ml virus added to each dish. Plates were rocked every 10 minutes (at room temperature) to insure complete coverage of the cell monolayers. Inoculum was removed by aspiration at the end of one hour and 2.5 ml of fresh media added to each plate. Plates were reincubated for 24 to 48 hours, depending upon the virus used(9).

Fixation. Medium was aspirated and 2 ml of 0.85% saline added to each plate. Each plate was washed in this manner 4 times, for a total wash time of 3-4 minutes per plate. The fixative, which consisted of 95 volumes of absolute methyl alcohol and 5 volumes of 0.85% saline, was added and the cells allowed to fix for 4 minutes. Fixative was removed, 2 ml of 0.1 M phosphate buffer, pH 7.1, was added and the buffer allowed to remain for

4 minutes. Plates could be stained immediately or stored indefinitely at —70°C. Rinsings and washings were expedited by use of a Cornwall syringe fitted with a self-filling valve. The stream, directed at the rear wall of the dish, washed back over the cell monolayer gently and rinsed it thoroughly without noticeable loss of cells.

Antisera. The details of antiserum production in rabbits have been described elsewhere (8,9). Antisera were absorbed twice with sonic treated normal HA or KB cells, then clarified by centrifugation for ½ hour at 12,000 × *g* prior to use. Fluorescein conjugated anti-rabbit γ globulin (sheep origin) was obtained from Baltimore Biological Laboratories, Baltimore, Md. Anti-adenovirus serum did not produce fluorescence in normal or reovirus infected cells, nor did anti-reovirus serum produce fluorescence in normal or adenovirus infected cells.

Staining. Stored plates were thawed, rinsed with phosphate buffer for 4 minutes, and buffer aspirated. The appropriate antiserum was added, 0.4 ml per dish, and allowed to stand for 30 minutes at room temperature. Dishes were rocked at 10 minute intervals. Antiserum was removed and plates rinsed 6 times with phosphate buffer, 2 ml per rinse. Total rinsing time was at least 4 minutes per plate. This was a critical step in the procedure because thorough rinsing was necessary to avoid carry-over of material which would give background fluorescence.

Following rinsing, 0.4 ml of the conjugated anti-rabbit γ globulin was added per plate and allowed to react at room temperature for 30 minutes (with 10 minute rockings). After removal of the conjugate, plates were rinsed 3 times with phosphate buffer. Plates were then ready for examination with the fluorescence microscope. No protective glass cover-slips were necessary if the cells were kept wet with buffer.

Examination of plates was with a Bausch and Lomb microscope fitted with a dark field condenser. Source of the UV light was an Osram HBO 200 high-pressure mercury arc lamp. Barrier filter used was T-2 and exciter filters 5-58 or 7-51. Scanning of fields and counting was done using a 10× eye-piece and a 10× objective. More detailed examination was done using the 43× or 97× objectives. A grid was inserted into the eye-piece to facilitate the counting of foci in fields of known area. The use of plastic dishes resulted in no noticeable loss of light transmission or color fidelity as compared with glass cover-slips and glass slides. Examination of plates was facilitated by using a dish holder attached to the microscope's mechanical stage. The inside diameter of a Petri plate base was 32 mm, giving a total area of 804 mm^2 for cell growth. The area viewed in the gridded space at 100 × was 0.27 mm^2. A single focus per grid space would, therefore, be equivalent to 3.0×10^3 foci per plate (804 mm^2/0.27 mm^2). The infectivity (FF) titer of undiluted virus could be determined by the following calculation:

FF/0.5 ml = (1/virus dilution) (number FF/field) (3.0×10^3).

Comparison of FF assay with plaque assay. Adenovirus 2 was serially diluted and inoculated onto Petri plate cell monolayers at appropriate dilutions (lower dilutions for FF assay and higher dilutions for plaque assay). Inoculation was for one hour, after which inoculum was removed by aspiration. Cells inoculated for plaque assays were overlaid with a mixture containing Eagle's medium, 4% fetal bovine serum and 1.4% Noble Agar (Difco). On days 6, 7 and 8 post-inoculation, Petri plate cultures were overlaid with agar containing neutral red, re-incubated for 2-3 hours, and plaques counted. FF assays were done as described above.

Results. The appearance of cells infected with adenovirus 1 (a nuclear virus) and reovirus (a cytoplasmic virus) is shown in Fig. 1.

To test the reproducibility of this FF assay method, adenovirus 12 was diluted 10^{-1}, inoculated onto several plates of HA cells and incubated for 44 hours. Plates were stained with adeno 12 antiserum in the usual way. The fluorescent foci in 3 random fields from each plate were counted and the average number of foci per field calculated. Table I, last column, shows the average number of FF counted per field in 6 different plates. The average number of FF in all plates was 89,

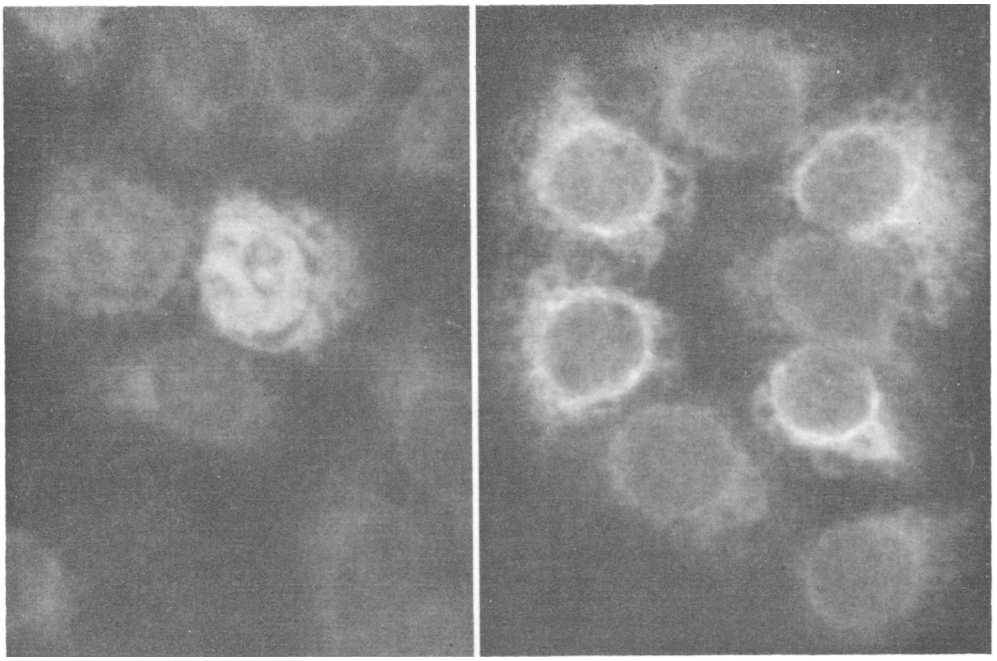


FIG. 1. Left: Adenovirus 1 (a nuclear virus). Right: Reovirus (a cytoplasmic virus). Micrographs were taken with an oil immersion lens on Kodachrome × film. Black and white reversed prints are shown here.

with a standard deviation of 7.4 (less than 10%). It was concluded on the basis of this and other experiments that this method is quite precise if large numbers of FF are counted (preferably over 100 per plate).

The validity of the method as a means for determining infectious virus concentration was studied. Five-fold dilutions of adenovirus 12 were inoculated on cell monolayers, incubated, fixed, stained and FF counted in each culture. Fig. 2 shows the results obtained in one such experiment. Over the range of virus concentrations inoculated, the number of FF counted was almost exactly proportional to the relative concentrations of the virus. Similar results were obtained using serially diluted reovirus 1 (Fig. 3) and AAV (see accompanying paper, 9).

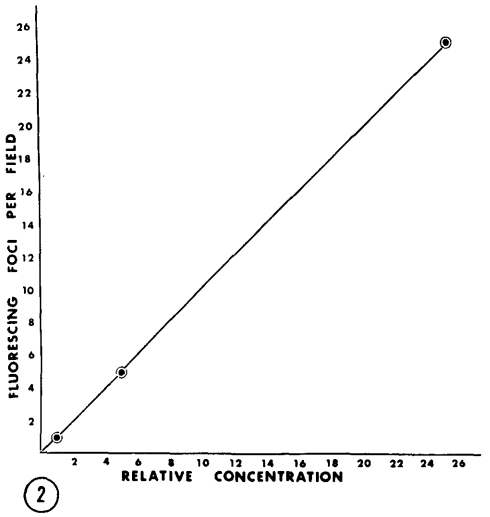
TABLE I. Study to Determine the Precision of Fluorescent Focus Assay Technique for Adenovirus Type 12.

Culture No.	Total fluorescing foci counted per 3 fields	Calculated No. of fluorescing foci/field
1	305	102
2	261	87
3	274	91
4	261	87
5	267	89
6	236	79
Avg	267	89*

* Standard deviation = 7.4.

We compared the sensitivity of the FF assay method with the plaque assay method for titrating adenovirus type 2 (see *Materials and Methods*). In one experiment, a sample titrating 9.0×10^6 FF/ml titrated 1.9×10^7 plaque forming units/ml. We conclude that the two methods are approximately equal in sensitivity for titrating this virus, the FF method possibly being slightly less sensitive. The FF method sensitivity was increased by overlaying infected cells with 0.25% agar, incubating for periods longer than 24 hours, removing the semi-solid medium, then proceeding as usual. Isolated groups of several infected cells were then counted as single foci.

We have also studied the morphology of cells infected with herpes simplex, vaccinia and influenza, using the fluorescent antibody technique in Petri dishes, but we have not attempted to quantitate these viruses. Acridine



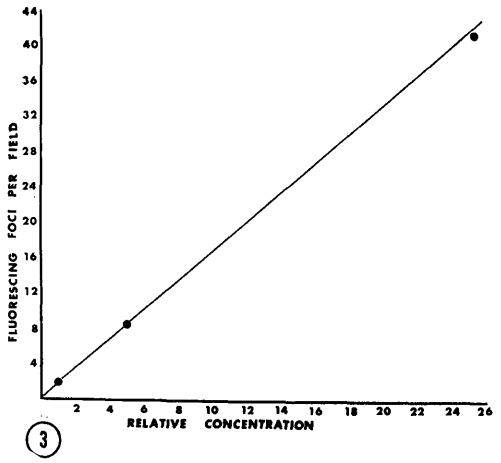


FIG. 2. A dose-response experiment to determine the validity of the FF assay technique for titrating adenovirus 12.

FIG. 3. A dose-response experiment to determine the validity of the FF assay technique for titrating reovirus type 1.

orange studies of virus infected cells were equally satisfactory in plastic Petri plates.

Discussion. The use of plastic Petri plates instead of conventional cover-slips for the cultivation of cell sheets resulted in several advantages. Foremost was the ease of handling and manipulation of the cell monolayers. There was no danger of losing the cells by dropping or breaking. Confusion concerning which surface of a cover-slip should be stained was obviated. Fixation and staining was much easier and more uniform because a large group of plates could be handled in the same way at the same time. The large surface area of the plate permitted the study and comparison of many representative fields, which resulted in higher precision than we found practical on cover-slips.

There were always many regions available for photographing which had not been examined and thereby bleached by the UV light. The optical qualities of the plastic Petri plates are sufficiently good to permit close scrutiny of cell morphology. The plate holder made examination rapid and systematic. The gridded eye-piece inserted into the ocular permitted precise counts of FF per unit area.

Larger amounts of reagents were necessary for staining cells in Petri plates, as compared with cells on cover-slips, because of the much larger surface area in the plates. However, plates can be marked off (subdivided) into several small areas with suitable marking materials, and only small portions used. This reduces the requirement for reagents and permits simultaneous testing of several sera on one plate.

Summary. The use of plastic Petri plates for cell growth and FA staining resulted in substantial savings in time and effort, and gave more uniform results than did glass coverslips. Our results suggest that this procedure is precise and valid for quantitation. The technique was used to titrate adenovirus 2, 12, AAV and reovirus.

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