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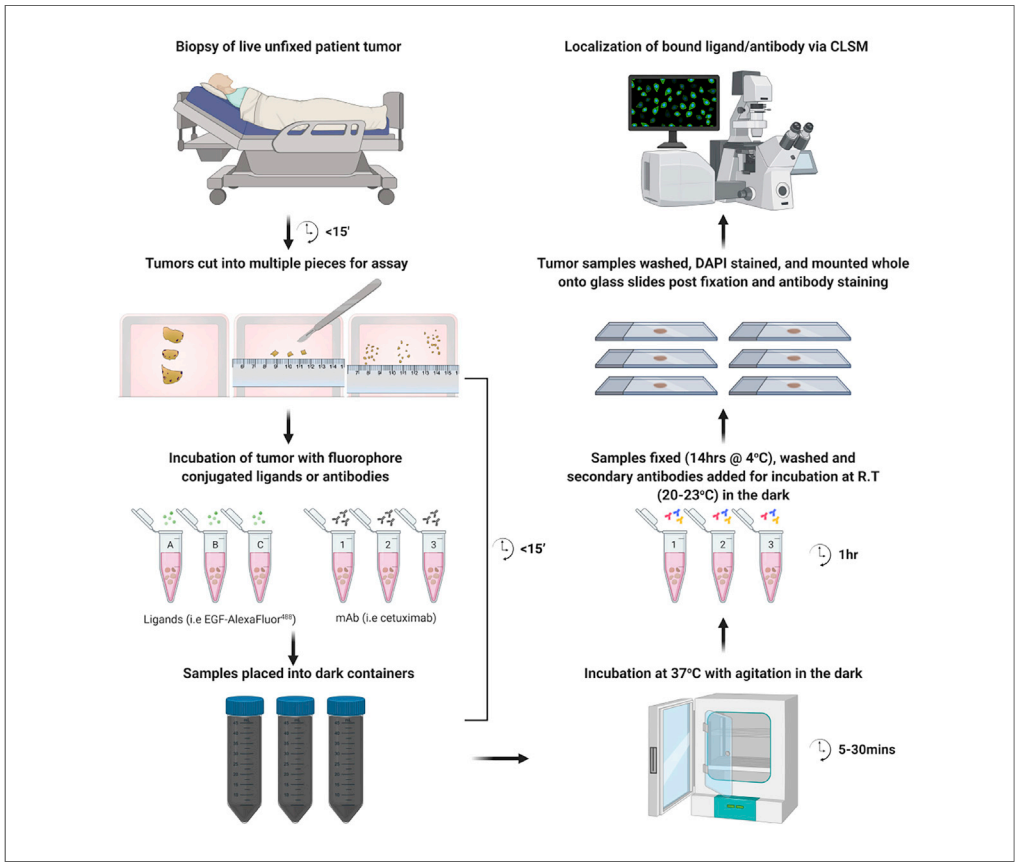
Antibody/Ligand-Target Receptor Internalization Assay Protocol Using Fresh Human or Murine Tumor *Ex Vivo* Samples



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HIGHLIGHTS

A robust protocol for spatiotemporal analysis of tumor surface receptors/ antigens

Can be used to analyze tumor surface target retention and internalization.

Can be used to analyze surface retention and turnover of tumor therapy/target

Can potentially be used as diagnostic tool for mAb response in patients

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Antibody/Ligand-Target Receptor Internalization Assay Protocol Using Fresh Human or Murine Tumor *Ex Vivo* Samples

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SUMMARY

We describe an *ex vivo* EGF ligand internalization assay using fresh patient tumor biopsies to determine how antigen targets will be trafficked before patients receive mAb treatment. This protocol facilitates a sensitive and reproducible indication as to mAbs surface retention times during treatment. EGF uptake protocols can also be used to analyze EGFR heterogeneity and localization of EGFR in both tumor and xenograft tissue. The technology can be adapted to analyze other receptors such as PD-L1 for which methods are provided.

For complete details on the use and execution of this protocol, please refer to Joseph et al. (2019) and Chew et al. (2020).

BEFORE YOU BEGIN

Human or Mouse Ethics Applications

Timing: weeks–months

1. Human ethics approval to collect and use human tissue needs to be obtained as per institute guidelines and according to Declaration of Helsinki protocols. Patient provided with Patient Informed Consent and given time to consider. Informed Consent given prior to start. For human sample, a surgeon and treating oncologist should determine tumor accessibility for removal. If the tumor sample is removed in operating theater during a routine procedure, consideration of potential effects of anesthesia should be given. If transfer is fast, sample can be accessed via pathology by qualified pathologist (e.g., head and neck dissection materials).
   Work shown in this protocol was done under the following ethics; HREC/11/QPAH/034; HREC ref: 2009/098; HREC/99/QPAH/34; HREC/15/QPAH/48.
2. Alternatively, animal ethics approval needs to be collected for mouse experiments.

Tumor Sample Collection

Timing: 30 min

3. Tumor needs to be fresh (i.e., high tissue viability) for assays, therefore laboratory needs to be ready to collect tumor immediately after excision.
   a. Tumor removed by clinician and placed in sterile tube. Ensure clinicians know that sample should not be put into formaldehyde.
   b. Tumor collected from clinic and placed in serum free media, taken to research laboratory. Alternatively collect tumor dry in tube, tube in ice. Ensure appropriate personal protective equipment is used.
4. Start tumor assay as per step by step method below
   a. Reagents need to be prepared fresh just prior to assay. Pre-warm media to 37°C and pre-heat an oven or incubator to 37°C in which samples can be rotated in during incubation.
   b. It is useful to have one laboratory member collect the tumor while others prepare in laboratory.

⚠ CRITICAL: Appropriate personal protective equipment (PPE, including laboratory coats, gloves, protective eyewear and P2/N95 respirators) to be worn and appropriate safety precautions (such as vaccinations) undertaken when dealing with live unfixed human tissues as per institute direction.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Cetuximab (Erbitux®)	Merck KGaA	CAS ID: 205923-56-4
EGFR (clone: 31G7)	Life Technologies	Cat#280005; RRID:AB_86904
Anti-mouse-IgG Alexa Fluor^{594}	Invitrogen	Cat#A-11005; RRID:AB_2534073
Anti-human IgG Alexa Fluor^{555}	Invitrogen	Cat#A-21433; RRID:AB_2535854
Anti-human IgG Alexa Fluor^{647}	Invitrogen	Cat#A-21445; RRID:AB_2535862
Biological Samples		
Human tumor samples	N/A	N/A
Chemicals, Peptides, and Recombinant Proteins		
EGF-AlexaFluor^{488}	Invitrogen	Cat#E13345
DAPI (4′,6-diamidino-2-phenylindole)	Invitrogen	Cat#D1306; CAS ID: 28718-90-3
DEAE-Dextran (10k)-Alexa Fluor^{555}	Invitrogen	Cat#D34679
Dent's Bleach	Dent et al, 1989	N/A
Dimethyl sulfoxide (DMSO)	Sigma-Aldrich	Cat#D8418; CAS ID: 67-68-5
10% Triton-X100	Sigma-Aldrich	Cat#93443; CAS ID: 9002-93-1
Hydrogen Peroxide Solution	Sigma-Aldrich	Cat#H1009; CAS ID: 7722-84-1
Paraformaldehyde (PFA)	Sigma-Aldrich	Cat#158127; CAS ID: 30525-89-4
Phosphate Buffered Saline (PBS) Tablets	Life Technologies, Gibco	Cat# 18912014
Methanol	Chem-supply Australia	Cat#MA004-20L-P; CAS ID:67-56-1
DMEM-F12	Life Technologies, Gibco	Cat#11320033
Horse serum	Invitrogen	Cat#16050130
Other		
Surgical Scalpel	KAI medical	Cat#515-A
Microscope slide single concave	Wiltonics	Cat#LG7103-PK50
Coverslip no 1 22 × 50 mm	Menzel	Cat# MENCS22501GP
ProLong™ Gold Antifade Mountant	Invitrogen	Cat#P36930; RRID:SCR_015961

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Premium Sterile Conical Centrifuge Tubes, 50 mL, Black	Thomas Scientific	Cat# 1213G12
1.5 mL Eppendorf Tubes	Eppendorf	Cat#0030125150
Hybridization heated rotisserie oven	Hybaid	Cat# 1211V79
Zeiss 510 Meta confocal microscope	Zeiss	N/A
Olympus FV3000 confocal laser scanning microscope	Olympus	N/A
Software and Algorithms		
Zen 2008 software	Zeiss	N/A
Fiji Image J	Schindelin et al. (2012)	https://imagej.net/Fiji

MATERIALS AND EQUIPMENT

Alternatives: This protocol has been used with 63× and 100× objectives on Zeiss 510 Meta confocal microscope and analyzed using Zen 2008 software (Carl Zeiss, Germany) or Olympus FV3000 confocal laser scanning microscope (Olympus Life Science) and analyzed using open-source software Fiji (Schindelin et al., 2012). Alternatively, imaging can be performed using different microscope configurations, e.g., light sheet microscopy. As the tissue sections are thick, it is important to use a microscope which allows you to control depth of field and eliminate or reduce background fluorescence away from the focal plane.

Alternatives: For incubation of tumors at 37°C, samples are typically placed in Eppendorf tubes in dark (contained in black 50 mL centrifuge tubes) in a rotating (15 rpm) heated rotisserie oven (e.g., Hybaid® Mini Hybridization Oven MK II). If oven is not available, samples can be incubated in 37°C incubator shaker (e.g., IKA® KS4000 i control). It is important using either alternative that the samples are constantly moving/rotating to ensure they do not dry out and even coverage in medium. Ensure that equipment used for incubation are well masked (e.g., glass windows covered with aluminum foil) or kept in a dark room to ensure samples are protected from direct light during incubation.

Dent's Bleach

Reagent	Final Concentration	Volume (mL)
Methanol (100%)	4 parts	40
DMSO (100%)	1 part	10
Hydrogen Peroxide (30%)	1 part	10
Total	**n/a**	**60**

Dent et al. (1989).

⚠ CRITICAL: Toxic! – Prepare and handle only in fume hoods with appropriate PPE. Contains hydrogen peroxide which causes severe skin burns and eye damage, is harmful if swallowed or inhaled and may cause respiratory irritation. Contains methanol which is a highly flammable liquid and vapor. Contains DMSO which is a combustible liquid, readily penetrates skin and may cause eye, skin and respiratory tract irritation.

PBTX

Reagent	Final Concentration	Volume (mL)
PBS (NaCl: 137 mM, KCl: 2.7 mM, Na_2HPO_4: 10 mM, KH_2PO_4: 1.8 mM)	1×	49.5
10% Triton-X-100	0.1% (v/v)	0.5
Total	**n/a**	**50**

Blocking Buffer

Reagent	Final Concentration	Volume (mL)
PBS (NaCl: 137 mM, KCl: 2.7 mM, Na_2HPO_4: 10 mM, KH_2PO_4: 1.8 mM)	1×	44.5
Horse serum	10% (v/v)	5
10% Triton-X-100	0.1% (v/v)	0.5
Total	n/a	50

Alternatives: If Horse Serum is not available, alternatives such as Bovine Serum Albumin may be tried and optimized.

STEP-BY-STEP METHOD DETAILS

Day 1 Fresh Tumor Sample Preparation, EGF Ligand Uptake, and Fixation

Timing: 1–2 h

Tumor samples are cut, washed in serum free media to remove serum before incubation with and without EGF-Alexa Fluor^{488}.

1. Tumor samples collected and transferred to research laboratory (Refer to "Before you Begin" and "Graphical Abstract").

Optional: This technique can also be used to assay monoclonal antibody uptake in human tissue or mouse xenograft tissue e.g., Anti-EGFR or anti-PD-L1 uptake in xenografts (Chew et al., 2020). Human or mouse xenografts are excised and transferred to research laboratory as soon as possible. Within 30 min as maximal time, 15 min preferred.

2. Slice the transverse sections of unfixed tumors into pieces of approximately 2 × 2 × 0.5–1.0 mm using sterile surgical scalpels (Figure 1) (Joseph et al., 2019). If available, keep matched normal tissue as control and treat under similar conditions.
   a. If it is a large tumor sample, some tumor pieces may be washed as per below (no treatments) and fixed in 4% PFA overnight (8–14 h), for sectioning at a later stage.
   b. Cut as many smaller sections as possible as it is recommended that multiple tumor sections be used in each condition (as listed below in step 5). Multiple tumor sections should be randomized into each condition to allow assessment of potential variation within the tumor samples.

⚠ CRITICAL: Appropriate PPE to be worn and appropriate safety precautions taken when dealing with live unfixed human or mouse tissues.

3. Wash tumor samples 3× in 5-min washes with serum-free media (Ham's F-12 media: DMEM) at 4°C.
4. While tumor samples are washing, prepare media containing EGF-AlexaFluor^{488}, cetuximab and DEAE-dextran as per below. Note, minimum 200 µL medium per condition in Eppendorf tube so that samples do not dehydrate.
5. After washing with serum-free media, incubate tumor lumps in separate Eppendorf tubes as per following conditions:
   a. Ham's F-12 media: DMEM media only, No EGF (no uptake control), 30 min, on ice.
   b. Ham's F-12 media: DMEM media containing EGF-AlexaFluor^{488} (2 µg/mL), 30 min, on ice.
   c. Ham's F-12 media: DMEM media containing EGF-AlexaFluor^{488} (2 µg/mL), 5 min, 37°C.
   d. Ham's F-12 media: DMEM media containing EGF-AlexaFluor^{488} (2 µg/mL), 15 min, 37°C.
   e. Ham's F-12 media: DMEM media containing EGF-AlexaFluor^{488} (2 µg/mL), 30 min, 37°C.

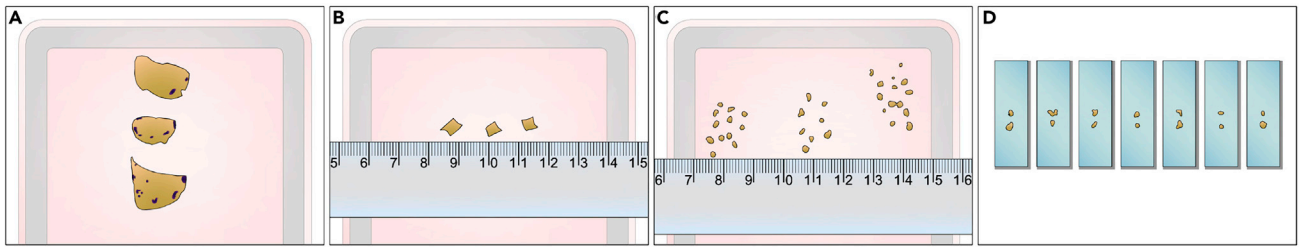

Figure 1. Method Illustrating the Live Tumor Uptake

Schematic representation of the processing method illustrated in Joseph et al. (2019). Three live unfixed tumor biopsies (A, step 1) were dissected to select for tumor only (B). Note that surrounding normal matched tissue (A and B) can be kept for control normal tissue for comparison. Tumor samples were further dissected into multiple smaller lumps (approximately 2 × 2 × 0.5–1.0 mm, C, step 2). Multiple lumps were incubated in each EGF uptake assay condition in Eppendorf tubes (as specified in step 5 of the protocol) and then mounted onto concave glass slides (D, step 21).

f. Ham's F-12 media: DMEM media containing cetuximab (25 μg/mL), 30 min, 37°C then add EGF-AlexaFluor^{488} (2 μg/mL), 15 min, 37°C (cetuximab competition).
g. Ham's F-12 media: DMEM media containing DEAE-Dextran-Alexa Fluor^{555} (60 μg/mL), 30 min, 37°C.

Note: Use multiple tumor sections/lumps in each condition listed above. This allows coverage across multiple tumor areas for each condition and randomizes the experiment.

Note: It is recommended that both positive and negative control samples are processed simultaneously. Multiple tumor samples in each condition allows analysis of different areas of the tumor. Condition (a) is a negative control for both EGF uptake and to be used for secondary antibody only control. Condition (b) is control for EGF ligand binding. Condition (c)-(e) are EGF ligand uptake test conditions and may differ with different tumor samples. These conditions can be co-immunostained with other antibodies if required. For example, total EGF receptor level could be analyzed by co-immunostaining EGF ligand uptake samples with an anti-EGFR antibody. This is described in more detail in step 12, optional (after step 12) and step 14a. Condition (f) is the cetuximab competition test condition. This control directly addresses the specificity of EGF ligand uptake. Cetuximab binds to the EGF ligand binding site on the EGFR therefore should block all EGF ligand binding. Thus, when analyzing by microscopy, this fluorescent level is used as the baseline over which fluorescent signal is judged to be specific, so only EGF-AlexaFluor^{488} staining above any fluorescence from a cetuximab competition control is used. Cetuximab can also be visualized by using fluorescent secondary antibody. Condition (g) with DEAE-Dextran-AlexaFluor^{555} for 30 min is used to assess tissue viability, some uptake should be visualized. **If you are limited by tumor amount, condition (a), (b), (e) and (f) are essential**, condition (c) and (d) can be omitted and condition (g), DEAE-Dextran-Alexa Fluor^{555}, can be added to condition (e). Ensure 37°C incubation is carried out with movement/rocking in oven or incubator. Ensure all samples kept in dark (covered aluminum foil or contained in black 50 mL centrifuge tubes) as fluorophores are used.

Optional: For antibody uptakes, instead of incubating with fluorescent EGF ligand (condition (c)-(e)), any monoclonal antibody of choice can be substituted (e.g., anti-PD-L1 for CT26 mice model). It is crucial to also include relevant controls; no antibody uptake control on ice (condition (a)), antibody uptake control on ice (condition (b)) and DEAE-Dextran-Alexa Fluor^{555} control (condition (g)) to check for tissue viability. Any antibody uptake conditions should be processed as for cetuximab control condition (f).

Optional: Fluorescently conjugated cetuximab may be used instead of cetuximab. This optional method would not require incubation with a secondary antibody and the type of fluorophore would need to be different to that used for ligand uptake or immunostaining.

6. After incubations, wash samples 5 times with cold PBS (total ~30 min)
7. Fix samples in 4% paraformaldehyde (PFA) for 14 h/overnight at 4°C.

⚠ CRITICAL: PFA is toxic. Prepare and handle PFA only in the fume hood, with appropriate PPE and discard appropriately.

Days 2–3 Whole Mount Tumor Tissue Immunofluorescence Staining, Secondary Antibody Incubation, and Mounting

Timing: 3–5 h, day 2 and 2–3 h, day 3

PFA is washed out after fixing tumor samples overnight (8–14 h). The following protocol describes steps for processing tissue with or without additional immunofluorescent staining prior to mounting tissue samples for microscopic analysis.

Note: Dent's bleach protocol, steps 9–11, is usually only used for human tumors which may have high autofluorescence, this is particularly an issue when imaging the epidermal layer or high keratin or collagen-containing tissue. Dent's bleach protocol is not usually required if using human or mouse xenografts. Tumors are incubated with Dent's bleach to reduce autofluorescence but this may also decrease EGF ligand fluorescence. These steps have been provided as part of the normal protocol but are optional, see below. Alternative methods to reduce the effect of sample autofluorescence on image quality include using conjugated far-red fluorophores (e.g., AlexaFluor^{647}) for labelling proteins of interest as less autofluorescence is seen at longer wavelengths when compared to visible wavelength fluorophores. Sudan Black B has also been suggested to be used as an alternative for autofluorescence reduction.

8. Wash fixed samples twice in PBS.

Optional: This assay can also be used to compare total antibody uptake or antigen (surface and internal) vs cell surface (antibody bound to the surface only) for both antibody uptakes or post-fixation immunofluorescence staining. To do this, all permeabilization steps need to be omitted including the use of Dent's bleach protocol (steps 9–12), permeabilization (step 13) and any Triton-X containing buffers (for washes and incubations use Blocking buffer or PBS without Triton-X). This will mean secondary antibody will not have access to internalized antibody or antigen and will only bind to surface exposed antibody. DAPI is cell permeable so this does not interfere with this step. This condition should be done alongside permeabilized samples for comparison.

Optional: The following Dent's bleach protocol is used to minimize autofluorescence of tissue. If Dent's bleach is not required, steps 9–11 can be omitted, going straight from washing in PBS after fixation (step 8) to blocking step (step 12). However, if microscopy imaging displays a high level of autofluorescence it is recommended that Dent's bleach is used.

9. Place samples in 100% methanol at 4°C for 2 h.
10. Incubate samples in Dent's bleach (4 parts methanol:1 part DMSO:1 part 30% H_2O_2) (Dent et al., 1989) for 2 h at room temperature (21°C–25°C) in dark.

⚠ CRITICAL: Toxic! – Prepare and handle Dent's bleach only in fume hood, with appropriate PPE and discard appropriately. Dent's bleach is toxic. See safety instructions listed in Materials and Equipment section.

11. Remove Dent's bleach and add 100% methanol. Samples now need to go through a methanol series to rehydrate. Incubate samples in MeOH series (10 min each): 75% MeOH, 50% MeOH, 25% MeOH, PBS ×2
12. For samples that require antibody incubation continue to step 13. For samples that do not require antibody staining (conditions (b)-(e)) go to step 19 and continue to end of protocol.

Optional: Samples that have undergone EGF ligand uptake (Conditions (b)-(e)) can be co-immunostained for other proteins if required and this is described in step 14.

13. Incubate samples in blocking buffer (PBTX with 10 % horse serum) for 2–4 h rocking at room temperature (21°C–25°C).

Optional: PBTX is optional. If surface only staining is being examined it can be omitted. If both surface and internalized staining is to be analyzed it should be included. While protocols for monolayer immunofluorescence staining include triton only in the initial steps, standard protocols for tissues keep PBTX throughout primary, washes and secondary staining. This is especially the case for SCC as extremely high collagen secretion occurs in these tumors.

14. For conditions that are being immunostained, incubate with primary antibody diluted in blocking buffer, overnight (8–14 h) at 4°C. Go to step 15 (day 3) and continue to end of protocol.
    a. Concentration of primary antibody required depends on tissue type, and antigen density. Check with manufacturer's protocol or perform a titration test prior to running the assay. Post-fixation labelling of EGFR (anti-EGFR (e.g., Clone 31G7, Life Technologies) or alternative) should be completed to enable analysis of EGFR expression and localization.

Pause Point: Incubation of primary antibody occurs overnight (8–14 h), then go to step 15 and continue to end of protocol.

    b. For cetuximab competition (condition (f)) and secondary only control, incubate tumor sample with secondary antibody (Anti-human IgG Alexa Fluor^{555}) for 1 h with rocking at room temperature (21°C–25°C). Go to step 17 and continue to end of the protocol.

Note: The secondary only control is very important as this is used to determine the baseline voltages of laser channels during image acquisition via confocal microscopy, making sure that any signal detected is not from the autofluorescence of the tissue, which may lead to false positives.

Optional: For antibody uptakes, they should be incubated with appropriate secondary antibody as described in step 14b.

15. Wash tumor samples 5× in PBTX for 20 min each wash.
16. Add appropriate secondary AlexaFluor antibodies diluted in blocking buffer and incubate for 1 h with rocking at room temperature (21°C–25°C).
17. Wash tumor samples 3× in PBTX for 10 min each wash.
18. Wash tumor samples in PBS twice.
19. Incubate tumor samples with DAPI (50 mmol/L in PBS) for 10 min at room temperature (21°C–25°C) to stain cell nuclei.

Note: DAPI staining of the nuclei is crucial to be able to identify individual cells and tumor regions by microscopy.

20. Wash tumor samples 3× in PBS.

21. Mount tumor samples onto concave microscope slides using ProLong™ Gold Antifade Mountant (see mounting Methods Video S1).

    a. Add drop of ProLong Gold mounting media to center of concave section of slide.
    b. Place tumor sections on top of mounting media in center of concave slide.
    c. Place coverslip (22 × 50 mm) over tumor sections at angle to avoid formation/accumulation of bubbles around tumor sections. Ensure ProLong Gold mounting media covers tumor sections.
    d. Press coverslip down firmly on tumor section and seal surrounding coverslip with nail polish. While ProLong Gold and other hardening media work well the nail varnish on the larger coverslips helps reduce any potential drying out prior to media hardening.

Note: Correct mounting of tumor samples is crucial for successful imaging. Bubbles will interfere with imaging. Tumor sections need to be in center of concave slide such that the sample is in correct plane for microscopy imaging.

22. Leave mounting media to cure overnight (8–14 h) in dark before image acquisition.

Day 4 Microscope Analysis and Image Acquisition

Timing: 1–2 h per condition

23. Use confocal microscope to image tumor sections. Microscopy imaging should occur as soon as possible. While fluorescence from secondary antibody post-fixation staining is relatively stable, the directly conjugated ligands fluorescent intensity can decrease to levels difficult to detect against background if not imaged in a timely manner, ideally within a week.

Note: Controls discussed above are important for both imaging and interpretation of results. For antibody uptake and immunostaining, use secondary only controls to set the imaging settings on the microscope. For EGF ligand uptake staining, use cetuximab competition control to set the imaging settings on the microscope, as described above.

Note: Post-acquisition analysis methods vary depending on experimental question that is being asked and are described further below (Quantification and Statistical Analysis).

EXPECTED OUTCOMES

Microscopy analysis of EGF ligand uptake will allow assessment of the endosomal activity of EGFR in response to ligand stimulation in tumors. Researchers will be able to assess if EGF ligand is internalized or if EGF ligand is blocked on the surface and uptake is dysregulated (Joseph et al., 2019). It is important to recall that for a correct comparison of all conditions the same acquisition parameters (e.g., laser power, pixel dwell time/exposure time) should be used for all samples.

This is demonstrated in more detail in Joseph et al, which showed heterogeneous EGF ligand uptake, having both internalizing and blocked EGF ligand within the same dysplastic tissue of some samples (Joseph et al., 2019). In advanced squamous cell carcinoma (SCC) we showed that EGF ligand uptake was either internalizing or blocked.

Heterogeneity in the EGF or antibody uptake signal across tumor sections is expected in the internalization assay. This can be seen in the top panels of Figure 2. In Chew et al, we demonstrated that EGF ligand uptake became more homogenous across the tumor tissue by comparing patient tumor biopsies pre- and post-infusion with the dynamin inhibitor (prochlorperazine, PCZ) (Chew et al., 2020). An example of multiple fields of view (14 per condition) of EGF ligand uptake are shown

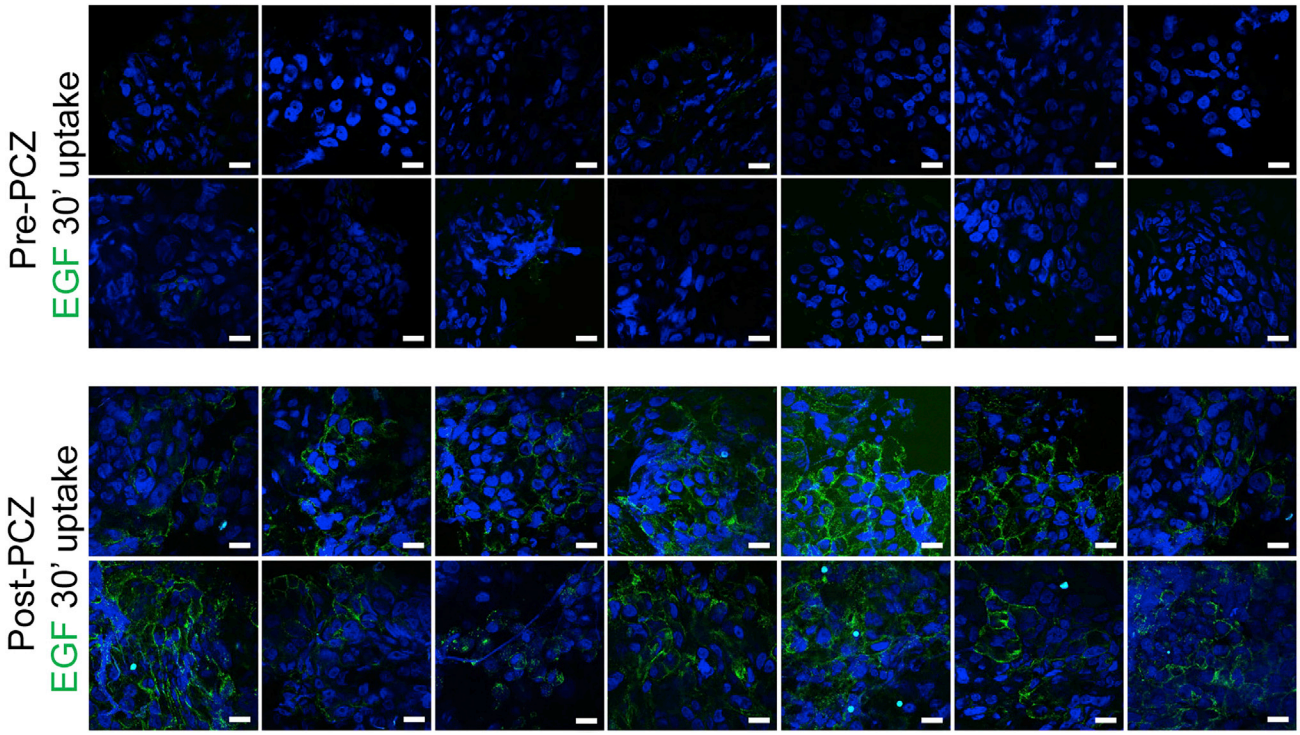

Figure 2. Heterogeneity in the EGF Uptake across Tumor Sections from Tumor Biopsies Taken Pre- and Post PCZ-Infusion and Incubated with EGF-Alexa^{488} (Green)

Scale bar, 20 μm.

for Pre- and Post-prochlorperazine treated patient tumor biopsies from Patient 4 biopsy reported in (Chew et al., 2020) (3 per condition) (Figure 2). Statistical analysis can be performed by measuring the mean fluorescence intensity for all fields of view for each patient biopsy as shown in (Chew et al., 2020). The EGF ligand binding site of EGFR is the binding site for the humanized monoclonal antibody, cetuximab, therefore we show using this assay that we increase drug binding sites in the tumor.

For cetuximab competition (condition (f)), pre-incubation with cetuximab should block any EGF ligand binding or uptake (Figure 3A). A secondary antibody can be used to immunostain for cetuximab uptake. Note, this is not total EGFR staining. Post-fixation immunofluorescent staining using an alternative EGFR antibody can be used to look at total EGFR immunofluorescence which can also be used as a marker of tumor cells in SCC (Chew et al., 2020; Joseph et al., 2019). DEAE-Dextran-AlexaFluor^{555} is internalized after 30 min if the tissue samples are live and displays a heterogeneous pattern (Chew et al., 2020; Joseph et al., 2019) (Figure 3B).

Antibody uptake experiments in both human tumor or human/mouse xenograft experiments can give insight into endosomal trafficking of the monoclonal antibody in the tissue. It can be used for proteins or receptors that do not have a measurable ligand. Total antigen levels (internal and surface) can be compared to surface antigen levels alone by omitting permeabilization steps for either antibody uptakes or post-fixation immunofluorescent labelling (Chew et al., 2020). This assay can therefore be used to analyze monoclonal antibody or drug accessibility at the cell surface.

QUANTIFICATION AND STATISTICAL ANALYSIS

Multiple single plane images are acquired across each tumor sample at the highest fluorescence intensity level of the field of view in the total volume of the Z-series. In order to analyze total EGF ligand

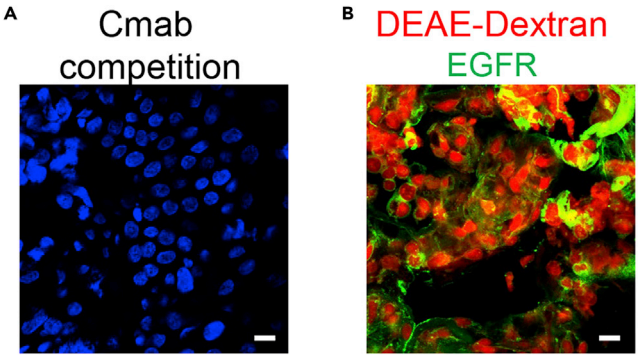

Figure 3. Control Conditions Used in the EGF Internalization Assay

(A) Pre-incubation with anti-EGFR monoclonal antibody (cetuximab) to block EGF-Alexa^{488} binding (green, not detected). DAPI, blue.

(B) Incubation with DEAE-Dextran-Alexa^{594} (red) to show that cells are viable and capable of fluid-phase uptake and not necrotic areas of the tumor. Samples were co-stained for EGFR (green). Scale bar, 20 μm.

uptake, the overall mean fluorescence intensity can be measured for all fields of view for each patient biopsy sample image using the Image J function (Chew et al., 2020). When fluorescence intensity is very variable across the sample, 3D Z-stacks can be acquired. In this case, the mean intensity should be measured at each Z-section and averaged over a total of 20 μm Z-stack with the optimal Z-separation given by the Nyquist's equation.

However, it is important to consider that the localisation of the target rather than its total levels should be considered when performing the imaging analysis. This assay was originally used to identify different EGF ligand uptake patterns, EGF ligand blocked on the plasma membrane or EGF ligand that was internalized (Joseph et al., 2019). SCC with recurrent or metastatic disease was classified as blocked (plasma membrane staining, cobblestone pattern) or internalizing (endosomal staining, punctate pattern) and these patterns correlated to monoclonal antibody therapy outcomes. Interestingly, it was found that early-stage lesions (eg AK, IEC, and SCC samples) had variation in EGF ligand internalization, having both internalizing and blocked patterns within tissue samples. Due to the lack of software with the ability to differentiate plasma membrane against internalized EGF staining at that time, multiple fields of 63× images from each tumor were expanded until individual pixels were available. All pixels were counted, whereby linear arrays of pixels in lines of 4 or more pixels versus pixels in punctuate, non-linear arrangements were individually scored by researchers who had no knowledge of the experiment. This independent quantification worked well and linear arrays were scored as plasma membrane EGF distribution and punctuate arrays as internalized EGF. Efficacy of method was checked visually by expert cell biologists experienced in distribution analysis. These were quantified as described in Joseph et al. (2019) or as explained in Methods Video S2. Current computer software analysis programs may be able to automate this process.

For antibody uptake experiments, total surface levels can be measured by performing the ex-vivo assay in non-permeabilizing conditions and measuring total fluorescence levels using the Image J function.

LIMITATIONS

The preservation of the live tumor samples is crucial for the success of EGF ligand internalization assay. Tumor biopsies must be live (therefore they should not be frozen or fixed prior to the assay), and the assay must be carried out within 15 min from tumor excision. The protocol may be unsuccessful if these conditions are not met.

Tumor samples are sometimes limited in size or tissue amount. It is advisable to have multiple tumor chunks in each assay condition. Minimal conditions are listed above. Not all tumor samples will be

optimal for the assay because cutting up of the tumor samples is random and some pieces may not contain tumor or just be connective tissue. (More details in Troubleshooting section, Problem 2 (below), on how to differentiate between tumor and connective tissue)

This assay has potential to be used for analysis of other ligand and receptor combinations. However, antibody/ligand and/or receptor function may be a limitation to this assay and should be considered when designing experiment as antibody binding may alter endogenous antigen trafficking. EGF ligand is amenable to this assay as it binds directly to the receptor and is clustered as a function of its normal processing allowing visualization by microscopy (Pinilla-Macua and Sorkin, 2015). Additionally, cetuximab is monovalent. Bivalent or multivalent antibodies may cluster receptors on the cell surface and therefore antibodies may aggregate targets on the surface and thus increase retention time as normal endocytosis of the receptor is inhibited or delayed, or alternatively may decrease retention time leading to increased endocytosis. In this case, it may show antibody effects on target receptor, but it does not infer anything about normal endocytosis of receptor without the antibody binding.

TROUBLESHOOTING

Problem 1

No EGF uptake or fluorescence can be seen in any of the conditions during the imaging.

Potential Solution

The control samples are crucial in order to identify the success of the experiment. If no EGF uptake can be visualized with the confocal microscope is important to:

- Check that the tumor tissue expresses EGFR. Do this by analyzing the post-fixation immunofluorescent staining of the EGFR in control tumor samples.
- Check if the Dextran uptake has been successful. If the Dextran uptake has been successful, this confirms that the biopsies are not necrotic.
- The preservation of the EGF ligand is also crucial for the success of the assay. Fluorescently conjugated ligands are sensitive to light and freeze-thaw processes (the ligand must be preserved frozen in small aliquots which must not be re-used). To check the quality of the EGF ligand, a positive EGF uptake control in tissue culture cells (e.g., A431 cells) should be performed.
- Dent's bleach protocol can be omitted from protocol. Dent's bleach can decrease both autofluorescence and ligand fluorescence.
- Use nuclei staining to locate tumor tissue sample. Tumor sample needs to be in center of slide, so that sample is in correct plane for microscopy imaging.
- Use multiple tumor sections in each condition so you get randomized samples from the initial tumor section.

Problem 2

I do not know what is tumor tissue and what is normal tissue?

Potential Solution

When analyzing samples by microscopy it may be difficult to identify normal versus tumor tissue. EGFR immunostaining can be used to localize tumor tissue as it is specific to tumor cells consistent with EGFR overexpression and is used in pathology for SCC identification (Cañueto et al., 2017; Ch'Ng et al., 2008; Grandis and Tweardy, 1993). Alternatively, tumor samples could be additionally co-stained with accepted markers for selected tumor type. As part of this assay development, nuclei in confocal images were compared and there was obvious variation in the nuclei shape and density between normal and SCC samples. In normal tissue, nuclei were regular, widely spaced, and smaller than that seen in tumor tissue (Figure 4). Hence, it is crucial to have DAPI staining as a control for tissue samples. We do not see EGF ligand uptake in normal tissue (receptor-ligand not concentrated enough for confocal imaging).

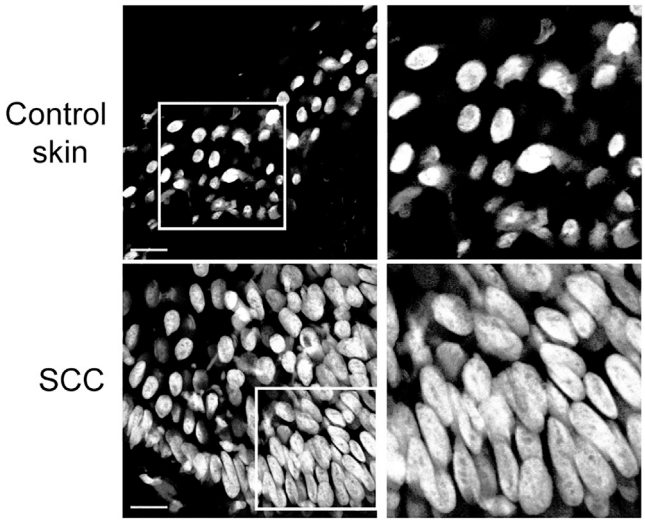

Figure 4. Normal (Control Skin; Top) and Dysplastic Tumour (SCC; Bottom) Keratinocytes Differ in Nuclear Size and Aspect and Can be Used to Distinguish between Tissue Types
Nuclei stained with DAPI. Scale bar, 20 μm.

If possible, it is advisable to have matched normal tissue to analyze alongside tumor tissue for comparison in this assay. For histology purposes, if enough tissue is available, sequential H&E and wholemount immunofluorescent of FFPE sections may also be done as seen in Joseph et al (Joseph et al., 2019) to enable better characterization of tumor vs normal tissue. **Consultation with a pathologist** is also invaluable for assessing tumor histology. Pathologist can also advise of histology markers for specific tumor types.

Problem 3
My sample is made up of blue matrix and I cannot see clear nuclei.

Potential Solution
This is due to the tissue sample, it is autofluorescence of extracellular matrix and collagen with minimal actual tumor cells (Figure 5). Autofluorescence is not as common in xenograft tissue samples as they have less collagen and matrix. It is therefore advisable to put multiple tumor chunks in each condition to ensure you have a larger randomized sample size. Dent's bleach protocol should be performed for these samples in future but will not completely remove this fluorescence. An alternative nuclear stain such as SYTOX Deep Red Nucleic Acid Stain may be used which fluoresces in far red range using traditional filter set or 647 laser line (Cy5) and may have less autofluorescence.

Problem 4
I cannot see any antibody uptake in any of the conditions during the imaging.

Potential Solution
It is useful to check that the tumor or xenograft sample expresses the antibody target. No staining in the assay may result from the antibody target not being expressed in the tissue or it may indicate that the drug target is all inside the cell and not accessible to the monoclonal antibody in the *ex vivo* live tissue. In order to check this, it is recommended that a post-fixation labelling of control tissue samples (no antibody uptake) is completed using the antibody of interest to analyze total levels and localization of the monoclonal antibody target in the tissue. It is also crucial to make sure that the permeabilization step is performed sufficiently to allow the secondary antibody binding.

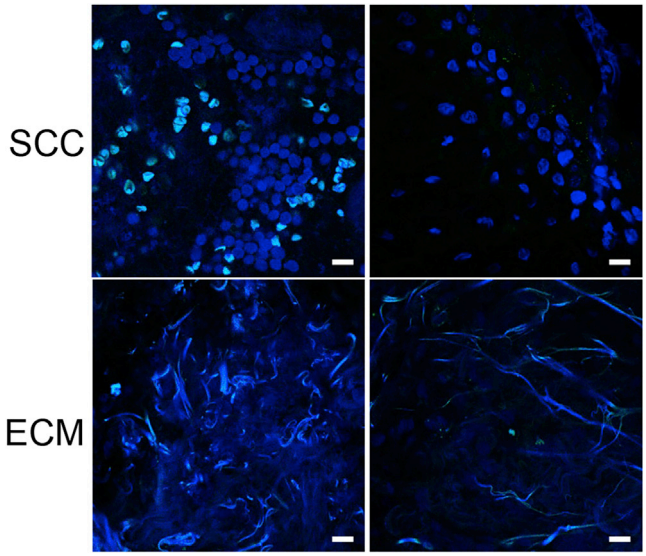

Figure 5. Optimal (Top) and Non-optimal (Bottom) DAPI Staining of Nuclei
The bottom panels show a high level of extracellular matrix (ECM) with minimal tumor cells. Scale bar, 20 μm.

RESOURCE AVAILABILITY

Lead Contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Fiona Simpson (f.simpson@uq.edu.au).

Materials Availability

This study did not generate new unique reagents.

Data and Code Availability

This study did not generate any unique datasets or code.

SUPPLEMENTAL INFORMATION

Supplemental Information can be found online at https://doi.org/10.1016/j.xpro.2020.100087.

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AUTHOR CONTRIBUTIONS

Writing – Review & Editing, S.R.J., B.L., and B.B.; Figures, S.R.J. and B.B.; Graphical Abstract, B.L. and B.B.; Funding Acquisition, S.R.J., B.B., and F.S.; Writing – Editing & Supervision, F.S.; Clinical

Advice, Patient Consent, and Tumor Biopsies, B.P. and E.W.; Protocol Development, R.B., S.R.J., and F.S.; Invention of Technique: F.S. (as per patent).

DECLARATION OF INTERESTS

This work utilizes technology subject to the following patents: Simpson F, Saunders NA (2015) Classifying epidermal growth factor receptor positive tumor into subtype, e.g., epidermal growth factor receptor antagonist sensitive subtype, involves analyzing ligand-induced epidermal growth factor receptor internalization status of tumor. WO2014063206-A1; Simpson F, Saunders NA. (2015) Composition useful in kit for treating tumor, preferably cell surface antigen positive tumor, e.g., cancerous tumors, comprises antibody that binds to cell surface antigen of tumor and inhibitor of receptor mediated endocytosis. WO2014063205-A1; Simpson F, Leftwich SR (2016) Methods for classifying tumours and uses therefor. WO 2016/127220-A1.

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