injected per hamster. All hamsters became infected and in those inoculated with the same number of sporozoites the prepatent period did not vary by more than ± 8 h. The infectivity of sterile sporozoites did not differ appreciably from that of nonsterile ones (correlation coefficient: 0.99).

Discussion. Several years ago, there was considerable research interest in the cultivation of the sporogonic stages of *Plasmodium* spp.[8]. With the exception of the in vitro formation of ookinetes of *P. berghei* from rodent blood[9], attempts to cultivate further developed mosquito stages of malaria of mammals have been frustrated, firstly, by the large numbers of fungi and bacteria associated with the digestive tract of mosquitoes, and, secondly, by the unavailability of culture systems that support extended parasite development in vitro. Recently, we have cultured guts and abdomens individually in antibiotic-free medium for up to 10 days (unpublished). Incidents of contamination with either fungi or bacteria were neither observed in these cultures nor in the rearing containers of the experiments described here. The samples taken from larval cultures equally remained sterile. In later experiments, contamination of adult cages was sometimes experienced when it was necessary to offer two consecutive infective blood meals. This was evident from the condition of the sucrose-hypoxanthine solution in the U-tube, and the filter paper. This problem can be alleviated by the use of autoclaved membrane feeders equipped with surface-sterilized membranes, rather than hamster. Keeping the mosquitoes at the lower temperature shortly before and after the infective blood meals resulted in a higher proportion of infected females. Presumably, this treatment reduced the activity of the proteolytic enzymes in the midgut[10], thus allowing a greater number of ookinetes to develop into oocysts. This contention is supported by the work of Gass[11, 12] and Gass and Yeates[13] who demonstrated that malarial ookinetes can be subject to considerable damage by digestive enzymes in the mosquito midgut. Even with high levels of infection, mosquito mortality was usually lower than in the conventionally reared colony where up to 90% of the females may not survive the 18 days required for complete sporogony. We attribute this to the lack of microbes which in nonsterile mosquitoes can aggrevate the delicate condition of heavily infected females[14]. We feel that the wide variations in the percentage of infected female mosquitoes are due to the problems of handling material in a newly developed system. The modification of Leibovitz's L-15 medium described here readily supports the growth of a wide variety of mammalian and invertebrate cells in vitro (Munderloh and Kurtti, unpublished). In conjunction with mosquito cells, it permits the rearing of axenic *A. stephensi* that are efficient vectors of rodent malaria, and are likely to transmit other species as well.

1 Supported in part by Public Health Service research grant AI 18345 from the National Institute of Allergy and Infectious Diseases, by a grant from the Agency of International Development DSPE-5542-G-SS-3042-00, and by a Charles and Johanna Busch award.

2 Boorman, J., Nature, Lond. *213* (1967) 197; Rosales-Ronquillo, M.C., Simons, R.W., and Silverman, P.H., Ann. ent. Soc. Am. *66* (1973) 949; Hamilton, D.R., and Bradley R.E., Sr, J. Invert. Pathol. *30* (1977) 318.
3 Sneller, V.-P., and Dadd, R.H., Exp. Parasit. *51* (1981) 169.
4 Munderloh, U.G., and Kurtti, T.J., part of this work was presented as a poster at the 2nd International Conference on Malaria and Babesiosis, Annecy, France, September 1983.
5 Vanderberg, J.P., Nussenzweig, R.S., and Most, H., J. Parasit. *54* (1968) 1009.
6 Munderloh, U.G., Kurtti, T.J., and Maramorosch, K., J. Parasit. *68* (1982) 1085.
7 Warhurst, D.C., and Folwell, R.O., Ann. trop. Med. Parasit. *62* (1968) 349.
8 Schneider, I., and Vanderberg, J.P., in: Malaria, vol. 2, p. 235. Ed. J.P. Kreier. Academic press, New York 1980.
9 Weiss, M.M., and Vanderberg, J.P., J. Parasit. *63* (1977) 932.
10 Clements, A.N., The Physiology of Mosquitoes, p. 154. Pergamon Press, New York 1963.
11 Gass, R.F., Acta trop. *34* (1977) 127.
12 Gass, R.F., Acta trop. *36* (1979) 323.
13 Gass, R.F., and Yeates, R.A., Acta trop. *36* (1979) 243.
14 Maier, W.A., Z. ParasitKde *41* (1973) 11.

0014-4754/85/091205-03$1.50 + 0.20/0

A simple method for counting nuclei in the preimplantation mouse embryo[1]

K.M. Ebert, R.E. Hammer and V.E. Papaioannou

Tufts University School of Medicine, 136 Harrison Avenue, Boston (Massachusetts 02111, USA), and Laboratory of Reproductive Physiology, School of Veterinary Medicine, University of Pennsylvania, Philadelphia (Pennsylvania 19104, USA), 23 October 1984

Summary. An easy and rapid method of counting the number of cells in the preimplantation mouse embryo is described. The procedure increases the speed with which large numbers of embryos can be processed using a simple squash technique. Cell numbers are determined by exposing the embryos to the fluorescent DNA-binding dye, Hoechst 33258, removing the zona pellucida and simply squashing the embryo and counting the number of fluorescent nuclei. An increase in fluorescent intensity and maintenance of nuclear conformation of the squashed preparations are greatly improved by the use of the non-ionic detergent Triton X-100. Viability of dye-treated fertilized one-cell and blastocyst stage embryos is maintained at least up to day 13 of pregnancy following transfer of the embryos to the uteri of pseudopregnant recipients. Additional uses for this staining technique are discussed.
Key words. Morula; blastocyst; nuclei; vital stain; Hoechst 33258.

The most generally used technique for counting blastomeres in the preimplantation embryo is the air-drying method of Tarkowski[2] or various modifications of this method as outlined by Kinsey[3]. More recently an improved method for chromosomal preparations from preimplantation mammalian embryos was reported by Dyban[4]. The common features of these methodologies are 1) they were primarily designed as chromosomal preparations, 2) they involve fixing, spreading and staining of the embryos, and 3) they require considerable experience for consistent high quality preparations.

For cell counting, the present method using a fluorescent DNA-binding dye has several advantages over earlier techniques.

1) Embryos are rapidly processed since no exposure to hypotonic solution is required.

2) Embryos are treated in the same medium used for culture and are therefore easily handled.

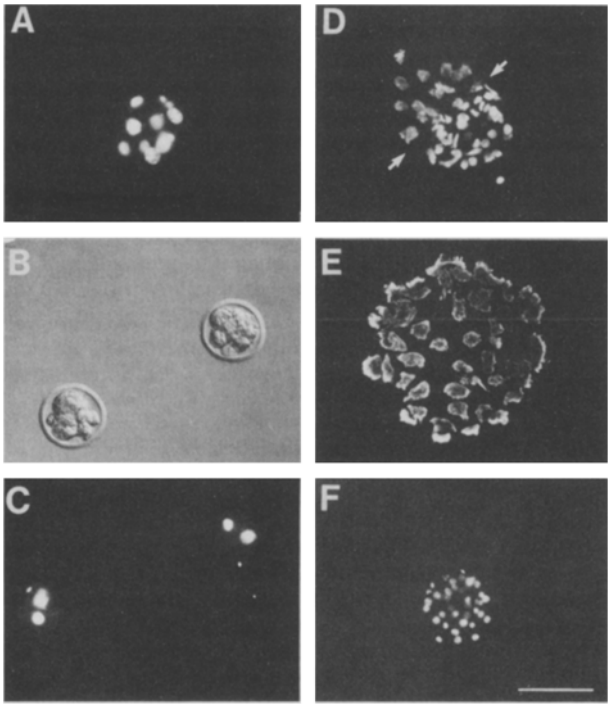
3) No fixation is required to prepare the embryos prior to fluorescent observations.
4) The DNA-binding dye is a vital dye and can be used with fertilized one-cell ova and blastocyst stage embryos without apparent loss of viability.

Material and methods. Mouse embryos were collected from either random bred CD-1 of F_1 (C57 X SJL) females. The embryos were flushed from the reproductive tract at various times after fertilization to recover different preimplantation stages (i.e. fertilized one-cell, morula, blastocyst) or were recovered and cultured in vitro using an established protocol[5]. For determination of the number of nuclei, the embryos were exposed to culture medium containing 10 µg/ml of Hoechst 33258 (Polysciences) for 30 min. The embryos were then transferred to a small droplet (10 µl) of medium on a clean microscope slide and covered with a glass cover slip with two opposite edges rimmed with vaseline. If embryos were to be recovered, the coverslip was gently lowered only until contact was made with the droplet. After brief exposure to uv light for determination of the number of nuclei, the coverslip was removed and the embryo returned to culture or transferred to a recipient female. If recovery of the embryo was not desired, the embryo was squashed by placing additional pressure on the coverslip until the droplet spread and the fluid surface tension drew the coverslip firmly down on the embryo. Blastocysts can be squashed with the zona pellucida intact or after the zona pellucida has been removed with acid Tyrode solution[6]. During the squashing procedure the nuclei have a tendency to distort due to the physical force of the coverslip. To eliminate this problem the embryos were exposed to a 1% Triton X-100 solution in culture medium for 20–30 s and immediately transferred to a drop of culture medium without the detergent prior to squashing. The Triton X-100 treatment also increases the intensity of fluorescence of the DNA-dye complex probably due to lose or clearing of the cytoplasm.

Stained embryos were examined using a Zeiss Standard RA fluorescent microscope with a HBO 200 W/4 lamp under transmittance illumination. Excitation filter UG 5 was used in combination with barrier filters 41 and 65 which gives a pass band between 410 and 650 nm resulting in a blue fluorescence.

Viability testing of the fertilized one-cell ova was carried out following exposure to the Hoechst dye with or without UV radiation using a Leitz Dialux 20 microscope equipped with epifluorescence. Two sources of the UV light, 50 and 100 W lamps, were used with dye concentrations from 0.01 to 1.0 µg/ml. An excitation filter BP 340–380 was used in combination with a suppression filter LP 430 resulting in a blue fluorescence. Following treatment and culture to the blastocyst stage the embryos were transferred to the uteri of Swiss albino females in the third day of pseudopregnancy and dissected out 10 days later for evaluation.

Blastocysts recovered at 3.5 days post coitum were stained with Hoechst 33258 at concentrations from 1.0 to 200 µg/ml without exposure to UV radiation to determine if the dye adversely effects the viability of the blastocyst. Embryos were then transferred to the uterus of CD-1 females in the 3rd day of pseudopregnancy and dissected out 11 days later.



A Intact mouse morula stained with Hoechst DNA-binding dye (33258) and observed with UV irradiation; *B* fragmented mouse ova stained and observed under Nomarski differential interference contrast optics; *C* the same fragmented ova observed with UV irradiation; *D* intact mouse 3.5-day blastocyst stained and squashed under a coverslip, note that the cells in the upper region (arrows) are still within the zona pellucida and fluoresce with less intensity than the nuclei that are away from the zona; *E* mouse 3.5-day blastocyst stained and the zona pellucida removed prior to squashing; *F* mouse 3.5-day blastocyst stained, the zona removed and the embryo treated with 1% Triton X-100 prior to squashing. The bar represents a distance of 100 µm and all photographs have equal magnification.

Table 1. Test of viability of fertilized one-cell mouse embryos (FIC) after treatment with Hoechst 33258 and UV radiation for fluorescent detection of nuclear DNA

UV source	Dye dosage (µg/ml)	UV exposure (min)	FIC	Number of blastocysts (%)	Normal fetuses[a] (%)
50 watt	1.0[b]	0.5	21	17 (81)	NT
	1.0[b]	1.0	23	13 (57)	NT
	1.0[b]	2.5	18	6 (33)	NT
	1.0[b]	5.0	20	0 (0)	NT
	1.0[b]	—	19	16 (84)	NT
	—	15.0	19	6 (32)	NT
	—	—	20	18 (90)	NT
100 watt	1.0	0.5	16	0 (0)	NT
	1.0	1.0	16	0 (0)	NT
	0.1	0.5	16	9 (56)	6 (67)
	0.1	1.0	16	1 (6)	NT
	0.01	0.5	17	13 (76)	9 (69)
	0.01	1.0	15	12 (80)	9 (82)[c]
	1.0	—	16	8 (50)	7 (88)
	—	0.5	16	9 (56)	4 (44)
	—	1.0	15	8 (53)	7 (88)

[a] Viable fetuses determined on the 13th day of gestation. [b] 15-min exposure to Hoechst 33258. [c] Only 11 blastocysts were transferred. NT = not transferred.

Table 2. Test of viability of mouse blastocysts after 30-min treatment with Hoechst 33258, transfer to pseudopregnant recipients and examination on the 14th day of gestation

Dye dosage (µg/ml)	Number of blastocysts	Implantation sites[a]	Normal fetuses
1	8	8	7
10	8	8	7
100	8	8	8
200	8	8	8

[a] Represents total number of normal and resorbed implantation sites.

Results and discussion. A typical morula stained with Hoechst dye and observed under fluorescent optics is shown in the figure, A. Although all the nuclei are readily counted in this focal plane they are not in sharp focus as the three-dimensional perspective is still maintained. Fragmented ova that may give the appearance of a morula can easily be distinguished by this approach (fig., B and C).

The squash technique is necessary to determine the number of cells in the mouse blastocyst since all the nuclei cannot be observed in a single focal plane. If the blastocyst is squashed with the zona pellucida intact, the nuclei may be partially expressed from the broken zona pellucida (fig., D), but the presence of the zona pellucida interferes with the intensity of fluorescence of nuclei left inside the zona. The figure, E, shows a blastocyst squashed after the zona pellucida was removed. The wavy appearance of the DNA is the result of the distortion of the nuclei under pressure. Exposure of the blastocysts to a 1% Triton X-100 solution for 20–30 s prior to squashing decreases the nuclear distortion as shown in the figure, F.

The results in table 1 show that fertilized one-cell ova remain viable up to the blastocyst stage in culture when exposed to Hoechst 33258 at a concentration of 1.0 µg/ml. Additionally, transfer of the treated ova to pseudopregnant females shows that the embryos are viable and normal at least to day 13 of gestation. Exposure to only the UV light from either the 50 or 100 W lamp at the times used does not adversely effect the survivability of the fertilized ova. However, the intensity of the UV light in combination with either 0.5 or 1.0 min of UV exposure has a dramatic effect on the number of blastocysts that develop from the one-cell stage, following treatment with 1.0 µg/ml of the DNA-binding dye. 81 and 57% of the ova exposed to the 50 W lamp for 0.5 and 1.0 min respectively, developed to the blastocyst stage in vitro compared to 0% when the ova were exposed to the 100 W lamp for similar periods of time. Lower concentrations of dye result in improved embryonic viability, but do not give intense images of fluorescence with the present optical equipment. It may be possible, however, to visualize nuclei with lower dye concentration if image intensifying equipment is employed[7]. Blastocysts exposed to the dye and UV light showed increased fluorescent intensity as the dye concentration increased.

The dye alone at various concentration does not appear to effect the viability of the blastocyst. Embryos exposed to the dye at concentrations as high as 200 µg × ml for 30 min showed no decrease in viability up to day 14 of pregnancy (table 2). In both experiments testing viability, fetuses were found to be normal on dissection, thus providing no evidence that dominant mutations effecting gross fetal morphology were caused by the dye or UV, either alone or in combination. However, it would be necessary to use a multiple recessive stock of mice and to allow their development to term to obtain a more complete test of the possible mutagenic effect of such treatment.

This technique for detecting nuclei can be useful as a quick and easy method to:

1) Count the number of cells at the compact morula or blastocyst stage of embryo development.

2) Distinguish between early cleavage stages and abnormal fragmented ova.

3) Detect the number and location of pronuclei particularly in the ova of species such as the cow and pig where it is difficult to visualize pronuclei under phase or Nomarski optics.

4) Determine that sperm penetration has occurred during in vitro fertilization of oocytes.

1 Acknowledgment. Financial support was provided by NIH Grant HDD-06210 (KME) and by Basil O'Connor Starter Research Grant No. 5-379 from the March of Dimes Birth Defects Foundation (VEP). We thank Steven Halpern for help with the photography and Jon Flax for expert technical assistance.

2 Tarkowski, A. K., Cytogenetics *5* (1966) 394.

3 Kinsey, J. P., in: Methods in Mammalian Embryology, p. 260. Ed. J. C. Daniels Jr., W. H. Freeman, San Francisco 1971.

4 Dyban, A. P., Stain Tech. *58* (1983) 69.

5 Brinster, R. L., in: Growth, Nutrition and Metabolism of Cells in Culture, vol. 2, p. 251. Eds G. Rothblat and V. Gustofalo. Academic Press, New York 1972.

6 Nicolson, G. L., Yanagimachi, R., and Yanagimachi, H., J. Cell Biol. *66* (1975) 263.

7 Albertini, D. F., Biol. Reprod. *30* (1984) 13.

0014-4754/85/091207-03$1.50 + 0.20/0

A simplified method for isocratic HPLC analysis of polyamines

W. L. Whitmore and T. A. Slotkin*

Department of Pharmacology, Duke University Medical Center, Durham (North Carolina 27710, USA), 13 December 1984

Summary. A simple technique is described for the separation and analysis of polyamines in tissues and body fluids, utilizing precolumn clean-up on disposable CM-cellulose columns, followed by an automatable HPLC procedure. Complete separation and analysis takes 12–15 min per sample with sensitivity in the pmole range.

Key words. Polyamines; putrescine; spermidine; spermine; high pressure liquid chromatography.

Interest in the polyamines has expanded by major proportions due to the recognition that these substances serve as primary modulators of both normal and pathological cell growth, such as that seen during ontogeny, pathological or compensatory tissue hypertrophy, malignancy and infection[1–4]. Sensitive methods for the separation and analysis of polyamines by HPLC have been developed which typically require either multi-buffer elution with either postcolumn or precolumn derivatization[5–9], or an isocratic system requiring a prolonged period for column regeneration between samples[10]. We now describe a procedure which utilizes isocratic HPLC separation and thus does not require multiple pumps, column re-equilibration or other specialized equipment or techniques. The result is a complete separation of the polyamines in 12–15 min per sample with sensitivity comparable to that of more elaborate procedures.

Materials. Ortho-phthalaldehyde (OPA), CM-cellulose (0.72 meq/g, coarse grade), putrescine dihydrochloride, histamine dihydrochloride, spermidine phosphate, spermine diphosphate, agmatine sulfate, cadaverine dihydrochloride and Brij 35 were purchased from Sigma Chemical Corp. (St Louis, MO). 2-Mer-