may be a major principle of organization in other regions of the primate cortex as well.

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References and Notes

1. V. B. Mountcastle, *J. Neurophysiol.* **20**, 408 (1957).
2. D. H. Hubel and T. N. Wiesel, *Proc. R. Soc. London Ser. B* **198**, 1, (1977).
3. E. G. Jones, H. Burton, R. Porter, *Science* **190**, 572 (1975); M. Wong-Riley, *Brain Res.* **162**, 201 (1979); T. J. Imig and J. F. Brugge, *J. Comp. Neurol.* **182**, 637 (1978); T. J. Imig and R. A. Reale, *ibid.* **192**, 293 (1980); E. Kosar and P. J. Hand, *ibid.* **198**, 515 (1981); M. Sur, J. T. Wall, J. H. Kaas, *Science* **212**, 1059 (1981).
4. P. S. Goldman and W. H. J. Nauta, *Brain Res.* **122**, 393 (1977).
5. P. S. Goldman-Rakic, in *The Organization of Cerebral Cortex*, F. O. Schmitt, F. G. Worden, S. G. Dennis, G. Adelman, Eds. (MIT Press, Cambridge, Mass., 1981), pp. 69–97.
6. D. N. Pandya and H. G. J. M. Kuypers, *Brain Res.* **13**, 13 (1969); S. Jacobson and J. Q. Trojanowski, *ibid.* **132**, 209 (1977); E. G. Jones and T. P. S. Powell, *Brain* **93**, 793 (1970).
7. G. R. Leichnetz, *Neurosci. Lett.* **18**, 119 (1980).
8. M. M. Mesulam, *J. Histochem. Cytochem.* **26**, 106 (1978).
9. In terms of comparison with the ocular dominance system, the terminal fields of callosal and associational projections labeled in the present and similar studies represent only a small fraction of their total representations, whereas virtually the entire system of ocular dominance stripes can be labeled by injections into the eye.
10. N. Butters, D. Pandya, D. Stein, J. Rosen, *Acta Neurobiol. Exp.* **32**, 305 (1972); P. S. Goldman and H. E. Rosvold, *Exp. Neurol.* **27**, 291 (1970); ———, B. Vest, T. W. Galkin, *J. Comp. Physiol. Psychol.* **77**, 212 (1971).
11. Supported by PHS grants NS 16666 and MH 00298.

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Lipofuscin: Resolution of Discrepant Fluorescence Data

Abstract. *Lipofuscin granules (age pigments) emit yellow light under ultraviolet excitation in the fluorescence microscope. The reported blue emission maximum of extracts of lipofuscin-laden cells may result from instrumental bias. The major fluorescent components that accumulate with age in these lysosomal residual bodies of human retinal pigment epithelium are yellow-emitting fluorophores. Different age-related fluorophores, which do emit blue light, are derived from other intracellular sources. A reevaluation of the connection between blue-emitting lipid peroxidation products and the age-related lipofuscin granules of classical pathology is necessary.*

Postmitotic cells of various aging tissues accumulate heterogeneous nondegradable lipophilic materials in lysosomal residual bodies called lipofuscin granules. The residues are believed to be damaged cell membrane components that have been autophagized or phagocytized during the life of the cell (*1*). In situ, lipofuscin granules fluoresce upon excitation with ultraviolet light (*2*). The identity of the fluorophores is not established, however, in part because fluorescence microscopists have consistently reported the color of lipofuscin fluorescent emission to be yellow or red (*3*), whereas extracts of lipofuscin-rich cells have been reported to exhibit a blue fluoresence (*4*, *5*).

Retinal pigment epithelium (RPE) consists of postmitotic cells that phagocytize photoreceptor outer segment disks (*6*), the major source of the lysosomal residue in these cells. Human RPE accumulates lipofuscin with age (*7*) and is therefore a good model for our spectral studies.

To decide whether the fluorescence of lipofuscin is yellow or blue and whether the reported blue peak of extracts (*5*) could arise from unusually large solvent effects (*8*), we analyzed both intact cells and extracts in calibrated spectrofluorometers. In addition, high-performance thin-layer chromatography (HPTLC) was used to separate and detect the fluorescent materials.

The emission spectra were corrected for the overall spectral sensitivity of the instruments used. The microspectrofluorometer was a Leitz Dialux fluorescence microscope with appropriate detecting, amplification, and recording equipment (*9*). Interference filters were placed in front of the photomultiplier tube (PMT) to select specific wavelengths. For corrected spectral analysis, calibrations were made on the transmission of all components of the optical path and on the spectral sensitivity of the PMT (*10*). Extracts were measured in both the microspectrofluorometer and a spectrofluorometer (Turner model 430) whose emission monochromator and PMT unit were spectrally calibrated for subsequent correction of emission data (*11*) and whose monochromator wavelength settings were calibrated with a quinine sulfate standard.

The RPE samples were separated into two age groups, those from persons over 40 years of age and those from persons under 40 years of age. For microspectrofluorometry, sheets of RPE were dissected, placed on slides to dry and covered with immersion oil of very low fluorescence. Emission from about seven cells was analyzed. The RPE from a young person was matched with the RPE from an old person, and their respective emission spectra were measured. The spectrum of the RPE from the young donor was subtracted from that from the old donor. This difference spectrum eliminates background fluorescence (such as that from cellular flavinoids or stored retinol derivatives). In all, 11 matched pairs were analyzed, the difference spectra were calculated, and the results were pooled.

In the microscope, lipofuscin granules appeared to emit broad-band yellow light when excited with 366 nm. The age-related cellular emission showed a major peak at 540 to 640 nm (yellow-orange) and a minor peak at 460 nm (blue) (Fig. 1A). Thus, a substance accumulating with age in human RPE cells emits yellow light.

To separate the component fluorophores, we lyophilized human RPE cells (*12*) and extracted them by the Folch chloroform-methanol procedure (*13*). All solvents contained butylated hydroxytoluene (5 mg/100 ml), and tissues and HPTLC plates were stored under argon to retard peroxidation (*14*). In polar extracts, the chromatographic profile (*15*) showed a weak blue fluorescence, but only in the RPE from old donors (Fig. 2, left). However, most of the fluorescence was in the nonpolar extract.

The corrected emission spectrum of the highly fluorescent nonpolar extracts of RPE from old donors (Fig. 1B) was similar to that of the intact cells (Fig. 1A). A smaller blue emission peak was largely overshadowed by a broad peak at 540 to 580 nm. Chromatographic separation of this extract (*16*) revealed two intense blue bands that were not detected in similar extracts from young individuals (Fig. 2, middle). Several other bands common to samples from young and old donors were intensely fluorescent in the yellow and orange range of the spectrum. The RPE from young donors also contained some fluorescent granules, but quantitation of fluorophores was not attempted.

To determine whether the age-related blue-emitting fluorophores had originated in the lipofuscin granules, we fractionated the RPE homogenates from old donors (*17*) and isolated the lipofuscin granules. Analysis of nonpolar extracts of the lipofuscin granule fraction by HPTLC (*18*) revealed only yellow-emitting components, the major one being a band at the origin (Fig. 2, right).

Our study reemphasizes that the spectral characteristics of all components of

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spectrofluorometric equipment must be scrutinized so that long-wavelength fluorophores are not overlooked (*19*). The spectral efficiency of the emission monochromator and spectral sensitivity of the PMT combined in their effects to render our spectrophotometer at least an order of magnitude less sensitive to light at the yellow and red end of the spectrum than to the blue wavelengths (Fig. 1C). If these instrumental shortcomings had not been corrected, we would have failed to detect the fluorescent yellow components in the extracts (Fig. 1B). The same phenomenon has been encountered in other lipofuscin studies. For example, neuronal lipofuscin emission is yellow in the fluorescence microscope (*20*), and although an early spectral study reported dual emission peaks at 440 to 460 nm and 530 to 560 nm (*21*), the yellow components were not detected in more recent spectral analyses in situ (*22*) and in vitro (*23*). Difficulty in correlating morphometric data of lipofuscin granule accumulation with assays of the extractable blue fluorophore was reported in another study (*24*). Awareness of instrumental limitations may help to strengthen lipofuscin research.

Speculation as to the possible nature of the lipofuscin fluorophores in various tissues has centered on retinoids (*24*) and on by-products of lipid peroxidation, such as malondialdehyde, which can cross-link amine-containing compounds via *N,N'*-disubstituted 1-amino-3-iminopropene linkages (−NH−CH=CH−CH=N−) (*25*). This imine-conjugated Schiff base structure fluoresces with an emission of between 430 and 480 nm (blue) regardless of the amine donor molecule. No yellow-emitting products have been reported to originate by this mechanism. The formation of blue fluorescence in cell organelles under peroxidative stress has been well documented (*26*), as has the accumulation of blue fluorophores with age (*27*). Indeed, in our studies, at least two blue-emitting fluorophores were associated with old, but not young, whole cell extracts, and these may indicate membrane damage. However, the absence of blue-emitting fluorophores from extracts of isolated lipofuscin granules suggests that the blue fluorophores are not main contributors to the lipofuscin granule fluorescence in situ. The relationships, if any, between the blue-emitting fluorophores and the yellow-emitting fluorophores associated with lipofuscin granules remains to be determined.

Thus, while the term lipofuscin has come to be used for blue-emitting extracted fluorophores, we suggest that the word lipofuscin be reserved as a generic term for the age-related lysosomal residual bodies of classical pathology and cell biology. This will avoid implications as to the chemical structure of the fluorophores in the granules until they have been unequivocally identified and their etiology has been elucidated.

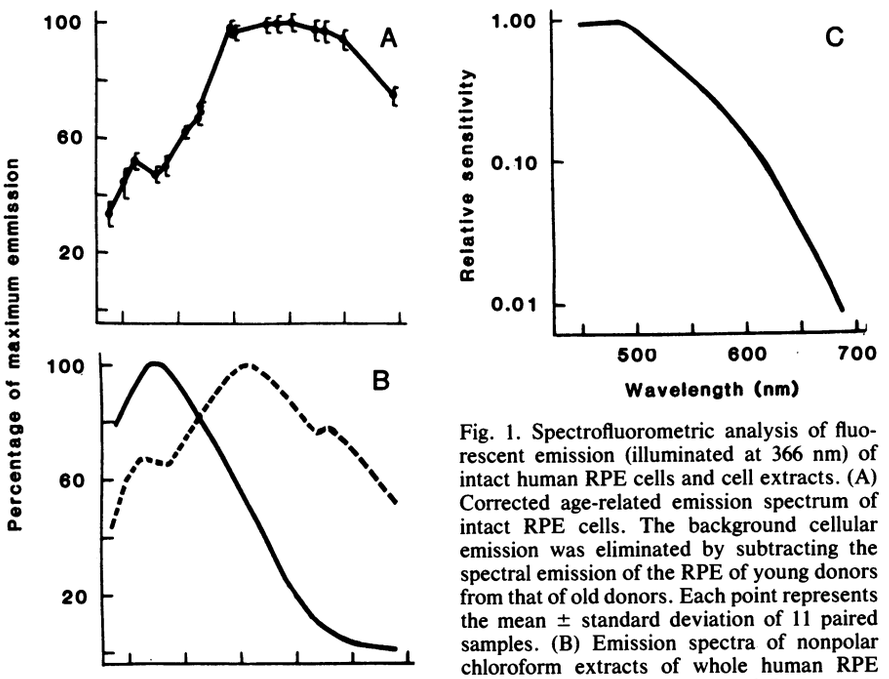

Fig. 1. Spectrofluorometric analysis of fluorescent emission (illuminated at 366 nm) of intact human RPE cells and cell extracts. (A) Corrected age-related emission spectrum of intact RPE cells. The background cellular emission was eliminated by subtracting the spectral emission of the RPE of young donors from that of old donors. Each point represents the mean ± standard deviation of 11 paired samples. (B) Emission spectra of nonpolar chloroform extracts of whole human RPE from donors over 40 years of age. Dashed curve indicates spectrally corrected fluorometric equipment (Leitz and Turner); solid curve, no spectral correction (Turner). (C) Spectral sensitivity curve of the Turner spectrofluorometer. The curve combines the spectral efficiency of the emission monochromator and the spectral sensitivity of the PMT.

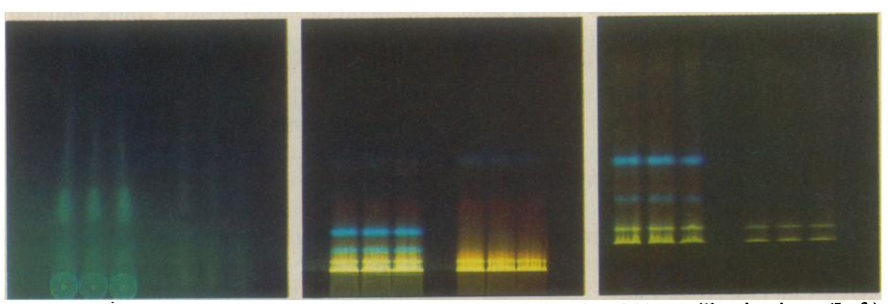

Fig. 2. Fluorescence chromatograms of RPE extracts under 366-nm illumination. (Left) Fluorophores of polar extracts of (left to right in groups of three) human RPE from donors over 40 years of age and human RPE from donors less than 40 years of age (5 mg each). Cellulose HPTLC plate developed with butanol, acetic acid, and water (*15*). (Middle) Fluorophores of nonpolar extracts of (left to right in groups of three) human RPE from donors over 40 years of age and human RPE from donors less than 40 years of age. Silica gel HPTLC plate developed with *n*-heptane, chloroform, methanol, and acetic acid (*16*). (Right) Fluorophores of nonpolar extracts of whole human RPE from donors over 40 years of age (left three columns) and isolated lipofuscin granules from human RPE of the same age group (right three columns). Silica gel HPTLC plates developed with hexanes, chloroform, methanol, and acetic acid (*18*).

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References and Notes

1. P. Gedigk and E. Bontke, *Z. Zellforsch. Mikroskop. Anat.* **44**, 495 (1956); E. Essner and A. B. Novikoff, *J. Ultrastruct. Res.* **3**, 374 (1960).
2. H. Popper, *Arch. Pathol.* **31**, 766 (1941); B. L. Strehler, D. D. Mark, A. S. Mildvan, M. V. Gee, *J. Gerontol.* **14**, 430 (1959); E. A. Porta and W. S. Hartroft, in *Pigments in Pathology*, M. Wolman, Ed. (Academic Press, New York, 1969), p. 191.
3. R. D. Lillie and H. M. Fullmer, *Histopathologic Technic and Practical Histochemistry* (McGraw-Hill, New York, ed. 4, 1976), p. 12.
4. A. S. Csallany and K. L. Ayaz, *Lipids* **11**, 412 (1976).

5. L. Feeney-Burns, E. R. Berman, H. Rothman, *Am. J. Ophthalmol.* **90**, 783 (1980).
6. R. W. Young and D. Bok, *J. Cell Biol.* **42**, 392 (1969).
7. B. W. Streeten, *Arch. Ophthalmol.* **66**, 391 (1961); H. Kolb and P. Gouras, *Invest. Ophthalmol.* **13**, 487 (1974); L. Feeney, *Invest. Ophthalmol. Visual Sci.* **17**, 583 (1978); G. L. Wing, G. C. Blanchard, J. J. Weiter, *ibid.*, p. 601.
8. J. S. Bellin, *Photochem. Photobiol.* **4**, 33 (1965); R. S. Becker, *Theory and Interpretation of Fluorescence and Phosphorescence* (Wiley-Interscience, New York, 1969), p. 34.
9. Microscope photometer system (Leitz MPV 1.1); photomultiplier tube (Hamamatsu R928) in a Pacific system (housing, amplifier, and model 204 power supply); light source, 100-W mercury arc (Osram, HBO); Ploempak 2.3 epi-illumination unit with filter modules; ×40 oil immersion apochromatic objective (numerical aperture, 1.00).
10. The PMT was cross-calibrated to a multiply calibrated photodiode (EG&G HUV4000B); filters were calibrated on a spectrophotometer (Cary 210). See also V. J. Ferrans, L. M. Buja, W. C. Roberts, D. S. Fredrickson, *Am. J. Pathol.* **64**, 67 (1971).
11. The emission unit of the Turner 430 spectrofluorometer, including the PMT (Turner Associates, No. 430-340), was calibrated with an L-101 spectral irradiance standard (Electro Optics Associates) [J. Lee and H. H. Seliger, *Photochem. Photobiol.* **4**, 1015 (1965)].
12. Human RPE cells were brushed from fresh, unfixed eyes within 24 hours of the donor's death and pooled in distilled water under nitrogen and lyophilized.
13. A. L. Tappel, in *Free Radicals in Biology*, W. A. Pryor, Ed. (Academic Press, New York, 1980), vol. 4, p. 8.
14. C. C. Farnsworth and E. A. Dratz, *Biochim. Biophys. Acta* **443**, 556 (1976).
15. Cellulose HPTLC plates (Eastman, No. 13255) were developed with a mixture of butanol, acetic acid, and water (12:3:5).
16. Silica gel HPTLC plates (E. Merck, No. 11845) were activated and developed with a mixture of *n*-heptane, chloroform, methanol, and acetic acid (76:49:13:2).
17. The postnuclear fraction was layered on a discontinuous density sucrose gradient and centrifuged; the yellow fluorescent band was collected. Electron microscopy showed virtually pure lipofuscin granules (G. E. Eldred and L. Feeney-Burns, in preparation).
18. Silica gel was activated and developed with hexanes, chloroform, methanol, and acetic acid (76:49:13:2).
19. C. E. White and R. J. Argauer, *Fluorescence Analysis, A Practical Approach* (Dekker, New York, 1970), p. 30; C. A. Parker, *Photoluminescence of Solutions* (Elsevier, New York, 1968), p. 252.
20. K. R. Brizzee, J. M. Ordy, B. Kaack, *J. Gerontol.* **29**, 336 (1974).
21. H. Hyden and B. Lindstrom, *Discuss. Faraday Soc.* **9**, 436 (1950).
22. H. Barden, F. Aviles, W. Rivers, in *Pigment Cell*, vol. 4, *Biologic Basis of Pigmentation*, S. N. Klaus, Ed. (Karger, Basel, 1979), p. 263; H. Barden, *J. Neuropathol. Exp. Neurol.* **39**, 598 (1980).
23. R. D. Taubold, A. N. Siakotos, E. G. Perkins, *Lipids* **10**, 383 (1975).
24. J. G. Bieri, T. J. Tolliver, W. G. Robison, Jr., T. Kuwabara, *ibid.* **15**, 10 (1980); W. G. Robison, Jr., T. Kuwabara, J. G. Bieri, *Invest. Ophthalmol. Visual Sci.* **19**, 1030 (1980).
25. K. S. Chio and A. L. Tappel, *Biochemistry* **8**, 2827 (1969); V. G. Malshet and A. L. Tappel, *Lipids* **8**, 194 (1973); B. L. Fletcher, C. J. Dillard, A. L. Tappel, *Anal. Biochem.* **52**, 1 (1973); V. G. Malshet, A. L. Tappel, V. M. Burns, *Lipids* **9**, 328 (1974).
26. C. J. Dillard and A. L. Tappel, *Lipids* **6**, 715 (1971); M. Minssen and K. D. Munkres, *Biochim. Biophys. Acta* **291**, 398 (1973); I. D. Desai, B. L. Fletcher, A. L. Tappel, *Lipids* **10**, 307 (1975); B. D. Goldstein and E. M. McDonagh, *J. Clin. Invest.* **57**, 1302 (1976).
27. A. Tappel, B. Fletcher, D. Deamer, *J. Gerontol.* **28**, 415 (1973); A. S. Csallany, K. L. Ayaz, L. C. Su, *J. Nutr.* **107**, 1792 (1977).
28. Supported by NIH grants EY 05456 (G.E.E.), EY 03408 (W.S.S.), and EY 03274 (L.F.B.); by NSF grant BNS 76-11921 (W.S.S.); and by grants-in-aid from Research to Prevent Blindness, Inc. (L.F.B.) and the National Society to Prevent Blindness (G.E.E.). Tissues were provided through the Eye Research Foundation and the Lions Tissue Eye Bank, Columbia, Mo.

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Early Chronic Low-Level Methylmercury Poisoning in Monkeys Impairs Spatial Vision

Abstract. *Five monkeys were treated from birth with oral doses of mercury as methylmercury (50 micrograms per kilogram of body weight per day); concentrations in the blood peaked at 1.2 to 1.4 parts per million; and declined after weaning from infant formula to a steady level of 0.6 to 0.9 part per million. There were no overt signs of toxicity. When tested between 3 and 4 years of age under conditions of both high and low luminance, treated monkeys exhibited spatial vision that was impaired compared with that of control monkeys.*

In the outbreaks of human poisoning from methylmercury in Japan and later in Iraq, one of the most consistent signs in adults was deficits in visual function. The deficit that has received the most emphasis is constriction of the visual fields, although other visual deficits, particularly changes in visual acuity, have been reported with equal frequency (*1*). Human data suggest that the fetus or neonate exposed to methylmercury in breast milk may be at greater risk from methylmercury poisoning than the adult and that the signs may be different from those of adult methylmercury poisoning (*2*, *3*).

The visual system of macaque monkeys resembles that of humans (*4*) and exhibits the same signs and pathological lesions as that of humans when exposed to methylmercury (*5*–*7*). Macaques are therefore excellent models for testing the effects of methylmercury on the visual system. We separated cynomolgus monkeys (*Macaca fascicularis*) from their mothers within 12 hours after birth and raised them in a primate nursery (*8*). They were given oral doses of mercury (0 or 50 μg per kilogram of body weight per day) as methylmercury starting at birth and continuing throughout the period of testing. Blood concentrations peaked at approximately 1.2 to 1.4 ppm and dropped after withdrawal of infant formula at 200 days of age to a steady level between 0.6 and 0.9 ppm (*9*). None of the monkeys showed any overt signs of toxicity. Food intake and weight gain were indistinguishable from those of control subjects and routine hematological measures were normal. No abnormalities were detected during regularly scheduled clinical neurological examinations, and treated monkeys were as agile in the large exercise cages as controls.

Optic head and macula appeared nor-

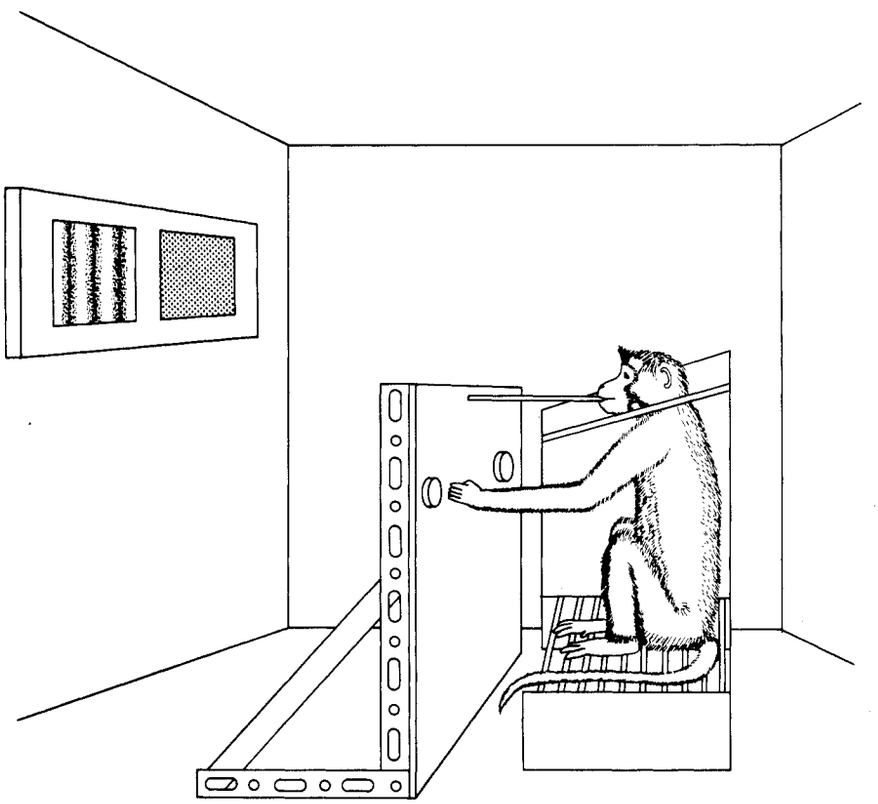

Fig. 1. Diagram of the apparatus.

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