Supplementary Materials for

Double-kill mechanism of artemisinin against *Plasmodium*

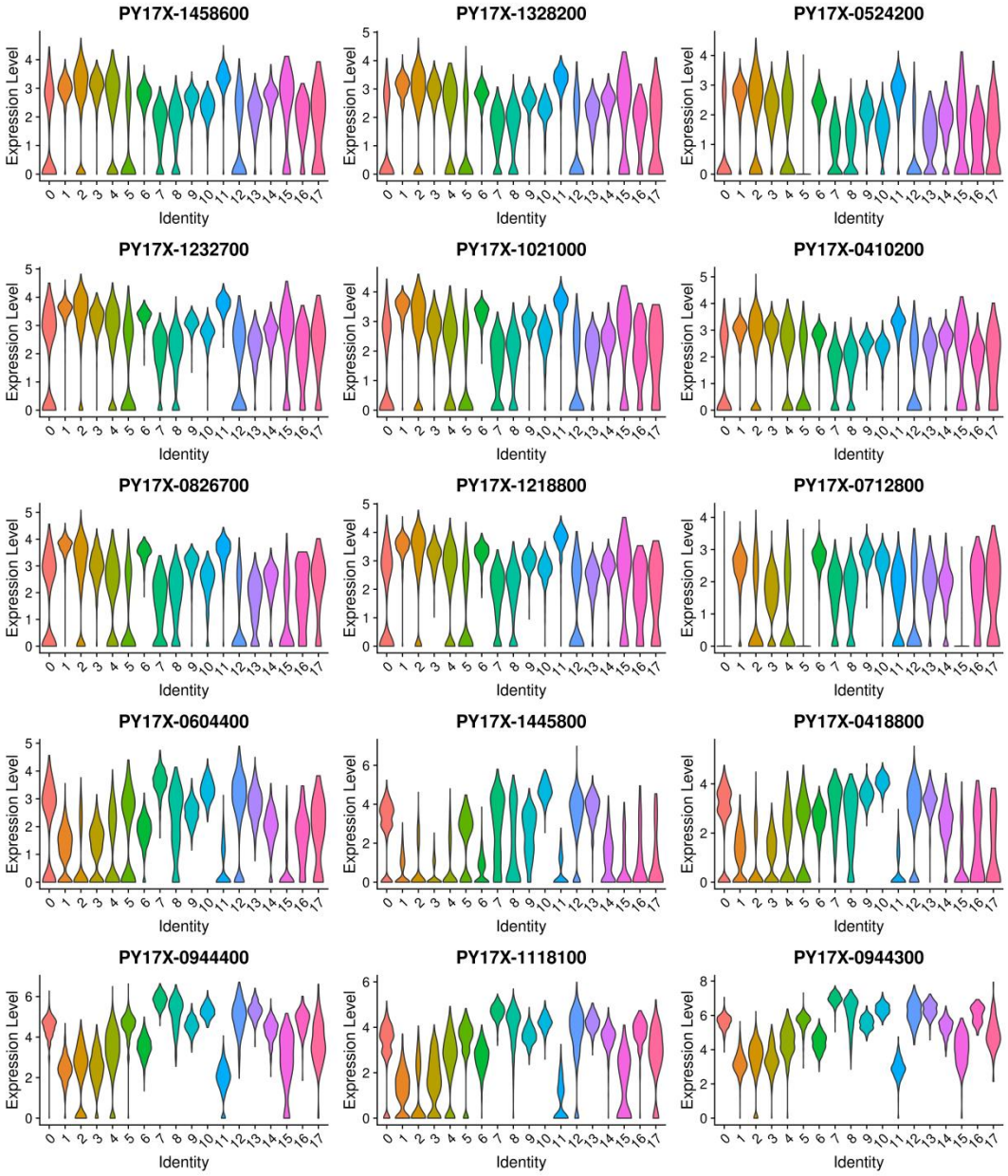
Jun Sun* , Fujiang Guo, Chuang Liu, Bo Chen, Wenyue Xu, Jia Li, Xiling Du, Yixian Liu, Yu Wang

*Corresponding author. Email: swksj@tongji.edu.cn

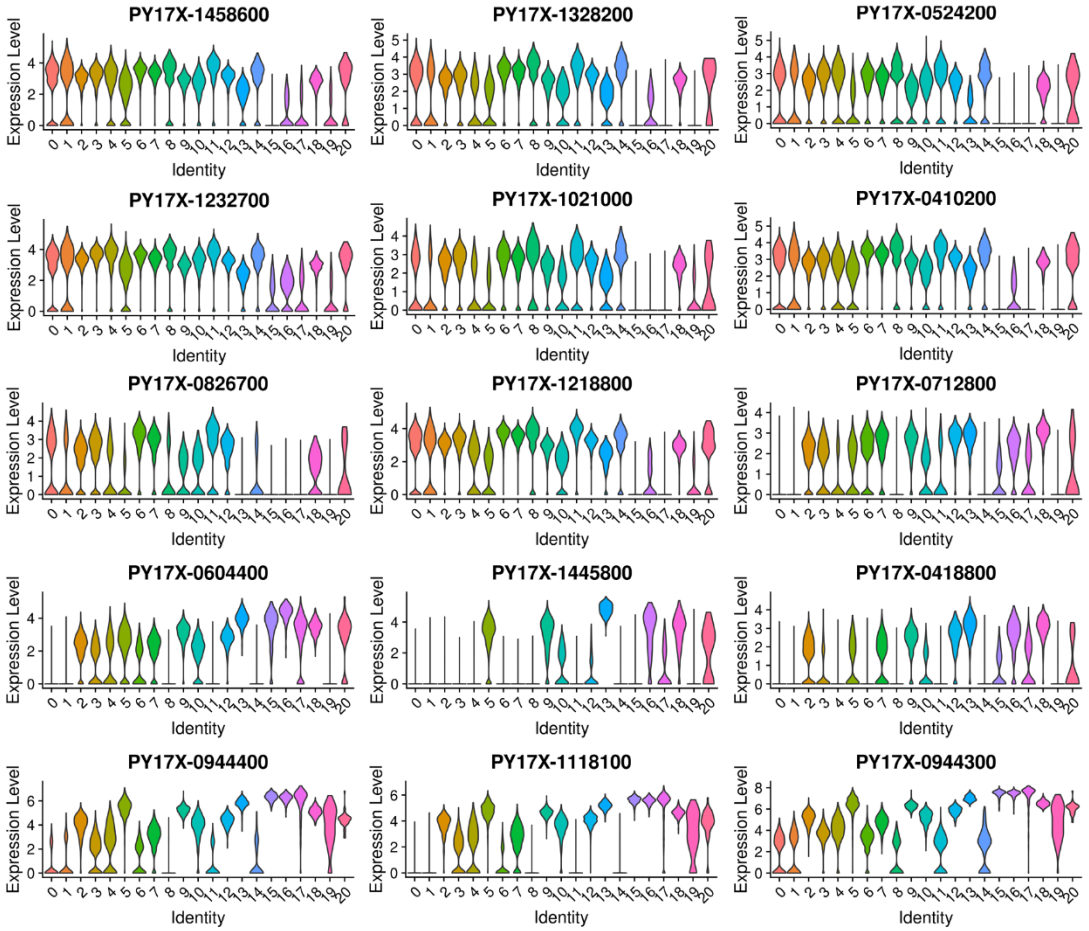
This PDF file includes:

Extended data Figs. 1 to 12 and figure legends

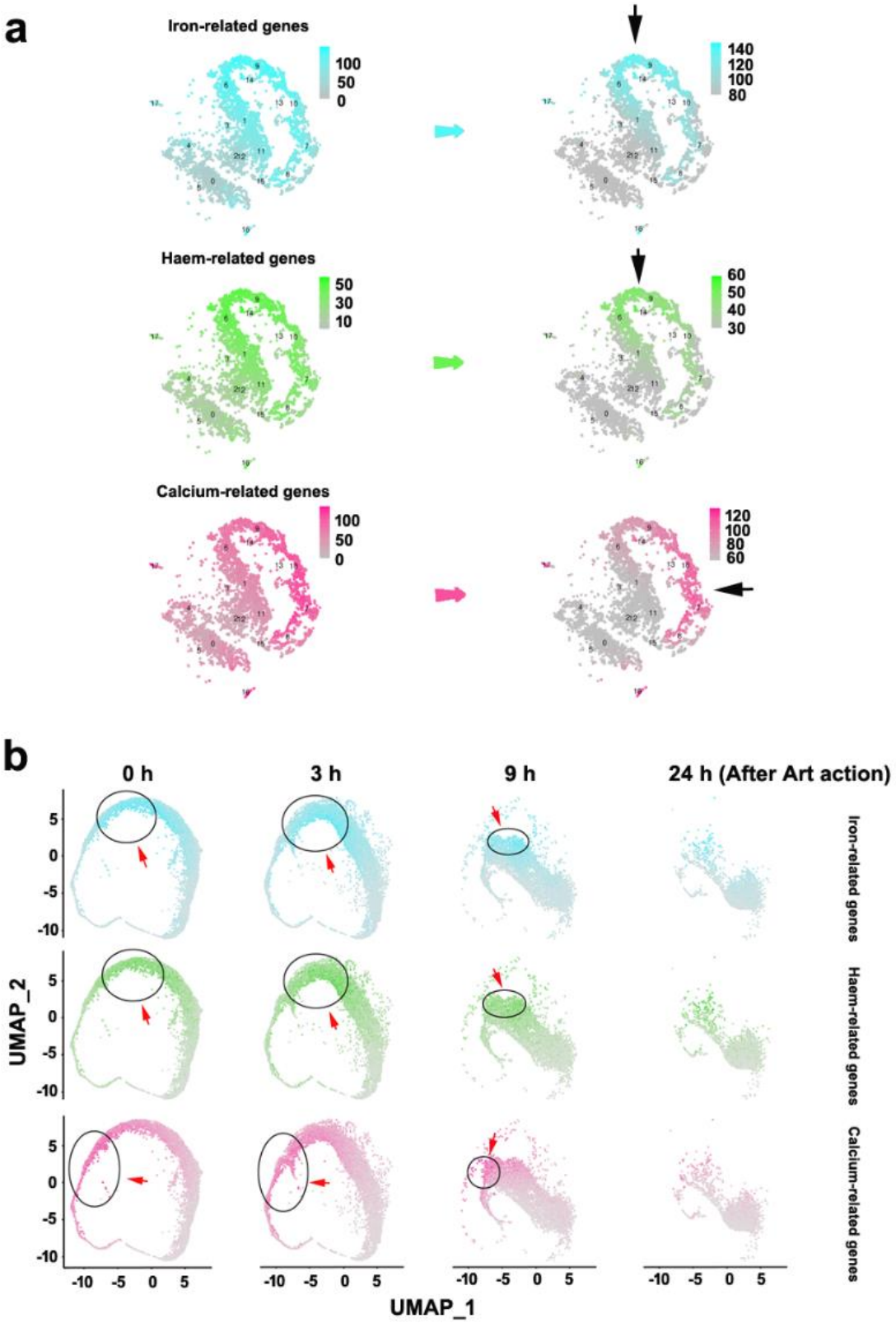
Extended data Table: Tables 1 to 2 and table legends



Extended data Fig. 1 | Violin plots showing the expression characteristics of the 15 marker genes in 18 clusters from the control and test groups at 6 h after artemether treatment. Based on the expression characteristics of marker genes, the relationship between clusters and infection stages of malaria parasites were determined.



Extended data Fig. 2 | Violin plots showing the expression characteristics of 15 marker genes in 21 clusters from the samples at 0 h, 3 h, 9 h and 24 h after artemether treatment. Based on the expression characteristics of marker genes, the relationship between clusters and infection stages of malaria parasites were determined.

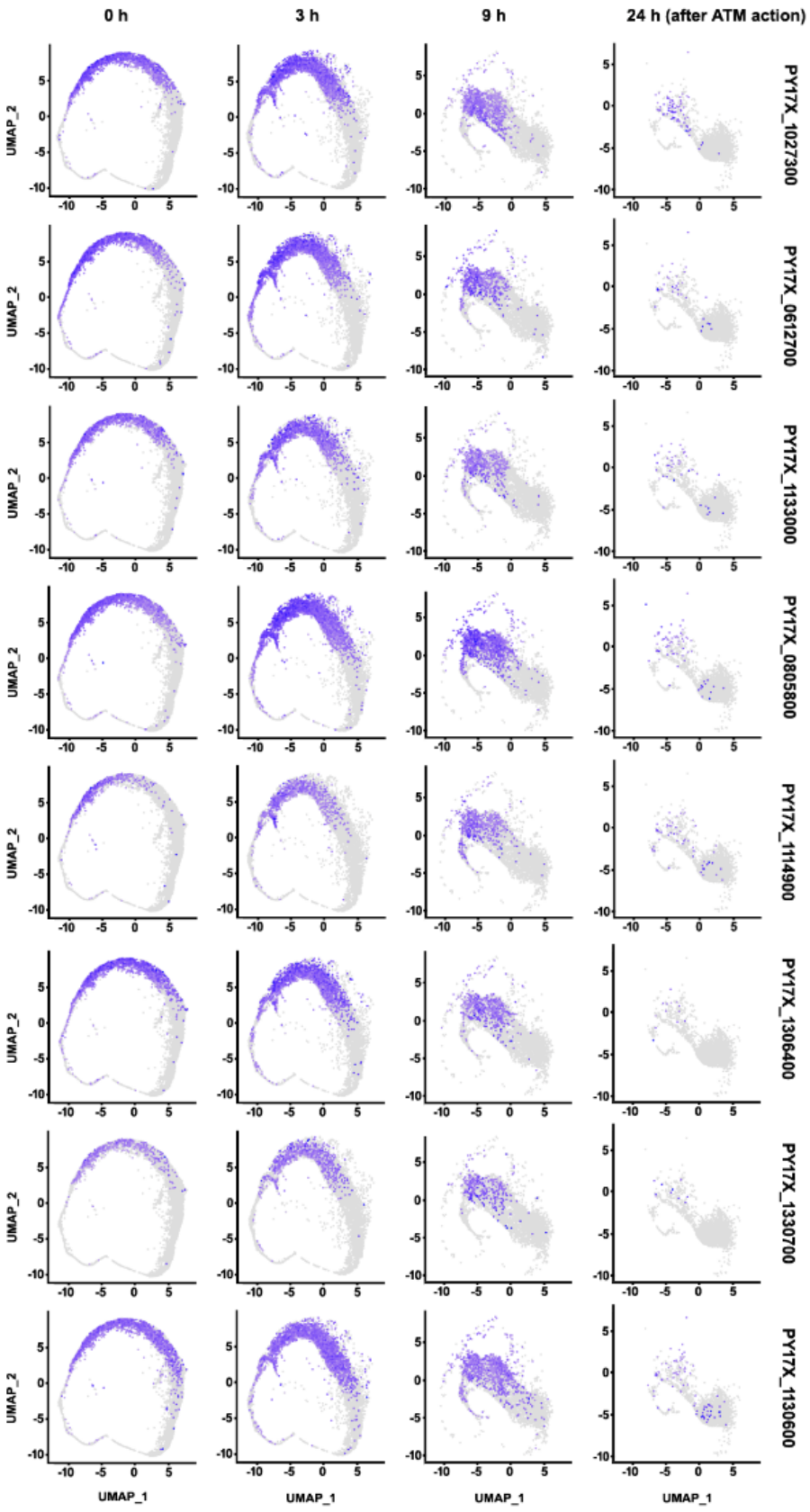


28

29 **Extended data Fig. 3 | Distribution of parasites that expressed iron-, haem- or Ca-related genes in all**

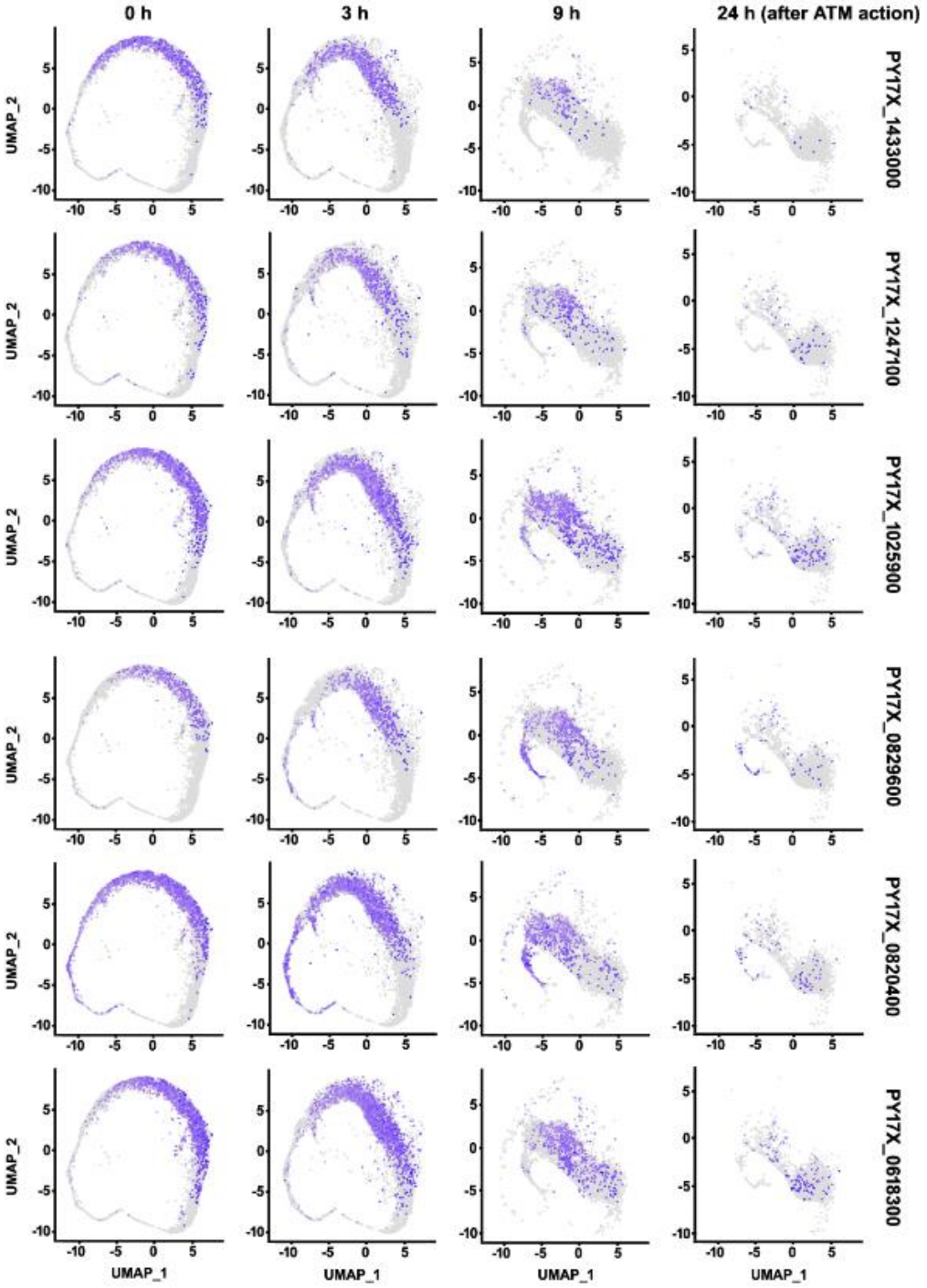
30 **clusters. a**, t-distributed stochastic neighbour embedding (tSNE) plots showing the distribution of parasites

that expressed iron-, haem- and Ca-related genes in all clusters at 6 h after artemether treatment. The parasites with highly-expressed iron- and haem-related genes were distributed in similar clusters (black arrows), different from those with highly-expressed Ca-related genes (black arrow). **b**, Uniform Manifold Approximation and Projection (UMAP) plots showing changes in the distribution of parasites with expressed iron-, haem- and Ca-related genes in all clusters at 0 h, 3 h, 9 h and 24 h after artemether treatment. The distribution of parasites with expressed iron- and haem-related genes was similar, but different from those with expressed Ca-related genes. The red arrows and black circles indicate the areas or clusters in which iron-, haem- or Ca-related genes were highly expressed.



Extended data Fig. 4 | Distribution of parasites with highly-expressed DNA synthesis-related genes in all clusters on UMAP plots of SCTs from samples at 0, 3, 9 and 24 h after artemether treatment.

PY17X_1027300, DNA replication licensing factor MCM2, putative; PY17X_0612700, DNA replication licensing factor MCM5, putative; PY17X_1133000, DNA replication licensing factor MCM6, putative; PY17X_0805800, DNA replication licensing factor MCM7, putative; PY17X_1114900, ATP-dependent DNA helicase UvrD, putative; PY17X_1306400, DNA primase small subunit, putative; PY17X_1330700, DNA polymerase alpha subunit B, putative; PY17X_1130600, DNA polymerase epsilon catalytic subunit A, putative.



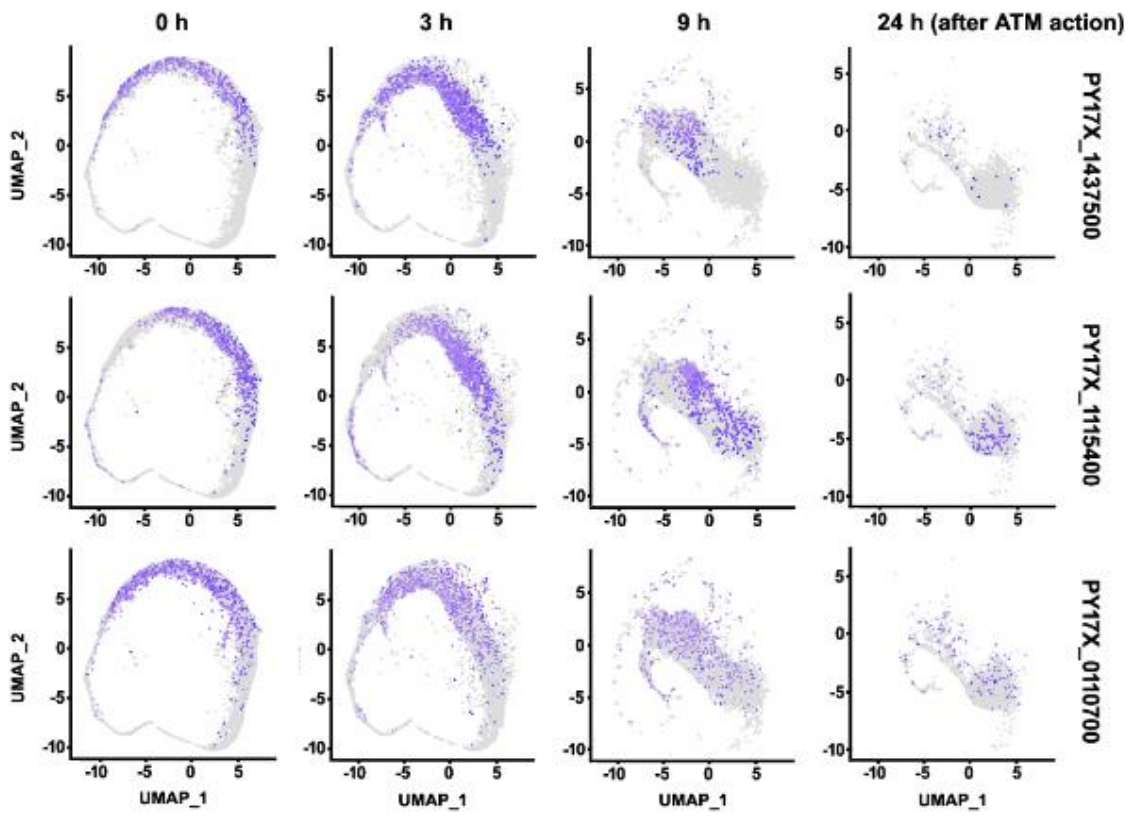
50

51 **Extended data Fig. 5 | The distribution of parasites with highly-expressed thioredoxin-related genes**

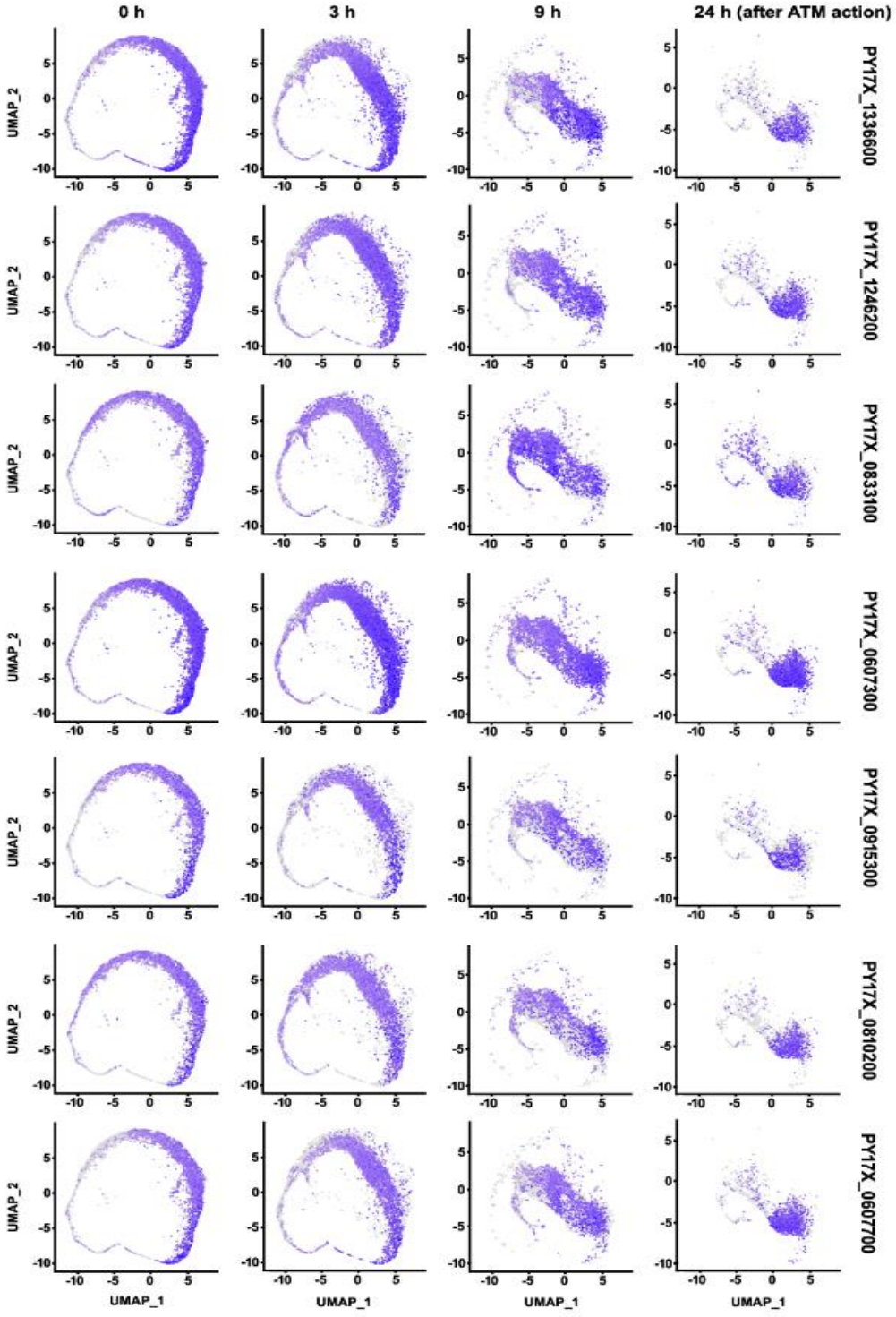
52 **in all clusters on UMAP plots of SCTs from samples at 0, 3, 9 and 24 h after artemether treatment.**

53 PY17X_1433000, thioredoxin peroxidase 2, putative; PY17X_1247100, thioredoxin-like protein, putative;

PY17X_1025900, thioredoxin-like protein, putative; PY17X_0829600, thioredoxin-like protein 2; PY17X_0820400, thioredoxin 3, putative; PY17X_0618300, thioredoxin-like protein.

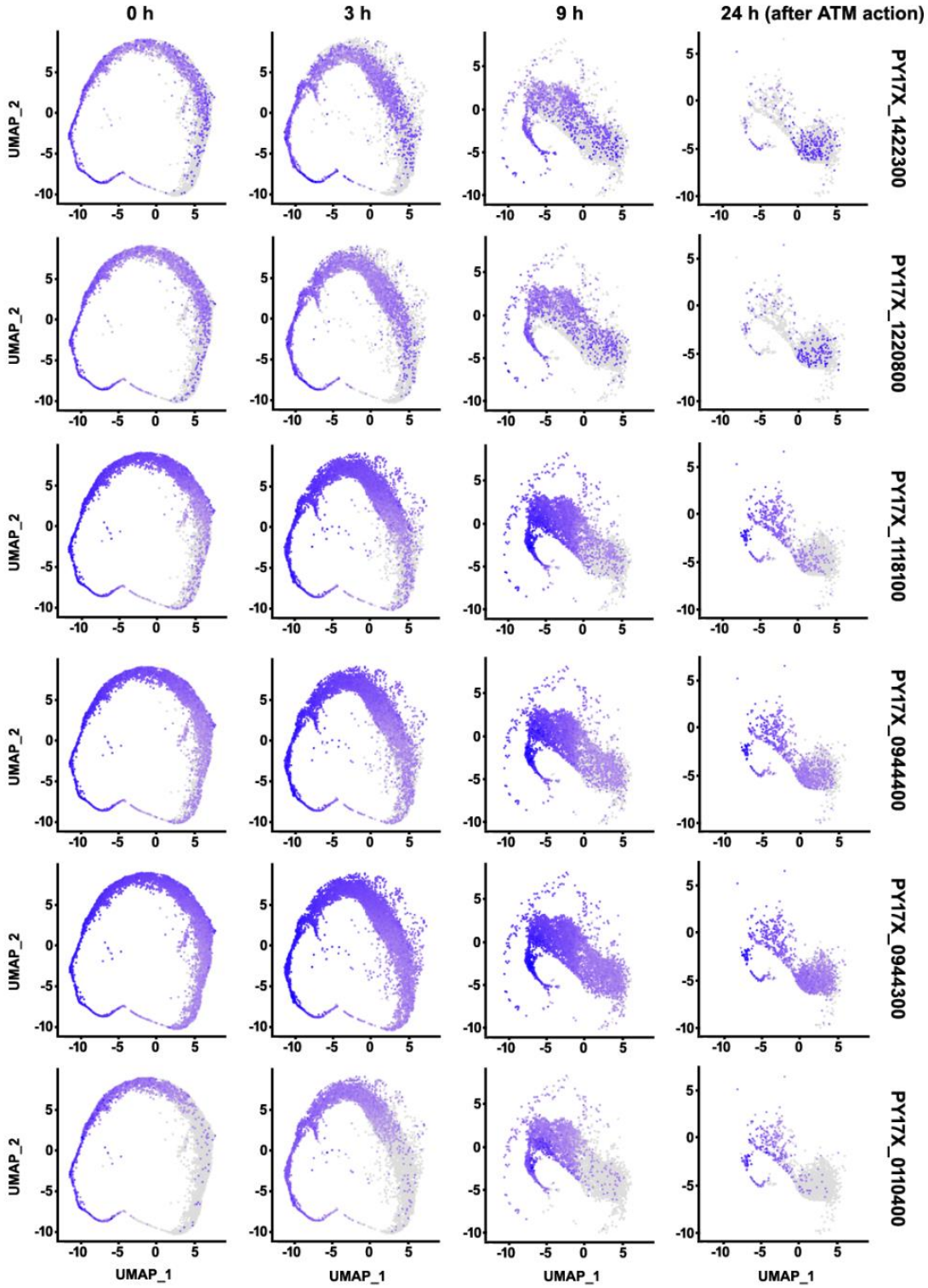


Extended data Fig. 6 | The distribution of parasites with highly-expressed pentose-phosphate-pathway-related genes in all clusters on UMAP plots of SCTs from samples at 0, 3, 9 and 24 h after artemether treatment. PY17X_1437500, ribulose-phosphate 3-epimerase, putative; PY17X_1115400, ribose-5-phosphate isomerase, putative; PY17X_0110700, transketolase, putative.



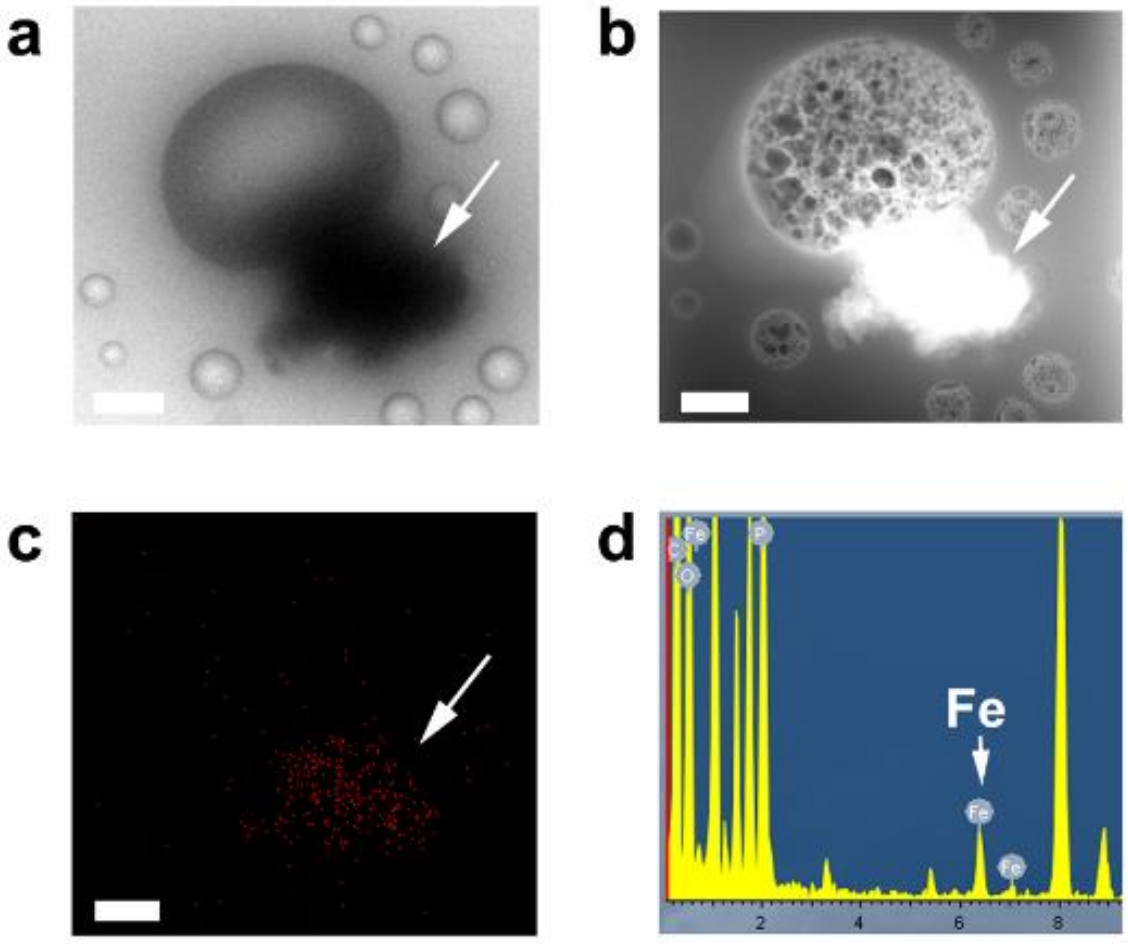
Extended data Fig. 7 | The distribution of parasites with highly-expressed protein synthesis-related genes in all clusters on UMAP plots of SCTs from samples at 0, 3, 9 and 24 h after artemether treatment. PY17X_1336600, eukaryotic initiation factor 4a, putative; PY17X_1246200, eukaryotic translation initiation factor 3 subunit E, putative; PY17X_0833100, transcription initiation factor TFIID

subunit 7, putative; PY17X_0607300, eukaryotic translation initiation factor 3 subunit C, putative; PY17X_0915300, DNA-directed RNA polymerase I subunit RPA2, putative; PY17X_0810200, DNA-directed RNA polymerase II subunit RPB1, putative; PY17X_0607700, DNA-directed RNA polymerase III subunit RPC2, putative.

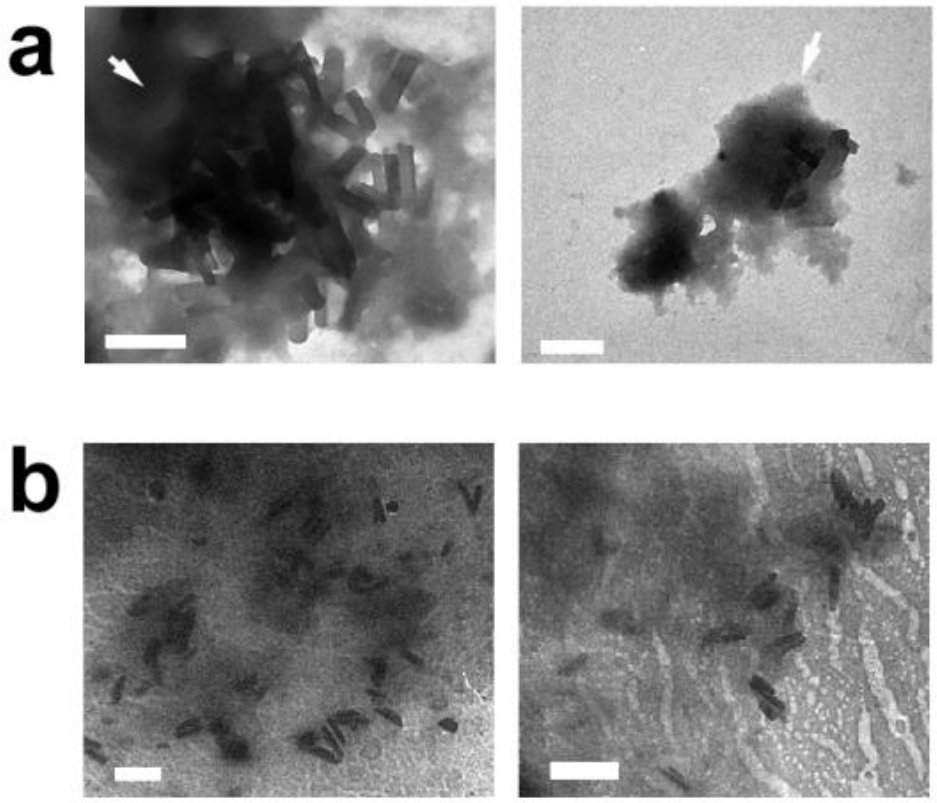


Extended data Fig. 8 | The distribution of parasites with highly-expressed histone synthesis - related genes in all clusters on UMAP plots of SCTs from samples at 0, 3, 9 and 24 h after artemether

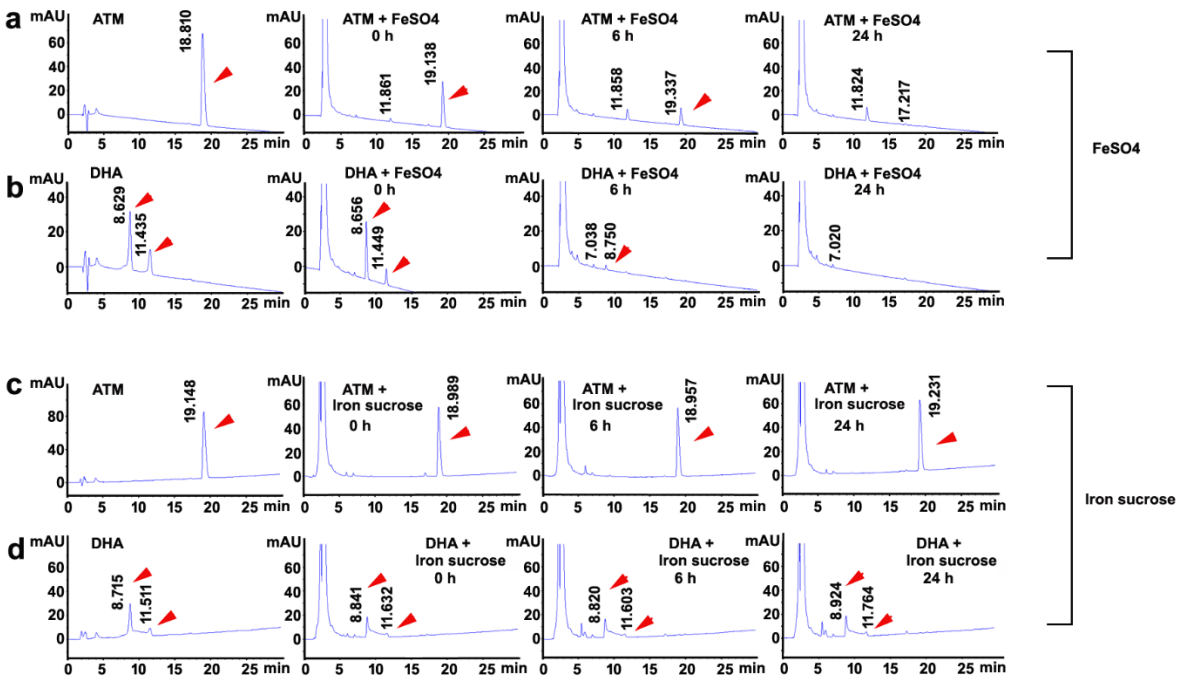
treatment. PY17X_1422300, histone H2B variant, putative; PY17X_1220800, histone H2A.Z, putative3; PY17X_1118100, histone H2A, putative; PY17X_0944400, histone H4, putative; PY17X_0944300, histone H2B, putative; PY17X_0110400, histone H3, putative.



Extended data Fig. 9 | EDS analysis of HAS under TEM. **a** and **b,** Bright- and dark-field image of HAS (arrow). **c** and **d,** EDS mapping and spectrum of HAS, indicating iron in the HAS. All scale bars indicate 1 μm.



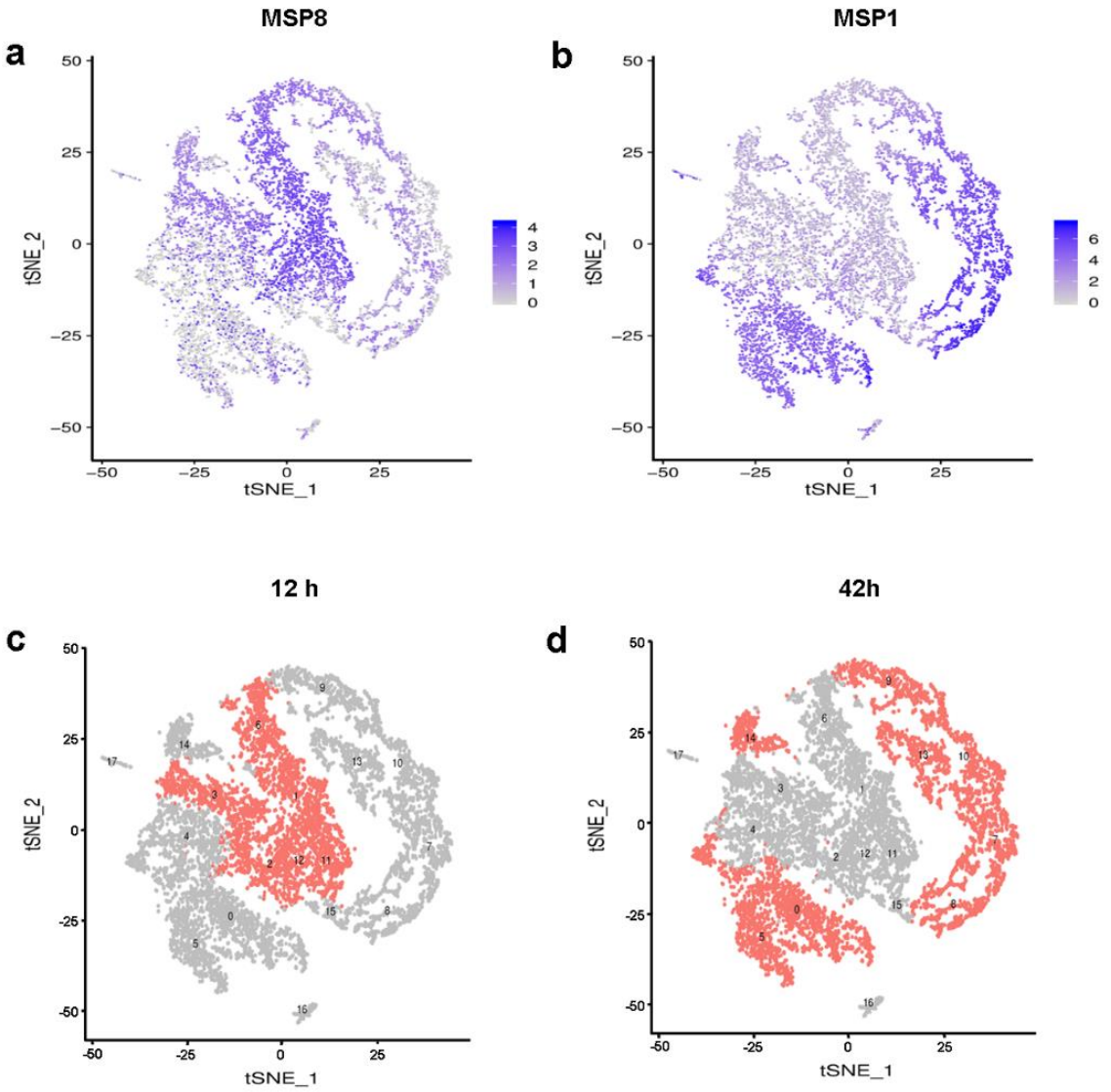
Extended data Fig. 10 | Degradation of haem-aggregate-sphere (HAS) in the presence of SDS under TEM. a, Haemozoin crystal formed in the HAS (arrows). **b,** After SDS treatment, non-crystal part of HAS was degraded, and crystal part remained. All scale bars indicate 1 μm.



Extended data Fig. 11 | The interaction between artemether (Art) or dihydroartemisinin (DHA) and iron sucrose or ferrous sulfate. **a,** The interaction between Art and ferrous sulfate 0 h, 6 h, and 24 h after they were mixed. **b,** The interaction between DHA and ferrous sulfate 0 h, 6 h, and 24 h after they were mixed. **c,** The interaction between Art and iron sucrose 0 h, 6 h, and 24 h after they were mixed. **d,** The interaction between DHA and iron sucrose 0 h, 6 h, and 24 h after they were mixed.

Method: Ferrous sulfate or iron sucrose was dissolved in DMSO reagent to prepare a 1000 mmol/L mother liquor, respectively. Then, the liquor was diluted with Tris-HCl buffer (50 mmol/L pH 8.5) to 100 mmol/L, respectively, and stored in darkness. Artemether and dihydroartemisinin were also weighed and dissolved in MeOH to prepare 100 mmol/L mother liquor, respectively. Subsequently, ferrous sulfate solution (100 mmol/L) was mixed with artemether (100mmol/L) and dihydroartemisinin (100 mmol/L) solutions at equal volumes, respectively, after which the solution was incubated at 37℃ in darkness for 0 h, 6 h and 24 h at

250 rpm /min, respectively, and then stored in darkness. Iron sucrose solutions (100 mmol/L) were mixed with artemether (100 mmol/L) and dihydroartemisinin (100 mmol/L) solutions at equal volume, respectively, then incubated at 37℃ in darkness for 0 h, 6 h and 24 h at 250 rpm/min, respectively, and then stored in darkness. HPLC analysis was conducted on an Agilent 1260 HPLC system (Agilent Corporation, USA). Next, a 5 μm Agilent Ecllipse SB-C18, 4.6 mmol/L × 250 mmol/L analytical column was used to analyse samples. The chromatographic conditions were as follows: flow rate of 1.0 mL/min; column temperature of 30 ℃; detection wavelength at 254 nm; solvent A, 0.1% acetic acid, v/v; solvent B, CH3OH; 0-40 min, 70% - 95% B (v/v); 40-50 min, hold at 95% B (v/v); injection volume: 10 μL.



Extended data Fig. 12 | Comparison of the involved cell clusters at the early and later asexual blood stages through two sets of marker genes. **a** and **b,** tSNE plots showing the expression characteristics of marker genes *MSP8* and *MSP1* in all clusters in the mixed sample of the artemether group and control group. *MSP8* and *MSP1* were reported to be expressed at the early and late blood stage of malaria parasites, respectively[1]. **c ,** tSNE plots as determined by the selected marker genes (PY17X_0826700 and PY17X_1218800) at 12 h post-infection, similar to that of *MSP8*. **d,** tSNE plots determined by the selected

marker genes (PY17X_0944400, PY17X_0110400 and PY17X_0944300) at 42 h post-infection, similar to that of *MSP1*. The result shows that the selected marker genes used in this manuscript are convincing.

Extended data Table 1 | Specially highly-expressed genes at different infection stages of malaria parasites were used as marker genes to determine the characteristic cluster of different infection stage.

6 h[a]	12 h	18 h	24 - 30 h[b]	36 h	42 - 48 h[b]
PY17X_1458600	PY17X_0826700	PY17X_0712800	PY17X_0604400	PY17X_1445800	PY17X_0944400
PY17X_1328200	PY17X_1218800			PY17X_0418800	PY17X_1118100
PY17X_0524200					PY17X_0944300
PY17X_1232700					
PY17X_1021000					
PY17X_0410200					

[a], These genes serve as marker genes to determine which clusters belong to a certain malaria parasite infection stage. These marker genes were obtained from the previous paper[2].

[b], The two stages share the common marker genes.

Extended data Table 2 | Ratios of parasites at different times or stages after different time of artemether treatment.

Samples (hours after artemether treatment)	total cell[a]	The ratios of parasites at different times (hours) post-infection with erythrocytes [b]					
		6 h	12 h	18 h	24 h	36 h	42 h
0 h	7114	4328(61%)	4307(61%)	4555(64%)	1458(20%)	1293(18%)	2574(36%)
3 h	8524	3813(45%)	1708(20%)	1518(18%)	1170(14%)	2002(23%)	2392(28%)
9 h	5854	3105(53%)	1712(29%)	39(1%)	524(9%)	1217(21%)	1710(29%)
24 h	2979	2689(90%)	2536(85%)	1(0%)	89(3%)	129(4%)	218(7%)

[a,] Total cell indicates all infected erythrocytes by *Plasmodium yoelii* 17XNL-EGFP, which was collected and analyzed by single-cell RNA sequencing.

[b,] The parasites at different infection time points can share common marker genes, which were used to determine different clusters and infection stages.

Reference

1 Howick, V. M. *et al.* The Malaria Cell Atlas: Single parasite transcriptomes across the complete Plasmodium life cycle. *Science* **365**, doi:10.1126/science.aaw2619 (2019).

2 Zhu, L. *et al.* New insights into the Plasmodium vivax transcriptome using RNA-Seq. *Sci Rep* **6**, 20498, doi:10.1038/srep20498 (2016).