Supplementary Fig. 5. Phagocytosis of S. aureus by the hemocytes of horseshoe crab in vivo and in vitro. Mid-log phase S. aureus cells were stained with fluorescent BacLight™ Red (Molecular Probes, Inc., Eugene, OR, USA) following the protocol provided. For in vivo analysis, ~1×10^7 cells were injected into the heart of a medium-sized horseshoe crab. Blood samples were collected 1 h later into tubes containing a fixative (10 % formalin, 0.5 M NaCl), at the final ratio (v/v) of ~ 1:5. After ~30 min of fixation in an ice bath, the samples were centrifuged at 150 × g for 10 min. The supernatant was removed, and the cells were re-suspended in fresh fixative and stored at 4 °C. For in vitro experiments, horseshoe crab hemolymph was bled into sterile falcon tubes and immediately used. Fluorescent labeled S. aureus cells were added at the final density of ~ 10^7 cells/ml into the fresh blood, or the blood supplemented with 1/10 (v/v) of 0.5 M EDTA or the complete Protease Inhibitor Cocktail (Roche). It should be noted that the horseshoe crab blood cell density is ~ 2 × 10^6 cells/ml (Ornberg & Reese, 1981, J Cell Biol 90: 40-54). The tubes were incubated at room temperature with very gentle shaking for 1 h. Thereafter, the blood samples were fixed as above. The fixed cells were analyzed by fluorescence microscopy at 400× magnification. Both bright field image and fluorescence image were taken for each view. (A) A typical view of hemocytes taken from the blood of a horseshoe crab injected with S. aureus. The internalization of red fluorescent bacterial cell(s) represents in vivo phagocytosis. In the pilot experiments, phagocytosis appeared to occur from 30 min after infection (not shown). (B) The typical views of in vitro experiment, which show that both EDTA and protease inhibitors appear to suppress the phagocytosis. It is noted that under the in vitro conditions, the labile horseshoe crab hemocytes tend to aggregate at prolonged incubation, thus posing a possibility that the bacteria may either be engulfed or associated or co-aggregated with the hemocytes. However, theoretically protease inhibitors should not inhibit the non-specific association of bacteria with hemocytes, thus favoring the process of engulfment/phagocytosis, which, in the absence of EDTA or protease inhibitor is possibly mediated by the protease-activated CrC3 opsonization.
A) *In vivo* phagocytosis

B) *In vitro* phagocytosis

Blood + *S. aureus*

Blood + Protease inhibitors + *S. aureus*

Blood + EDTA + *S. aureus*

Fluorescence views  Bright field views  Overlapped views

Supplementary Fig. 5