A TWO-LAYER TECHNIQUE FOR DETECTING SURFACE ANTIGENS IN THE SEA URCHIN EGG WITH FERRITIN-CONJUGATED ANTIBODY

JANE BAXANDALL, P. PERLMANN, and B. A. AFZELIUS. From the Wenner-Gren Institute for Experimental Biology, University of Stockholm, Sweden

Sea urchin gametes provide a favorable subject for investigation with ferritin-conjugated antibodies, since their surface layers are functionally and immunologically highly differentiated (1–3), and are well known from light and electron microscope studies (1, 4, 5). A closer knowledge of the egg surface is of great inherent interest as the surface reactions play an important role in fertilization. In the present study of these surface antigens the ferritin-conjugate technique (6, 7) has been

adapted for a two-layer, or *indirect*,[1] staining method, similar to that used in fluorescence immunology (8). The first layer of unlabeled specific antibody is applied to the tissue, followed by the second layer of labeled anti-γ-globulin prepared in a different species of animal. The unlabeled specific antibody then acts as an antibody in respect to its homologous antigen in the tissue on one side, and as an antigen in respect to the labeled anti-γ-globulin on the other. Since there is a larger number of active sites, on the unlabeled antibody, for reaction with the marker molecule, the sensitivity in locating antigen with labeled antibody is greatly increased (9).

MATERIAL AND METHODS

In the study to be described, antisera against *Paracentrotus lividus* gametes, prepared by injecting rabbits, were chosen in order to explore the potentialities of immune electron microscopy in this material. Antiserum was made against a lyophilized heat-stable (100°C, 2 hours) fraction of a homogenate of jellyless unfertilized eggs (antiegg serum). As learned from light microscopic studies, this serum affected the surface of the unfertilized eggs in different ways, without affecting the jelly coat (10, 2). A second antiserum made against isolated jelly substance (11), in a large proportion of treated eggs, caused a light microscopically visible contraction and precipitation of the jelly layer which is immunologically different from the egg surface (10, 2). An antiserum against lyophilized sperm homogenate was chosen as a control since previous light microscope studies showed that the surface of the sperm is immunologically very different from that of the egg (3, 10). Serum from normal rabbits was used as a further control.

The antirabbit γ-globulin serum was obtained from a sheep injected 3 times im with a total of 600 mg of isolated rabbit γ-globulin (in Freund's adjuvant) prepared by continuous flow electrophoresis and column chromatography (12). In an immune electrophoresis test this antiserum developed a single precipitate with the rabbit γ-globulin.

Isolated γ-globulin fractions were prepared from these whole sera by chromatography on DEAE cellulose, using elution with 0.0175 M phosphate buffer at pH 6.3 (13). The eluates were concentrated by dialysis against polyethylene glycol (14). The ferritin-globulin conjugates were prepared according to Singer's technique (7) with the modifications used by Smith *et al.* (15). The conjugates were stored frozen in small phials and before use were dialyzed against several changes of sterile sea water. Fresh unfertilized eggs with intact jelly coats taken from *P. lividus* (16) and washed in sea water were treated, unfixed, with sea water solutions of the isolated γ-globulins (conjugated or unconjugated).

For *direct* staining eggs were treated with the ferritin-globulin conjugate for 30 minutes and then washed with repeated changes of sea water. In the *indirect* method the eggs were treated as previously, but with unconjugated γ-globulin, and washed with sea water for 10 minutes. The ferritin-globulin conjugate of sheep antirabbit γ-globulin was then added and after 30 minutes the excess conjugate was washed away thoroughly.

The treated eggs were fixed for electron microscopy with 2 per cent osmium tetroxide in sea water, dehydrated in graded alcohols and propylene oxide, and embedded in Epon 812 (17). Sections were examined with a Siemens Elmiskop I at initial magnifications of 10,000 and 15,000.

RESULTS AND DISCUSSION

Unfertilized eggs treated *directly* with ferritin-labeled antiegg-γ-globulin had a thin layer of ferritin along the cell surface, underneath the jelly coat (Fig. 1). When treated *indirectly* the thickness of the ferritin layer on the surface was markedly increased while the jelly coat again remained essentially unaffected (Fig. 2). The reverse results were obtained with the antijelly-γ-globulin which precipitated the jelly coat to a relatively narrow contracted band (Figs. 3, 4). With the ferritin-labeled conjugate of the *direct* method, the ferritin molecules were distributed throughout the jelly coat and along the cell surface (Fig. 3). Again, in the *indirectly* stained preparations, the ferritin in the same areas was much more densely packed (Fig. 4).

In the ferritin-labeling technique there are several potentialities for unspecific staining. The staining solutions in the present investigation may be expected to contain unconjugated ferritin molecules and γ-globulins which are both known to be taken up by cells (18–20) and may thus be absorbed unspecifically. Non-covalent binding between these molecules may obscure the picture further (6). In the present case, the specificity of the surface staining was controlled by means of treating the eggs with either antisperm γ-globulin or γ-globulin from normal rabbits. Neither the

[1] The terms *direct* and *indirect* are used here, as in fluorescence immunology, to indicate one-layer and two-layer methods of antiserum treatment, respectively (8). This has no relation to Singer and Schick's (6) use of the terms to indicate covalent (direct) and noncovalent (indirect) linkage of ferritin to the antibody molecule.

direct nor the indirect technique showed any significant ferritin staining on the egg surface (Figs. 5 to 7). In contrast, the antisperm serum gave a heavy staining of sperm and fertilized eggs.

The results indicate that the distinct specificities of the antiegg and antijelly sera, as known from light microscopic studies (2, 10), were also retained in the electron microscope. Thus, the antiegg serum primarily stained the egg surface while the antijelly serum mainly affected the jelly coat. This confirms previous conclusions regarding the nature of the antigens in the different surface layers. Electron microscopy along these lines in combination with antiserum absorption techniques (2) should, therefore, become a valuable tool for the study of the molecular architecture of the egg surface.

Since uniform results were obtained with a number of different antisera combinations it seems unlikely that the stronger staining with the *indirect* method was due to smaller amounts of unconjugated (= inhibitory) antibody with the antirabbit conjugates as compared to the anti-sea urchin conjugates. Although final proof of this point has to await further purification of the conjugates by means of ultracentrifugation (6) and electrophoresis (21), the present results suggest that the *indirect* staining method greatly increases the sensitivity in detecting surface antigens. They also indicate that it is of a high degree of specificity since no normal γ-globulin seems to remain attached to the egg surface.[2] It thus represents a convenient technique to use when only small quantities of antiserum are available or when there are many different antisera to be tested, as it only necessitates one ferritin conjugation. Moreover, in immunological experiments made for the light microscope with unlabeled antisera (2) the same samples can subsequently be treated with ferritin anti-γ-globulin conjugate for electron microscopical examination. Any possible loss in resolution of detecting antigen with the larger marker unit of the indirect method is easily balanced by the increase in sensitivity.

The staining of intracellular structures by means of ferritin-conjugated antibodies provided a number of technical difficulties (6), while the staining of surface antigens gave satisfactory results. It does not seem possible at this stage to draw definite conclusions about specific intracellular antigens, especially as the antisera used in this study were made against heat-stable antigens and were, therefore, poor in antibodies against proteins within the cell. Ferritin was found intracellularly in all labeling experiments, including the controls, and was seen almost exclusively within the yolk granules. The bounding membrane of many yolk granules was broken during fixation, which allowed ferritin and the contents of the yolk granules to escape into the free cytoplasm (Figs. 1, 6). This was rarely seen in eggs prefixed with 10 per cent acrolein, but this fixation method gave micrographs of inferior resolution for detecting

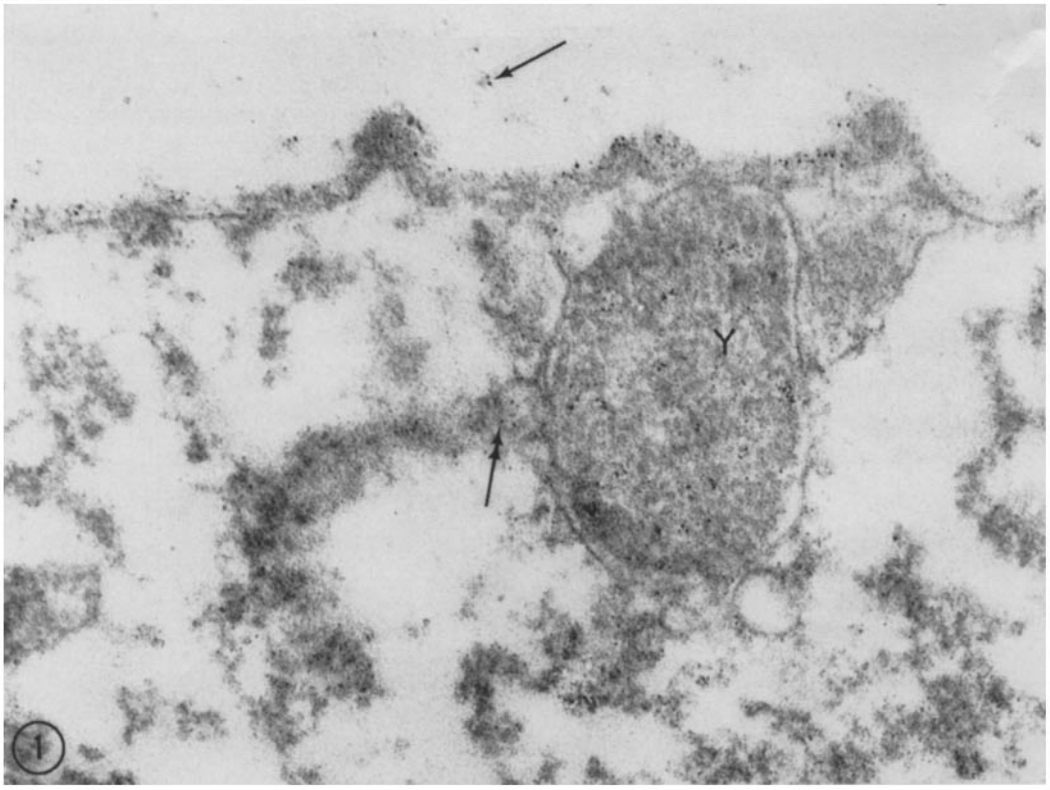
[2] In many micrographs of the *indirect* staining method, some of the ferritin molecules have an atypical appearance (Figs. 2, 4, 6.). This may be due to a loss of iron from the apoferritin in the samples of conjugate used in these experiments. As the control preparations with antisperm and normal rabbit γ-globulins do not show ferritin staining, it is considered that this atypical ferritin still indicates the presence of specific antirabbit γ-globulin.

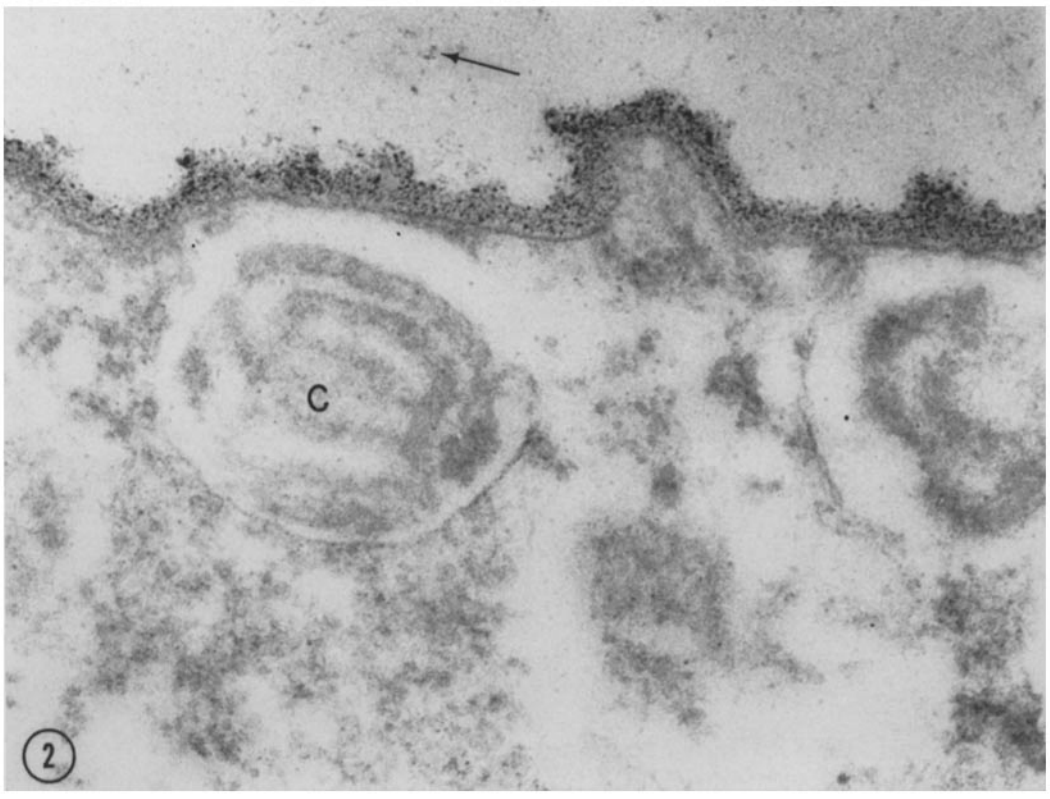
FIGURE 1

An unfertilized egg treated *directly* with ferritin conjugate from an antiegg serum. The ferritin molecules are arranged along the cell surface outside the plasma membrane. The yolk granule (*Y*), which is in close relation with the cell membrane, also contains a number of ferritin molecules, some having moved out through the broken bounding membrane (double-barbed arrow). The jelly substance is visible in places (arrow) where it has been slightly precipitated by the antiserum. × 80,000.

FIGURE 2

The two-layer or *indirect* staining method. An egg treated with an unconjugated sample of the same antiegg γ-globulin and later with a ferritin conjugate from an antirabbit γ-globulin serum. The layer of ferritin on the surface is greatly increased. The jelly has been weakly precipitated (arrow), but there is no ferritin in the jelly coat. *C*: cortical granule. × 80,000.





ferritin. It is likely that most, or all, of the intracellular ferritin represents non-specific staining, possibly caused by the uptake of either free ferritin or ferritin-globulin conjugate by the yolk granules. In order to achieve a specific staining of intracellular antigens, methods of rendering the cell permeable to the ferritin-antibody complex by freezing (22) or by treatment with complement (23) should probably be used. Experiments using sections of methacrylate embeddings, even with the methacrylate removed, treated with ferritin-globulin conjugate resulted in only unspecific staining.

SUMMARY

Electron microscopy of unfertilized eggs of the sea urchin *Paracentrotus lividus* was carried out after treatment of living eggs with ferritin-labeled γ-globulin from antisera against the surface layers (*direct* staining method). The *indirect* staining method of fluorescence immunology was also adapted for electron microscopy, the eggs being first treated with the unconjugated specific γ-globulin followed by a ferritin-globulin conjugate from a sheep antirabbit γ-globulin serum. This method resulted in a much stronger staining of the surface layers without any loss of specificity. With the direct and indirect methods the jelly layer of the unfertilized egg was seen to be immunologically different from the egg surface. Intracellularly, ferritin was taken up unspecifically in all experiments, and was seen mainly in the yolk granules.

This investigation was supported by a Post Graduate Studentship from the University of Edinburgh, Scotland, to one of us (J.B.), and by grants from the Hierta-Retzius Foundation of the Royal Swedish Academy of Science and from the Swedish Natural Science Research Council. We also wish to express our gratitude to the staff of the Stazione Zoologica, Naples, for their hospitality.

Received for publication, January 15, 1962.

REFERENCES

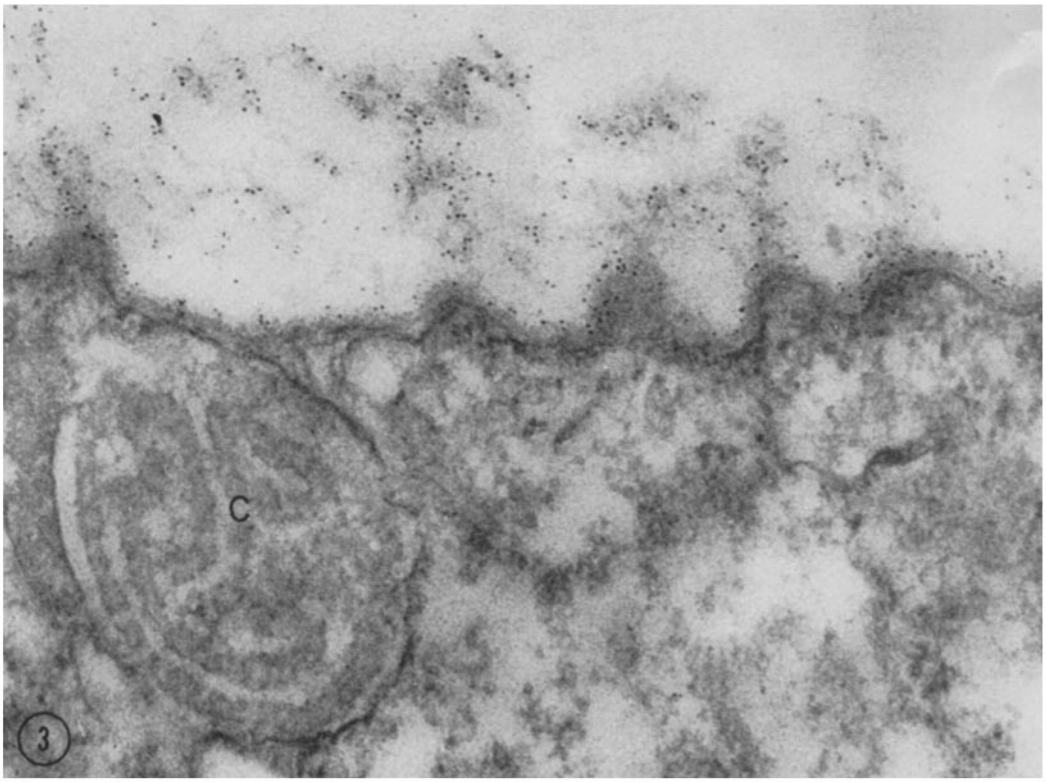
1. RUNNSTRÖM, J., HAGSTRÖM, B. E., and PERLMANN, P., *in* The Cell, (J. Brachet and A. E. Mirsky, editors), Academic Press, New York, **1,** 1959, 327.
2. PERLMANN, P., *Experientia*, 1959, **15,** 41.
3. METZ, C. B., *Internat. Rev. Cytol.*, 1961, **11,** 219.
4. AFZELIUS, B. A., *Z. Zellforsch. u. Mikr. Anat.*, 1955, **42,** 134.
5. AFZELIUS, B. A., *Exp. Cell Research*, 1956, **10,** 257.
6. SINGER, S. J., and SCHICK, A. F., *J. Biophysic. and Biochem. Cytol.*, 1961, **9,** 519.
7. SINGER, S. J., *Nature*, 1959, **183,** 1523.
8. COONS, A., *Internat. Rev. Cytol.*, 1956, **5,** 1.
9. MELLORS, R. C., Fluorescent antibody method, *in* Analytical Cytology, (R. C. Mellors, editor), New York, McGraw-Hill Book Company, 1959.
10. PERLMANN, P., *Exp. Cell Research*, 1957, **13,** 365.
11. VASSEUR, E., *Acta Chem. Scand.*, 1948, **2,** 900.
12. FAHEY, J. L., and HORBETT, A. P., *J. Biol. Chem.*, 1959, **234,** 2645.

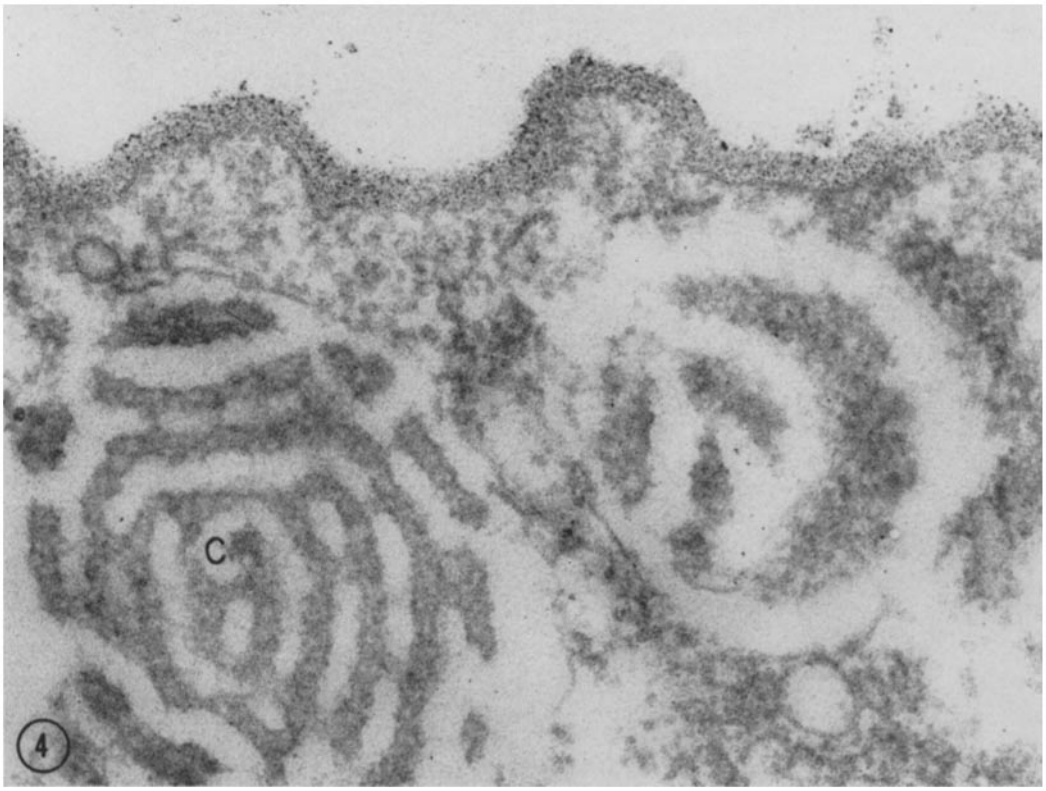
FIGURE 3

An egg treated with the ferritin conjugate from an antijelly serum in the *direct* method. The jelly coat has been precipitated to a thickness of approximately 0.5 μ and is labeled with ferritin. *C:* cortical granule (note a distinctive periodic structure). × 80,000.

FIGURE 4

The same antijelly serum unconjugated and applied to the egg which was later treated with the ferritin conjugate from an antirabbit γ-globulin serum (indirect method). The ferritin labeling of the highly contracted jelly is much stronger than in Fig. 3. *C:* cortical granule. × 80,000.





13. SOBER, H. A., and PETERSON, E. A., *Fed. Proc.*, 1958, **17,** 1116.
14. KOHN, J., *Nature*, 1959, **183,** 1055.
15. SMITH, C. W., METZGER, J. F., ZACKS, S. J., and KASE, A., *Proc. Soc. Exp. Biol. and Med.* 1960, **104,** 336.
16. HARDING, C. V., and HARDING, D., *Exp. Cell Research*, 1952, **3,** 475.
17. LUFT, J. H., *J. Biophysic. and Biochem. Cytol.*, 1961, **9,** 409.
18. BESSIS, M., and BRETON-GORIUS, J., *Rev. Hémat.*, 1959, **14,** 165.
19. NACHMIAS, V. T., and MARSHALL, J. M., JR., *in* Biological Structure and Function, (T. W. Goodwin and O. Lindberg, editors), New York, Academic Press, **2,** 1961, 605.
20. HOLTER, H., *Internat. Rev. Cytol.*, 1959, **8,** 481.
21. BOREK, F., and SILVERSTEIN, A. M., *J. Immunol.*, 1961, **87,** 555.
22. MORGAN, C., HSU, K. C., RIFKIND, R. A., KNOX, A. W., and ROSE, H. M., *J. Exp. Med.*, 1961, **114,** 825 and 833.
23. EASTON, J. M., GOLDBERG, B., and GREEN, H., *J. Exp. Med.*, 1962, **115,** 275.

FIGURE 5

A control preparation treated with ferritin conjugate from normal serum, showing unspecific staining with ferritin (arrows). (In most micrographs made of control eggs treated with this serum, there was less surface staining than shown in this figure.) × 80,000.

FIGURE 6

The *indirect* method used with normal γ-globulin and the ferritin conjugate from anti-rabbit γ-globulin serum. Again there is no significant surface staining with ferritin (arrow), but the yolk granules have taken up the ferritin, or ferritin conjugate, and are strongly stained. Both yolk granules have a broken bounding membrane which has allowed the contents to escape. × 80,000.

FIGURE 7

Another type of control experiment in which the unfertilized egg has been treated with antisperm γ-globulin serum from the same species in the *indirect* method. The amount of ferritin staining is negligible (arrow). × 80,000.