

# Sizing of Protein A-Colloidal Gold Probes for Immunoelectron Microscopy

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**ABSTRACT** Gold particles in colloidal solutions often vary considerably in size. The finest sols ( $\bar{\phi} < 15$  nm), especially, are very heterogeneous, as is indicated by coefficients of variance (CV) of 25–35%. We have complexed staphylococcal protein A with gold particles (PA/Au) and then fractionated the preparations by glycerol or sucrose gradient centrifugation into very homogeneous subfractions. In this way, PA/Au probes of almost any size between 4.5 and 15 nm could be prepared. The variation of the gold particles in these fractions resulted in CV's between 9 and 16%. The reactivity of the PA/Au complex was not affected by the gradient procedure, as was shown by single- and double-labeling immunocytochemistry of ultrathin cryosections of rat pancreatic tissue.

During the last ten years colloidal gold has become increasingly popular in cytochemistry (5). Gold particles are easily prepared and bound to lectins (7, 10), immunoglobulins (1, 3), staphylococcal protein A (8, 9), and various other proteins (3, 6). The particles can be detected with accuracy by transmission, as well as scanning, electron microscopy. An important feature also is that gold markers can be prepared in a variety of sizes, so that they are suitable tools for double-labeling experiments. By the reduction of gold chloride with white phosphorus, sodium ascorbate, or sodium citrate, sols can be prepared that contain spherical gold particles with average diameters of 5, 12, or 16 nm, respectively. Recently, we used protein A-colloidal gold (PA/Au) complexes for visualizing immunoreactions in ultrathin cryosections (4). We combined 5- and 16-nm gold markers for the simultaneous demonstration of two immunoreactions in one section. A shortcoming was that the larger gold particles were inferior with respect to sensitivity and resolution. The 12-nm gold would seem more appropriate, but the diameter of the gold particles prepared by ascorbate reduction varies considerably, ranging from 5 to 20 nm, so that they cannot be used together with 5-nm particles. This, possibly, is the reason why the ascorbate preparation is rarely used in cytochemical studies. We report here a simple method for the purification of uniform subfractions from ascorbate and white phosphorus gold preparations. This method enabled us to make gold labels of any size between 4.5 and 15 nm. Apart from a significant improvement of the double-labeling procedure, a larger assortment of particles makes the PA/Au technique much more flexible.

## MATERIALS AND METHODS

The fixation of rat pancreatic tissue, the cutting of ~100-nm thick cryosections,

the antibody preparations used, the immunocytochemical procedure, and the postincubation treatment of the sections have been previously described (4).

## Colloidal Gold Solutions

Colloidal gold solutions were prepared by reduction of chloroauric acid ( $\text{HAuCl}_4$ ; Drijfhout & Zn's, Amsterdam) with either white phosphorus (1), sodium ascorbate (11), or sodium citrate (2). The gold preparations obtained will be designated as  $\text{Au}_{\text{phos}}$ ,  $\text{Au}_{\text{asc}}$ , and  $\text{Au}_{\text{cit}}$ , respectively. Details of the procedure were as follows:

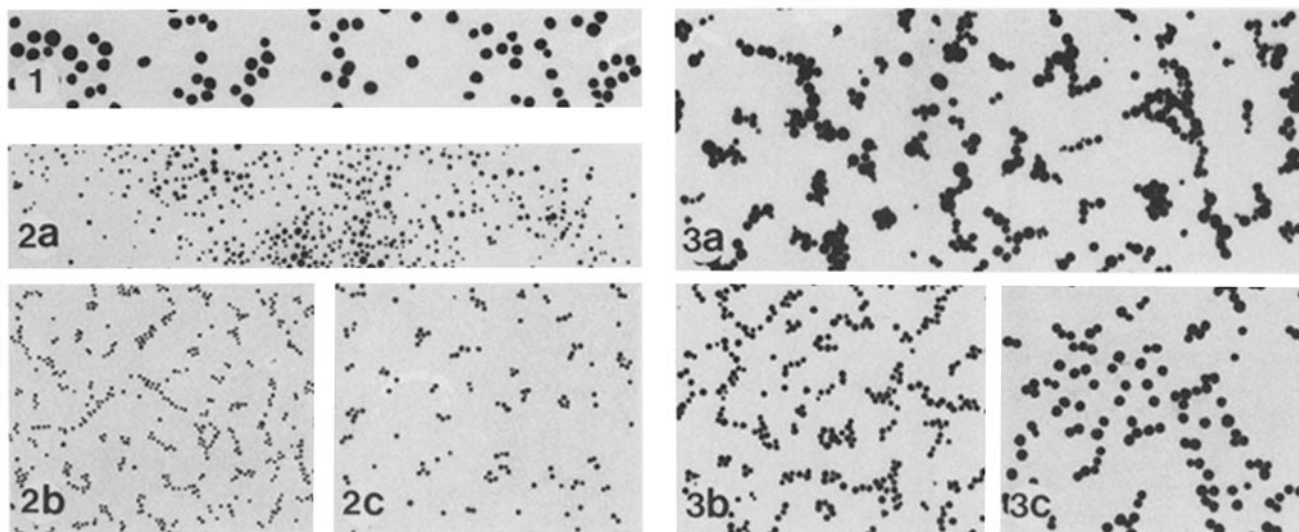
(a)  $\text{Au}_{\text{phos}}$ : 1.5 ml of a 1%  $\text{HAuCl}_4$  solution and 1.4 ml of 0.1 M  $\text{K}_2\text{CO}_3$  were added to 120 ml of distilled water. To this solution, 1 ml of white phosphorus (Merck, Darmstadt W. Germany) in diethylether was added and mixed well. The phosphorus solution was prepared by adding 1 part of ether saturated with white phosphorus to 4 parts of ether. The mixture was left for 15 min at room temperature and was then boiled under reflux until the color turned from brownish to red (~5 min).

(b)  $\text{Au}_{\text{asc}}$ : 1 ml of 1%  $\text{HAuCl}_4$  and 1 ml of 0.1 M  $\text{K}_2\text{CO}_3$  were mixed in ~25 ml of distilled water. While stirring, 1 ml of a 0.7% solution of sodium ascorbate (BDH Chemicals, Ltd., Poole, England) was added quickly. This reaction was done on ice. Higher temperature tended to increase the particle size. After adding the ascorbate, the color became immediately purple-red. Then, the volume was adjusted to 100 ml with distilled water and, finally, the solution was heated until boiling so that the color became red.

(c)  $\text{Au}_{\text{cit}}$ : 1 ml of a 1%  $\text{HAuCl}_4$  solution was added to 100 ml of distilled water, followed by 2 ml of 1% tri-sodium citrate  $\cdot 2\text{H}_2\text{O}$  (Merck). The mixture was boiled under reflux for 15–30 min, until the color became red. After cooling, 0.5 ml of 0.1 M  $\text{K}_2\text{CO}_3$  was added. All gold sols were made up and stored in clean glass vials.

## PA/Au Complex

Gold particles are easily precipitated from colloidal solutions by electrolytes, unless they are stabilized by proteins or other macromolecules which bind rapidly and spontaneously to their surface upon mixing. The binding of a protein is optimal at a pH close to or slightly above its isoelectric point (3), which is at pH 5.1 for PA. The amounts of  $\text{K}_2\text{CO}_3$  added in the gold sol recipes described above were adequate to bring the final pH of the sols between 5 and 6. Measurements of pH were done by pH paper. More accuracy was not needed and nonstabilized



FIGURES 1-3 Gold particles from various preparations. Carbon-coated Formvar grids floated for 1 h on drops containing a particular PA/Au complex. The grids were then rinsed quickly with distilled water and dried. Fig. 1,  $Au_{citr}$ ; Fig. 2,  $Au_{phos}$ : (a) crude preparation, (b) finest subfraction, and (c) medium-size subfraction; Fig. 3,  $Au_{asc}$ : (a) crude preparation, (b) finest subfraction, and (c) medium-size subfraction.  $\times 96,000$ .

sols are harmful to electrodes of pH meters. The PA preparation we used (Pharmacia Fine Chemicals, Uppsala, Sweden) was dissolved in distilled water (2 mg/ml). The amount of PA needed to stabilize a sol was determined as described by Horrisberger and Rosset (6). In small glass tubes, 0.25 ml of gold sol was mixed with various amounts of PA. After 5 min, 0.25 ml of 10% NaCl was added. As long as more PA could be bound, the solution turned blue. The lowest concentration of PA that prevented this color change was taken as the saturation point. We found that the saturation points for  $Au_{phos}$ ,  $Au_{asc}$ , and  $Au_{citr}$  were  $\sim 8$ ,  $\sim 6$ , and  $\sim 5$   $\mu\text{g}$  PA/ml of sol, respectively.

To prepare the PA/Au complexes, we added PA (2 mg/ml) to 30 ml of the gold sols so that the concentration exceeded the saturation point by 10%. After 5 min, 0.3 ml of 5% polyethylene glycol (Carbowax 20 M, Fluka, Buchs, Switzerland) was added, to be sure that the gold particles were stabilized maximally (6). Then the PA/Au was pelleted by centrifugation:  $Au_{phos}$ , 45 min at 125,000  $g_{av}$ ;  $Au_{asc}$ , 45 min at 50,000  $g_{av}$ , and  $Au_{citr}$ , 45 min at 15,000  $g_{av}$ . The pellet was composed of a large loose part and a small tightly packed part. The latter was not used and stuck to the wall of the tube during the further procedure. The supernate was removed without disturbing the pellet. The tubes were refilled with PBS (0.15 M NaCl, 2.5 mM KCl, 0.01 M phosphate, pH 7.2) in which the loose part of the pellet was resuspended and centrifuged again as described above. The supernate was removed carefully. The loose part of the second pellet, to be called "crude preparation," was resuspended in the remainder of the supernate (the small part that could not be removed without taking some of the pellet) and stored in siliconized caps.

To make uniformly sized subfractions, crude preparations of PA/ $Au_{phos}$  and PA/ $Au_{asc}$  were prepared as described above, except that the second centrifugation was omitted. The loose part of the first pellet was resuspended in the remainder of its supernate ( $\sim 0.5$  ml) and layered over a 10-30% continuous sucrose or glycerol gradient (volume 10.5 ml, length 8 cm) in PBS. The gradients were centrifuged in a SW41 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) for 45 min at 41,000 rpm in the case of PA/ $Au_{phos}$  and for 30 min at 20,000 rpm in the case of PA/ $Au_{asc}$ . From  $\sim 1$  cm under the top until the bottom, the gradient was stained red by PA/Au. The upper half of this red zone was collected in successive fractions of  $\sim 1$  ml and dialysed against PBS. Before use, the solution was diluted until the red color could just be seen. To minimize aspecific adherence to the sections, goat serum without immunoglobulins was added (4).

The separation was similar for both sucrose and glycerol. Glycerol may be advantageous because the samples can be stored at  $-18^\circ\text{C}$  after the glycerol concentration is raised to 50%. If glycerol is used on cryosections, the samples in glycerol should first be dialysed against PBS, because glycerol destroys the delineation of membranes. Furthermore, complexing of gold and protein can be done in glycerol. In sucrose, however, nonstabilized preparations tend to flocculate, possibly because of impurities of commercial sucrose preparations. Thus, on an aqueous glycerol gradient, gold preparations can be sized before stabilization with a protein. To this end, the nonstabilized sols are concentrated by pelleting (i.e., 20 min  $\times$  20,000  $g$  for  $Au_{asc}$ ) and then centrifuged over a 10  $\times$  30% glycerol

TABLE I  
Size of Gold Particles in Various Preparations

Reducing agent (particle code)	Average diameter $\pm$ coefficient of variance		
	Crude preparation	Finest fraction	Medium size fraction
	nm	nm	nm
White phosphorus ( $Au_{phos}$ )	$5.9 \pm 25\%$	$4.6 \pm 11\%$	$5.8 \pm 13\%$
Sodium ascorbate ( $Au_{asc}$ )	$11.3 \pm 35\%$	$7.8 \pm 16\%$	$12.7 \pm 9\%$
Sodium citrate ( $Au_{citr}$ )	$15.5 \pm 13\%$		

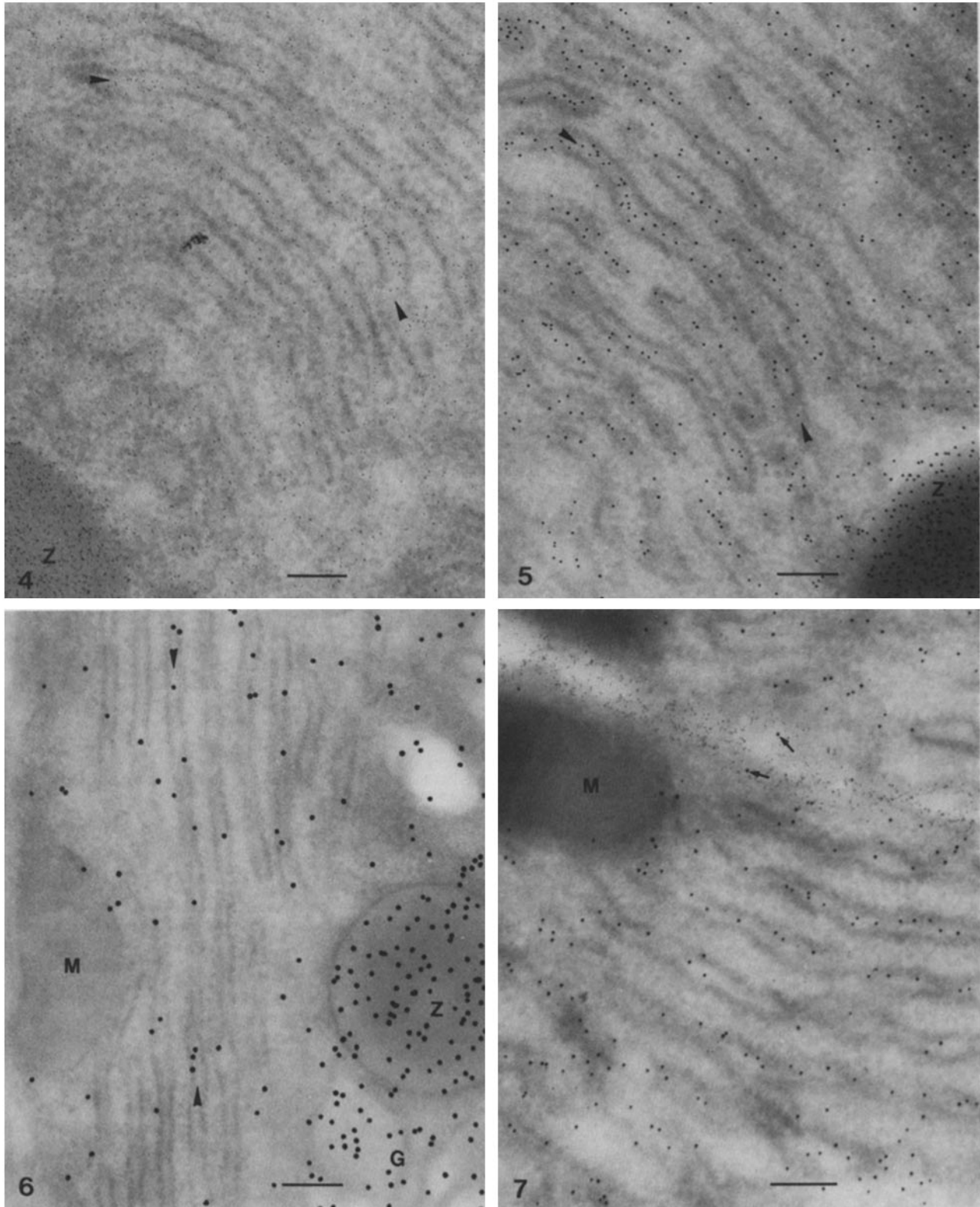
The values were determined on 50 gold particles from the same preparations as shown in Figs. 1-3.

gradient in distilled water (i.e., for  $Au_{asc}$  30 min  $\times$  15,000 rpm SW41). Subsequently, small homogeneous fractions from the gradients can be complexed with proteins. This is advantageous when very low amounts of a protein have to be bound to the gold.

## RESULTS AND DISCUSSION

Of three crude colloids that we prepared, only  $Au_{citr}$  contained particles of reasonably uniform size (Fig. 1). The average diameter was 15.5 nm with a coefficient of variance (CV) of 13% (Table I).  $Au_{phos}$  particles were much more variable (Fig. 2a), with an average diameter of almost 6 nm and a CV of 25%. However, this preparation yielded a suitable label for high-resolution cytochemistry when the largest granules were removed by differential centrifugation (4). The  $Au_{asc}$  colloid was even more heterogeneous (Fig. 3a). The average size of the granules was 11.3 nm with a CV of 35%. This made the crude  $Au_{asc}$  preparation unfavorable for use as an alternative for one of the other preparations or in combination with these in double-label experiments.

For immunocytochemistry, the size of the gold particles has several important implications. Large particles can easily be observed at the lower magnifications, so that a good overall impression of the labeling pattern is obtained. The large particles are also more distinct over electron-dense structures than



FIGURES 4-6 Frozen sections of 2% paraformaldehyde-/0.5% glutaraldehyde-fixed tissue from rat exocrine pancreas. Sequence of immunostaining steps: (i) rabbit immunoglobulins against rat pancreas amylase; (ii) finest subfraction of PA/Au<sub>phos</sub> (Fig. 4), or finest subfraction of PA/Au<sub>abc</sub> (Fig. 5), or PA/Au<sub>ctr</sub> (Fig. 6). Arrowheads, RER, intracisternal space. G, Golgi complex. Z, zymogen granules. M, mitochondrion. Bar, 0.2  $\mu$ m.  $\times$  53,000.

FIGURE 7 Rat pancreas. Sequence of immunostaining steps: (i) rabbit immunoglobulins against the major glycoprotein (GP-2) in membranes of exocrine pancreas cells; (ii) finest subfraction of PA/Au<sub>phos</sub>; (iii) rabbit immunoglobulins against rat pancreas amylase; (iv) finest subfraction of PA/Au<sub>abc</sub>. The small particles along the cell membrane can be clearly distinguished from the larger gold particles over the RER. The few large grains over the cell membrane give an impression of the minor interaction between both immunoreactions. M: mitochondrion. Bar 0.2  $\mu$ m.  $\times$  58,000.

the small ones. On the other hand, small particles are favorable because of a higher yield of label and, hence, of a more sensitive display of the immunoreaction. In addition, the resolution of the technique improves with decreasing particle size, and the chance that small cell structures are obscured by superimposed gold granules becomes smaller. The choice of a particular particle size is therefore a compromise depending on what information is wanted. Sometimes, more than one probe is needed to observe an immunoreaction at several magnification levels or to visualize more than one immunoreaction at a time in so-called double-label experiments.

These considerations demonstrate the need for a wider choice of labels with a well-defined size of narrow range. We achieved an important extension of the assortment of gold particles by fractionating the crude Au<sub>phos</sub> and Au<sub>asc</sub> preparations. Both were first complexed to PA, then concentrated and centrifuged over a gradient. Gold particles with increasing size were distributed in a red zone of the gradient from ~1 cm under the top down to the bottom. Medium-size particles (with a diameter close to the mean value of the crude preparation) were located at about the middle of this red zone. From this level upwards, five successive fractions of 1 ml each were collected. The heaviest of these, called "medium-size," and the lightest, called "finest," fractions are dealt with in Figs. 2 and 3 and Table I. Still larger particles in the lowest half of the gradient and in the pellet were contaminated with aggregates of 2 or 3 small particles. Such aggregates were absent from fractions in the upper half of the gradient. The variability of the particle size was considerably less in the finest and medium-size fractions of Au<sub>phos</sub> (Figs. 2*b* and *c*) and Au<sub>asc</sub> (Figs. 3*a* and *c*) than in the crude preparations (Table I). The differences in size between the medium-size Au<sub>phos</sub> and the finest Au<sub>asc</sub> fractions and between the medium-size Au<sub>asc</sub> and the Au<sub>citr</sub> particles were small, so that homogeneous preparations of PA/Au markers can now be made with almost any desirable size between 4.5 and 15.5 nm. In addition, any larger probe, up to 150 nm, can be prepared by modifying the citrate reduction of HAuCl<sub>4</sub> (2), but such large granules are not useful for high-resolution cytochemistry.

Apparently, the gradient procedure did not affect the reactivity of the PA/Au complex. When PA/Au<sub>phos</sub> subfractions were used for anti-amylase staining in ultrathin frozen sections of exocrine pancreas (Fig. 4), the yield of labeling was about the same as when the crude Au<sub>phos</sub> preparation was used (4). The density of the labeling decreased with increasing size of the gold particle used. The 8-nm Au<sub>asc</sub> fraction (Fig. 5) gave a lower yield than the 4.5-nm Au<sub>phos</sub> fraction, but it was much more sensitive than the 15.5-nm Au<sub>citr</sub> (Fig. 6).

Impairment of penetration into the sections is probably one factor that causes a lesser sensitivity for the larger particles.

Stereoscopic observations showed that the labeling of dense material, such as zymogen granule content, is restricted to the very surface of the sections. Apparently, there is hardly any penetration even if the smallest gold particles are used. Possibly, the anti-amylase binds only to superficial antigenic sites, so that the PA-binding sites are equally exposed to any PA/Au complex used. Yet, the zymogen granules are labeled most densely by the smallest probes. At such sites with intense labeling, sterical hindrance and repulsion between the gold particles possibly become limiting factors which may determine the sensitivity of the various gold probes. These factors and the penetration characteristics of particle and cell structures under study have to be considered when labeling patterns are interpreted quantitatively.

The availability of so many homogeneous PA/Au preparations enabled us to improve the PA/Au double-label technique as described before (4). Two separately distributed antigens in the basal part of the pancreatic cells, one in the cell membrane (GP 2) and the other in the RER (amylase), were labeled distinctly with the finest fraction of PA/Au<sub>phos</sub> (4.5 nm) and the finest fraction of PA/Au<sub>asc</sub> (8 nm). The interaction between both immunoreactions was very low (Fig. 7). Hence, for high-resolution double-labeling the combined application of the 8-nm and the 4.5-nm probes seems now to be the best choice.

With the modification described here, the preparation of PA/Au remains very simple. As a routine procedure it does not take more than half a day.

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