In vivo imaging of ligand receptor binding with Gaussia luciferase complementation

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Studies of ligand-receptor binding and the development of receptor antagonists would benefit greatly from imaging techniques that translate directly from cell-based assays to living animals. We used Gaussia luciferase protein fragment complementation to quantify the binding of chemokine (C-X-C motif) ligand 12 (CXCL12) to chemokine (C-X-C motif) receptor 4 (CXCR4) and CXCR7. Studies established that small-molecule inhibitors of CXCR4 or CXCR7 specifically blocked CXCL12 binding in cell-based assays and revealed differences in kinetics of inhibiting chemokine binding to each receptor. Bioluminescence imaging showed CXCL12-CXCR7 binding in primary and metastatic tumors in a mouse model of breast cancer. We used this imaging technique to quantify drug-mediated inhibition of CXCL12-CXCR4 binding in living mice. We expect this imaging technology to advance research in areas such as ligand-receptor interactions and the development of new therapeutic agents in cell-based assays and small animals.

Ligand-receptor binding initiates signal transduction, and therapeutic agents targeting cell-surface receptors predominantly block ligand binding. The central role of ligand–receptor binding in normal signaling, disease and drug development emphasizes the need for improved technologies to analyze ligand-receptor complexes in intact cells and living animals. Ligand–receptor binding is currently quantified using fluorescent or radioactive ligands. These labels produce signals independent of receptor binding, generating a background that limits the detection of ligand-receptor complexes. Furthermore, a labeled ligand detects all accessible receptors rather than the subset of receptors that are actively signaling. Developing an imaging assay to quantify ligand–receptor complexes under physiologic conditions will substantially advance studies of multiple diseases and accelerate drug development.

The receptors CXCR4 and CXCR7, both of which bind the chemokine CXCL12, are promising therapeutic targets for cancer and other diseases. CXCR4 promotes tumor growth and metastasis in more than 20 cancers, and recent preclinical studies have shown similar effects for CXCR7 (refs 1–3). Malignant cells in breast, ovarian and other cancers secrete CXCL12 and/or express CXCR4, CXCR7 or both. CXCL11, a second ligand for CXCR7, is also present in tumors4–6. Agents blocking chemokine binding to these receptors are currently being developed for cancer therapy, highlighting the need for improved methods to image ligand-receptor complexes in vivo.

We used Gaussia luciferase (GLuc) complementation, a fully reversible system, to image chemokine-receptor binding7. GLuc fragments are inactive, so using this system ensures minimal background bioluminescence. Because GLuc does not require ATP, this system detects ligand-receptor complexes intracellularly and in the extracellular space. GLuc is also smaller than other luciferases and fluorescent proteins, which minimizes the potential steric effects of fusing enzyme fragments to the proteins of interest. Using GLuc complementation, we quantified chemokine binding to CXCR4 and CXCR7 and its inhibition with small molecules in cell-based assays and living mice, providing a new method to link in vitro and in vivo testing of therapeutic agents.

RESULTS

GLuc complementation for ligand-receptor binding

To identify the optimal orientations of the fusion proteins, we fused N- or C-terminal fragments of GLuc (NGLuc and CGLuc) to the C-terminus of CXCL12 and the N-terminus of either CXCR7 or CXCR4. These fusions position NGLuc and CGLuc in the extracellular space (Fig. 1a). As controls for the non-specific association of GLuc fragments, we also generated secreted, unfused NGLuc and CGLuc. We transfected cells with a single reporter, control secreted NGLuc or CGLuc or vector and seeded an equal number of matched pairs of cells into 96-well plates. After co-culturing overnight, the combination of cells expressing CXCL12-CGLuc and NGLuc-CXCR7 generated bioluminescence more than tenfold above the background, which was more than that produced by any other combination (Fig. 1b).

Similarly, complementation between CXCL12-CGLuc and NGLuc-CXCR4 was higher than any other pair of co-cultured cells (Fig. 1c). Flow cytometry showed comparable expression of all the matched pairs of receptor fusion proteins (Supplementary Fig. 1). We selected the CXCL12-CGLuc and NGLuc-CXCR7 and the CXCL12-CGLuc and NGLuc-CXCR4 fusions for subsequent studies.

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To quantify bioluminescence after a pulse of CXCL12, we incubated cells expressing NGLuc-CXCR7, NGLuc-CXCR4 or control protein CXCR7-GFP for 15 min with CXCL12-CGLuc or CGLuc. Complementation between CXCL12-CGLuc and NGLuc-CXCR4 or NGLuc-CXCR7 produced more light than complementation between CGLuc and NGLuc-CXCR4 or NGLuc-CXCR7, which produced an amount of light comparable to CGLuc-GFP (Fig. 1d). CXCL12-CGLuc binding to NGLuc-CXCR7 produced more bioluminescence than the binding of CXCL12-CGLuc to NGLuc-CXCR4, likely as a result of greater binding affinity between the former pair than the latter, relatively higher concentrations of cell surface CXCR7 than CXCR4 that do not desensitize after ligand binding and prolonged intracellular association of CXCL12 with CXCR7 (refs. 8–11).

To test complementation between CXCR7 and CXCL11 and to identify conditions that maximize bioluminescence, we co-cultured cells expressing NGLuc-CXCR7 with various ratios of cells secreting CXCL11-CGLuc or CXCL12-CGLuc. CXCL12-CGLuc binding to NGLuc-CXCR7 produced approximately 20-fold more light than NGLuc-CXCR7 binding to CXCL11-CGLuc, corresponding to an approximately 20- to 50-fold greater binding affinity of CXCR7 for CXCL12 than CXCL11 (refs. 8,9). The peak bioluminescence occurred at 1:1 ratios of chemokine-secreting and NGLuc-CXCR7 cells (Supplementary Fig. 2a, b), and we therefore used this ratio for subsequent experiments. These data also showed that the assay is not restricted to CXCL12.

Inhibiting chemokine-receptor binding in cell-based assays

We generated cells from the breast cancer cell line MDA-MB-231 that secreted CXCL12-CGLuc (231–CXCL12-CGLuc) or CGLuc (231-CGLuc) or that expressed NGLuc-CXCR7 (231–NGLuc-CXCR7) or NGLuc-CXCR4 (231–NGLuc-CXCR4). 231–CXCL12-CGLuc and 231-CGLuc cells equivalently expressed CGLuc, as determined by quantitative RT-PCR (qRT-PCR) (data not shown). 231–CXCL12-CGLuc cells secreted approximately 1 ng of chemokine per cell per hour, which is sufficient to activate CXCR4 (ref. 12). 231–NGLuc-CXCR7 cells had slightly higher surface expression and binding sites for CXCL12 than 231–NGLuc-CXCR4 cells did (Supplementary Figs. 3a, b and 4a, b). NGLuc-CXCR7 retained the chemokine scavenging function of CXCR7 (Supplementary Fig. 5)10.

We co-cultured 231–CXCL12-CGLuc or 231-CGLuc cells and 231–NGLuc-CXCR7 cells for 16 h with CXCR7 inhibitors (CCX733 and CCX771), an inactive analog to the CXCR7 inhibitors (CCX704) or a CXCR4 inhibitor (AMD3100). CXCR7 inhibitors reduced the complementation between CXCL12-CGLuc and NGLuc-CXCR7 to the amount seen between the control pair of CGLuc and NGLuc-CXCR7 (Fig. 2a). Neither CCX704 nor AMD3100 had any effect on complementation. Both CXCR7 inhibitors also reduced the bioluminescence from the binding of CXCL11-CGLuc to NGLuc-CXCR7 without affecting the minimal signal from CXCL11-CGLuc and NGLuc-CXCR4 (Supplementary Fig. 6). CCX733 and CCX771 both produced dose-dependent inhibition of the complementation of CXCL12-CGLuc and NGLuc-CXCR7, whereas CCX704 did not produce any such inhibition (Fig. 2b). CCX733 was also more effective than CCX771 at blocking CXCL12 binding to cell-surface NGLuc-CXCR7 (Supplementary Fig. 7).

CXCR7 and chemokine ligands co-localize within cells, suggesting that ligand-receptor complexes remain intact for an extended time10,11. To investigate the kinetics of inhibiting CXCL12 binding to CXCR7, we incubated co-cultures of 231–CXCL12-CGLuc and 231–NGLuc-CXCR7 cells with increasing concentrations of CCX733 for 6 or 24 h (Fig. 2c). Treatment with 1 μM CCX733 for 24 h reduced the amount of bioluminescence produced by the binding of CXCL12-CGLuc and NGLuc-CXCR7 to that produced.
by CGLuc and NGLuc-CXCR7, whereas less inhibition of bioluminescence occurred after only 6 h of treatment compared to 24 h of treatment. Inhibition of the complementation of CXCL12-CGLuc and NGLuc-CXCR7 by 300 nM CCX733 required 4 h of treatment, and no compound altered the chemokines in the lysosomes.  

We normalized the bioluminescence to the total protein in each well and graphed the means ± s.e.m. (b) GLuc bioluminescence from co-cultures of 231–CXCL12-CGLuc and 231–NGLuc-CXCR4 cells treated with various concentrations of AMD3100, CCX773, or CCX771 for 4 h. The graphs show the means ± s.e.m. relative to untreated cells. (c) Quantified GLuc activity from pairs of co-cultured cells (231–CXCL12-CGLuc and 231–NGLuc-CXCR7) or the control pair 231-CGLuc (G) and 231–NGLuc-CXCR7 (NG) after incubation for 6 or 24 h with increasing concentrations of CCX733. The graph shows the means ± s.e.m. relative to untreated cells. (d) The bioluminescence from co-cultures of 231–CXCL12-CGLuc and 231–NGLuc-CXCR7 cells treated for various periods of time with 300 nM CCX773 or 300 nM AMD3100. The graphs show the means ± s.e.m. for bioluminescence relative to those from cells treated with vehicle alone. *P < 0.05, **P < 0.01, ***P < 0.005.

 Imaging CXCL12-CXCR7 binding in vivo  
We orthotopically implanted 231–NGLuc-CXCR7 cells with 231–CXCL12-CGLuc or 231–CXCR7 cells in the mammary fat pads of mice, which reproduced the expression of CXCR7 or the secretion of CXCL12 seen in human breast tumors. 11, 12 231–NGLuc-CXCR7 cells also expressed firefly luciferase (FLuc) and GFP for in vivo and ex vivo imaging, respectively. Tumors containing 231–CXCL12-CGLuc and 231–NGLuc-CXCR7 cells had robust GLuc bioluminescence, whereas the bioluminescence from tumors containing 231–CXCL2 and 231–NGLuc-CXCR7 cells was undetectable (Supplementary Fig. 8a,b). By comparison, FLuc imaging performed 4 d before GLuc imaging showed comparable numbers of 231–NGLuc-CXCR7 cells in the two tumor groups. Consistent with results from cell-based assays, tumors containing 231–CXCL12-CGLuc and 231–NGLuc-CXCR7 cells...
showing fluorescence from eqFP650 and GFP. These reporters allow for independent imaging of both cell types. We implanted orthotopic tumors with 231–CXCL12-CGLuc and 231–NGLuc-CXCR4 cells (Supplementary Fig. 9). These data establish that Gluc complementation imaging detects quantitative differences in ligand-receptor binding in vivo.

To image 231–CXCL12-CGLuc and 231–NGLuc-CXCR7 cells independently, we transduced 231–CXCL12-CGLuc cells with the far-red fluorescent protein eqFP650 (ref. 14). The fluorescence from eqFP650 in 231–CXCL12-CGLuc cells and the bioluminescence from FLuc in 231–NGLuc-CXCR7 cells co-localized in primary tumors. Because the light from FLuc in the primary tumors shone over the primary tumors and metastatic foci in omentum and lungs from the mouse shown in b. The red arrows point to lung metastases with co-localized eqFP650 fluorescence and GLuc bioluminescence. The green arrow points to eqFP650 fluorescence from a metastasis containing only 231–CXCL12-CGLuc cells. P, photons; sr, steradians.

also produced more GLuc bioluminescence than tumors containing 231–CXCL12-CGLuc and 231–NGLuc-CXCR4 cells (Supplementary Fig. 9). These data establish that GLuc complementation imaging detects quantitative differences in ligand-receptor binding in vivo.

To image 231–CXCL12-CGLuc and 231–NGLuc-CXCR7 cells independently, we transduced 231–CXCL12-CGLuc cells with the far-red fluorescent protein eqFP650 (ref. 14). The fluorescence from eqFP650 in 231–CXCL12-CGLuc cells and the bioluminescence from FLuc in 231–NGLuc-CXCR7 cells co-localized in primary tumors. Because the light from FLuc in the primary tumors shone over the entire mouse during longer imaging times, we removed the primary tumors and analyzed the metastases in internal sites. eqFP650 fluorescence and FLuc bioluminescence co-localized in multiple metastases, which we confirmed by fluorescence microscopy (Fig. 4a,b). Flow cytometry showed an approximate 2:1 ratio of 231–NGLuc-CXCR7 to 231–CXCL12-CG cells in both the dissociated primary tumors and metastases.

Co-localization of 231–CXCL12-CGLuc and 231–NGLuc-CXCR7 cells suggested that intercellular chemokine-receptor binding occurs in metastases. We identified metastases that had both eqFP650 fluorescence and GLuc bioluminescence, which was evidence of CXCL12-CXCR7 binding in sites containing both 231–CXCL12-CGLuc and 231–NGLuc-CXCR7 cells (Fig. 4c). We verified the co-localization of fluorescence and GLuc complementation from the binding of CXCL12-CGLuc to NGLuc-CXCR7 in some metastases (Fig. 4d and Supplementary Fig. 10). Although the maximum distance for intercellular CXCL12-CXCR7 binding has not been determined in vivo, CXCR7 cells do not generate a chemotactic gradient when they are separated from CXCL12-secreting cells by >400 µm in vitro. Diffusion of CXCL12 is restricted by proteoglycans, so intercellular ligand-receptor binding probably occurs over much smaller distances in tumors. These data show that 231–CXCL12-CGLuc and 231–NGLuc-CXCR7 cells may metastasize to the same sites and establish intercellular ligand-receptor binding.

We attempted to inhibit CXCL12-CGLuc and NGLuc-CXCR7 complementation in mice using CCX771. We were unable to significantly reduce complementation in vivo, probably because blocking this interaction requires prolonged exposure to relatively high amounts of CCX771.

**Imaging inhibition of CXCL12-CXCR4 binding in vivo**

We implanted orthotopic tumors with 231–CXCL12-CGLuc and 231–NGLuc-CXCR4 cells. 231–CXCL12-CGLuc cells also express eqFP650, and 231–NGLuc-CXCR4 cells express firefly luciferase and GFP. These reporters allow for independent imaging of both cell types.
populations. After obtaining baseline imaging data, we implanted mice with subcutaneous osmotic infusion pumps that delivered either AMD3100 or PBS. The AMD3100 pumps delivered 600 μg of AMD3100 per day, producing serum concentrations of the compound of approximately 1.25 μM (ref. 16). GLuc complementation decreased by approximately 85% after 5 d of treatment with AMD3100, which was comparable to the approximately 80% inhibition of 231–CXCL12-GLuc binding to intact 231–NGLuc-CXCR4 cells seen after treatment with 1.25 μM AMD3100 (Fig. 5a,b). Treatment with AMD3100 reduced the bioluminescence from tumors containing 231–CXCL12-CGLuc and 231–NGLuc-CXCR4 cells to amounts comparable to control tumors containing 231–CGLuc and 231–NGLuc-CXCR4 cells (Supplementary Fig. 11). Relative to pretreatment values, GLuc bioluminescence increased by approximately 50% in mice treated with PBS. After removing the AMD3100 infusion pumps, the bioluminescence from CXCL12-CXCR4 binding in the mice from this group increased within 2 d to amounts comparable to those seen in mice treated with PBS.

AMD3100 limited the growth of MDA-MB-231 cells in primary tumors, as quantified by the decreased eqFP650 fluorescence of 231–CXCL12-CGLuc cells and a trend toward reduced FLuc activity in 231–NGLuc-CXCR4 cells (Fig. 5c,d). The loss of the GLuc signal was caused primarily by inhibition of the binding of CXCL12 to CXCR4, as the reductions in GLuc bioluminescence greatly exceeded the effects of AMD3100 on tumor burden. Mice treated with AMD3100 had fewer metastases than control mice treated with PBS (approximately 30% of mice treated with AMD3100 had metastases compared to 70% of control mice, as determined by FLuc imaging) (Fig. 5a). We also detected multiple metastases in 231–CXCL12-CGLuc and 231–NGLuc-CXCR4 cells by GLuc bioluminescence (Supplementary Fig. 12). These data establish that GLuc complementation can quantify the pharmacodynamics of therapy in vivo.

DISCUSSION

We developed a GLuc complementation assay to image ligand-receptor complexes under physiologic conditions in cell-based assays and living mice. The same imaging reporters are used in both cell cultures and animal studies, which enables direct comparisons of ligand binding and the targeting of potential therapeutic agents in vitro and in vivo. GLuc bioluminescence is proportional to the number of ligand–receptor complexes, which provides a quantitative assay to establish the targeting of therapeutic agents. Because many preclinical drug development studies are designed to achieve defined amounts of receptor inhibition at a target site, GLuc complementation provides essential information about receptor coverage in living animals17,18. Combining GLuc complementation with additional imaging reporters allows real-time analysis of receptor targeting and the resultant effects on tumor growth and metastasis.

Although we focused on chemokines and chemokine receptors, this imaging system is directly applicable to any ligand-receptor pair with a peptide ligand that can be fused genetically to a fragment of GLuc. Recombinant GLuc has previously been purified and fused to other molecules19,20, so it should be feasible to chemically link a fragment of GLuc to non-peptide ligands. This approach would further extend the applications of the technology for cell-based assays and animal imaging. In vivo imaging of GLuc complementation in internal organs is currently limited by its peak emission at 480 nm, which has limited penetration through tissues. Developing red-shifted mutants of GLuc, as has been done for other luciferases, will improve the detection of ligand-receptor complexes and improve drug targeting in intact mice21. We expect this imaging system will have a widespread effect in areas such as intercellular signaling in tumors and development of therapeutic agents targeting ligand-receptor binding.

METHODS

Methods and any associated references are available in the online version of the paper at http://www.nature.com/naturemedicine/.
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AUTHOR CONTRIBUTIONS
K.E.L., L.A.M., B.T.S., S.A.L., P.R. and G.D.L. performed the cell culture and animal experiments and analyzed the data. K.E.L., D.S., D.M.C. and G.D.L. provided new reagents. K.E.L., B.T.S., D.M.C. and G.D.L. prepared the manuscript. K.E.L. and D.S. performed the cell culture and animal experiments and analyzed the data. K.E.L., D.S., D.M.C. and G.D.L. provided new reagents. G.D.L. supervised the project.

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Plasmids. Construction of secreted chemokine fusion vectors. We amplified mouse CXCL12-α with the PCR primers 5′-ATGCCCTGAGGCCACCATG 5GACGCCGCAAAAGTCCGTCGLuc-3′ and 5G-5CATGAATTCCTCCGCGTTGGAAGCGCCTGTCGAGC-3′ and 5G-5CATGAATTCCTCCGCGTTGGAAGCGCCTGTCGAGC-3′. We used the same procedures for the assays with stably transduced reporter cell lines. In selected experiments, we incubated co-cultured cells with various concentrations of an inhibitor of CXCR4 (AMD3100; Sigma), a small-molecule inhibitor of CXCR7 (CCX73 and CCX71; ChemoCentryx), or an inactive analog of these CXCR7 inhibitors (CCX704; ChemoCentryx). We measured GLuc activity on an IVIS system (Caliper) immediately after adding coelenterazine. We used field-of-view (FOV) B, large binning and a 30–120 s acquisition time for imaging. We normalized the data for photon flux to the relative amount of total protein per well measured by sulforhodamine B staining. 

In vivo imaging. All mouse procedures were approved by the University of Michigan Committee for Use and Care of Animals. We implanted equal numbers of paired GLuc complementation reporter 231 cells (1 × 10^6 total cells) in the bilateral fourth inguinal mammary fat pads of female nude or nonobese diabetic severe combined immunodeficiency mice (Tac1). We used an IVIS Spectrum instrument (Caliper) for the optical imaging studies. We injected 4 mg per kg body weight of coelenterazine (Promega) intravenously into the tail vein to image GLuc. We imaged mice within 30 s of injection using FOV B, a 3 min acquisition and large binning for all mice. For firefly luciferase, we injected 15 mg per kg body weight of luciferin (Promega) intraperitoneally and began imaging 10 min later using FOV C, a 20–30 s acquisition and small binning. We quantified the data as photon flux, which accounts for differences in acquisition time for firefly luciferase. We set standard minimum and maximum values for the pseudocolor display before quantifying bioluminescence by a region-of-interest analysis. We used 605-nm emission filters, a 1–3 s acquisition and small binning for fluorescence imaging of eqFP650. We quantified the fluorescence data as radiant efficiency using standardized pseudocolor displays, as was described for the bioluminescence imaging data. To inhibit CXCL12-CXCR4 binding in mice, we implanted subcutaneous osmotic infusion pumps (ALZET) containing 25 mg ml^-1 AMD3100 in PBS. These pumps released 1.0 µl of AMD3100 per hour. Control mice received pumps containing PBS only. We removed the pumps after 6 d. To image internal metastases, we injected mice with either coelenterazine or luciferin for Gaussia or firefly luciferase, respectively, and killed the mice 5 min later. We removed the primary tumors and exposed the internal organs for imaging. We identified metastases based on fluorescence imaging, bioluminescence imaging or both and excised these sites for imaging ex vivo.

Fluorescence microscopy. We performed fluorescence microscopy of cells with an Olympus epifluorescence microscope and a 40× objective. We used an Olympus MPE Twin system and a 25× objective for two-photon microscopy of excised tissues.

Statistical analyses. We performed cell culture experiments two to five times each with four replicate samples per data point. We performed animal imaging experiments two or three times each with five to ten mice per group. To determine statistically significant differences (P < 0.05), we analyzed the data using a Mann-Whitney U test (GraphPad Prism).
Additional methods. Detailed methodology is described in the Supplementary Methods.

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