Supplemental Material:

Cetylpyridinium chloride (CPC) reduces zebrafish mortality from influenza infection: Super-resolution microscopy reveals CPC interference with multiple protein interactions with phosphatidylinositol 4,5-bisphosphate in immune function

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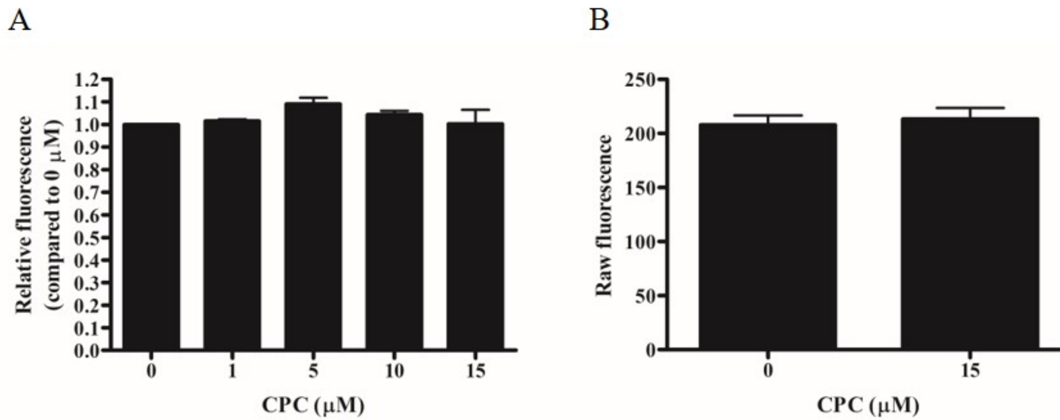


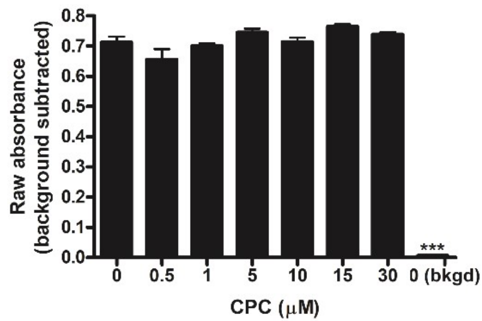
Figure S1. Effect of CPC on the ability of β-hexosaminidase to cleave fluorogenic substrate and effect of CPC on background fluorescence. (A) This is a control to test for CPC interference with the enzyme-substrate reaction between β-hexosaminidase and 4-methylumbelliferyl-N-acetyl-ß-D-glucosaminide (4-MU) in the assay used to quantify mast cell degranulation. **(B)** This is a control to test for CPC interference with background fluorescence in the degranulation assay.

Methods: (A) Supernatant from a flask of degranulated cells, which consists of β-hexosaminidase but no cells, was combined in equal proportions with CPC at the doses indicated and incubated for 30 min at 37 °C. This way, CPC was present only during the enzyme-substrate step of the degranulation measurement. Triplicate 25-μL samples of each supernatant-CPC mixture were plated into a Grenier 96-well black-bottom plate containing 100 μL per well of 1.2 mM 4-MU in sodium acetate buffer (Hutchinson *et al.*, 2011). Tyrode's-bovine serum albumin was used for background wells. The plate was incubated for 30 min at 37 °C, and 200 μL of glycine carbonate buffer was subsequently added to each well. Fluorescence was quantified using a Synergy2 microplate reader (Biotek). Background-subtracted fluorescence values were normalized to 0 μM CPC. **(B)** CPC at the doses indicated were plated in triplicate into background

wells (no cells, no IgE, no Ag), and raw fluorescence values were measured in the microplate reader (Biotek).

Results: (A) Values presented are means ± SEM for three independent experiments. No statistical significance was demonstrated by a one-way ANOVA followed by Tukey's post-test ($p < 0.05$), compared to 0 μM CPC. CPC had no effect on β-hexosaminidase activity, indicating that the mast cell degranulation assay measuring β-hexosaminidase release is sound. **(B)** No statistical significance was demonstrated by a one-way ANOVA followed by Tukey's post-test ($p < 0.05$), compared to 0 μM CPC.

A



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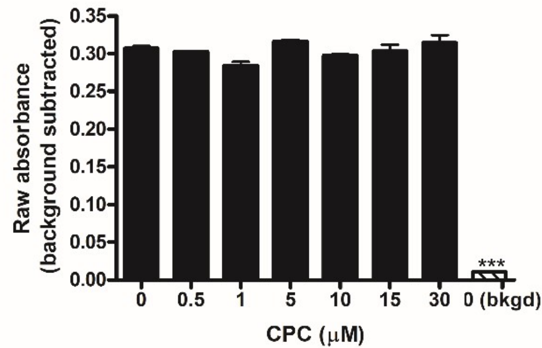

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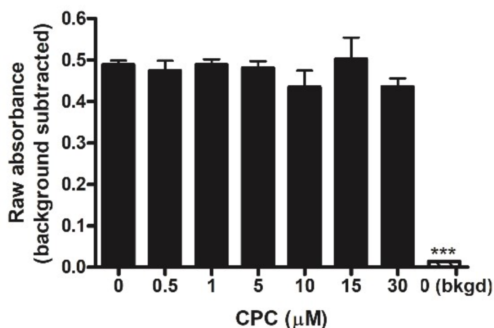

Figure S2. No-cell control to assess whether CPC interferes with the LDH enzymatic reaction that is used as a cytotoxicity readout. CPC at the doses indicated was plated with **(A)** 0.0003125 U/mL or **(B)** and **(C)** 0.000625 U/mL of purified LDH enzyme (Cayman chemical company, Ann Arbor, MI, USA). In a 96-well plate, 50 μL of a 2X solution of CPC (in BT) was plated with 50 μL of 2X LDH solution. Only BT (no LDH or CPC) was plated into the background wells. Following a 1-h incubation at 37 °C, the LDH cytotoxicity kit (Roche) was used per manufacturer's instructions to quantify LDH enzyme activity. Absorbance values at 490 nm and background absorbance at 630 nm were recorded, and the background values were subtracted from the 490 nm values. Values represent the averages of triplicate samples for individual experiments. Error bars represent SEM. Significance determined using a Tukey's post-test and one-way ANOVA. *** $p < 0.0001$.

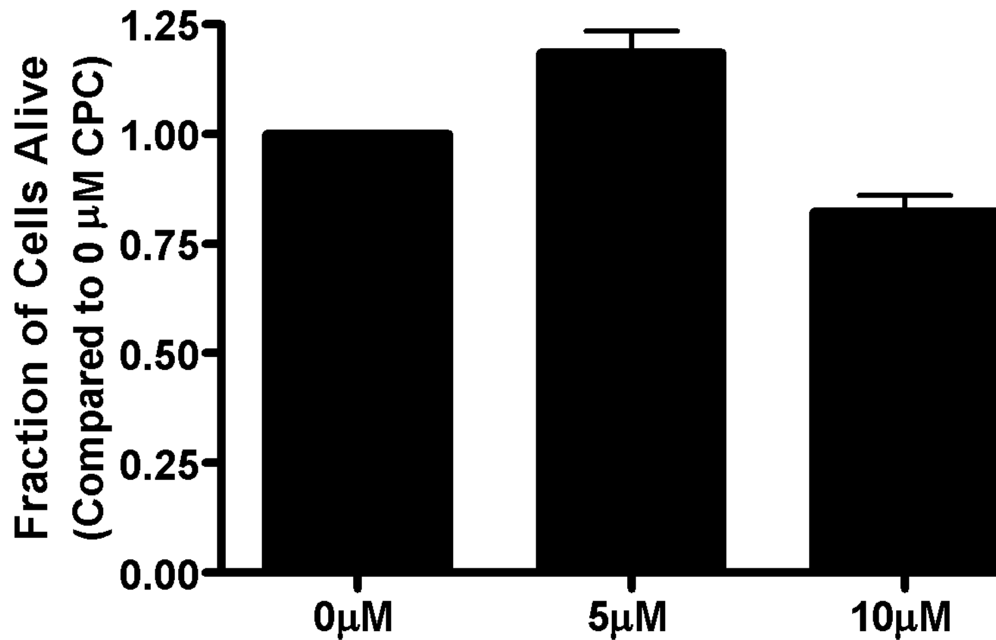

Figure S3. Cytotoxic effects of CPC on NIH-3T3 cells.

CPC cytotoxicity was tested in NIH-3T3 cells to add context to microscopy data utilizing this cell line.

CPC was prepared as in Methods. NIH-3T3 cells were cultured as in (Weatherly *et al.*, 2016). Cells were plated at 200,000 cells per well into each of 3 wells of a 6 well plate (Greiner) and grown overnight at 37 °C/5% CO_2. Next day, cells were exposed to micromolar doses of CPC (0 µM, 5 µM, and 10 µM) and incubated for 1 hour at 37 °C/5% CO_2. Cell viability was assessed using a trypan blue exclusion assay (as in section 2.1.4). Values presented are means ± SEM of three independent experiments. Analysis by the nonparametric Kruskal-Wallis test followed by Dunn's post test found no statistical significance of either CPC dose compared to control. CPC (up to 10 µM, 1 hr) exposure does not cause cytotoxicity of NIH-3T3 cells.

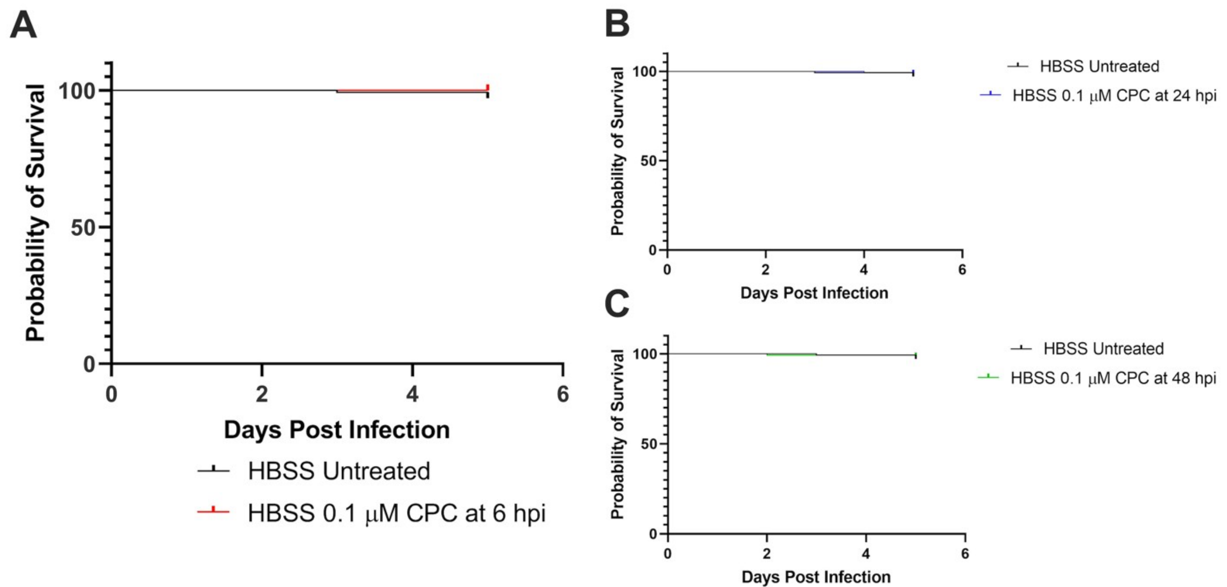

Figure S4. No significant mortality for HBSS-injected embryos. (A) Survival curve between 0.1 μM CPC treated and untreated, HBSS-injected zebrafish with a one-hour CPC treatment administered at 6 hpi. **(B)** Survival curve between 0.1 μM CPC treated and untreated HBSS injected zebrafish with a one-hour CPC treatment administered at 24 hpi. **(C)** Survival curve between 0.1 μM CPC treated and untreated HBSS injected zebrafish with a one-hour CPC treatment administered at 48 hpi. By 5 dpf the average survival of CPC treated zebrafish and untreated plates showed no significance in mortality within a 2.5% range. All graphs represent an n of 3 with ~150 zebrafish compared.

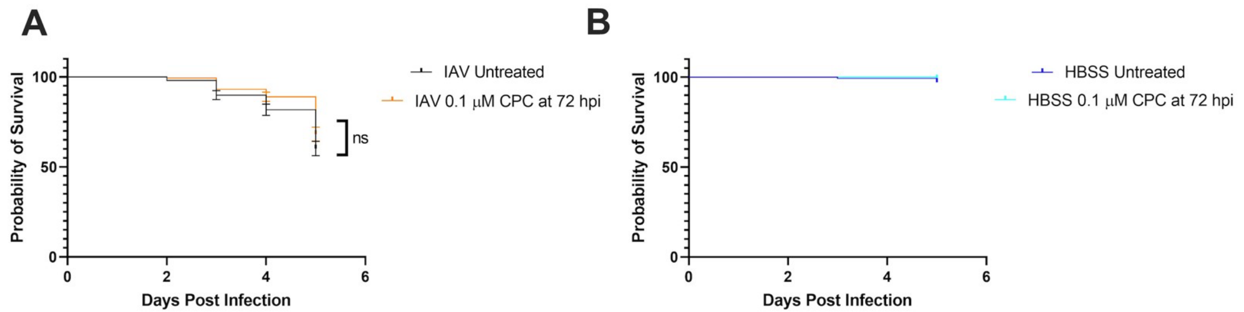

Figure S5. No significant mortality between HBSS and IAV at 72 hpi. (A) Survival curve between 0.1 μM CPC treated and untreated IAV infected zebrafish with CPC treatment administered at 72 hpi. There was no significant change in mortality between untreated and treated IAV infected groups. **(B)** Survival curve between 0.1 μM CPC treated and untreated HBSS injected zebrafish with CPC treatment administered at 72 hpi. There was no significant change in mortality between untreated and treated HBSS groups.