Profiling protein-protein interactions *in vivo* by cross-linking and mass spectrometry

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Part 1 – Global

Novel chemical cross-linking strategy for global identification of protein-protein interactions

Part 2 – Targeted

Method for investigating protein-protein interactions related to *Salmonella typhimurium* pathogenesis
Global cross-linking strategy

**Low complexity approach**

1. React with Cross-Linker
2. Trypsin digestion
3. LC-MS/MS
4. Data analysis

- List of cross-linked peptides
  - A-XXXX
  - B-XXXX
  - C-XXXX
  - D-XXXX
  - E-XXXX
  - F-XXXX

**High complexity approach**

1. Trypsin digestion
2. Enrichment
3. LC-MS/MS
4. Data analysis

- List of cross-linked peptides after enrichment
  - A-XXXX
  - B-XXXX
  - C-XXXX
  - D-XXXX

**Cross-Linker**

- Inter
- Dead-end
- Intra
Cross-linker design: enrichment-based strategy

Cross-linker spacer chain 9-10Å

Small cross-linker for cell permeability

Enrichment reagent

Reactive group

Primary amine reactive groups

Small molecule tag

Secondary reactive tag chemical moiety not present in proteins
Enrichment strategy using CLICK chemistry

React with azido compound containing biotin support Cu(I)

- Capture with avidin beads
- Wash to remove non-binders
- Elute

LC-MS/MS
Challenge:
Automated identification of cross-linked peptides

Xlink-Identifier aids automated data analysis

Du et al., manuscript in preparation
Challenge:

Manual validation of cross-linked species

Xlink-Explorer aids manual validation
Initial application:
Low complexity approach using ubiquitin in solution digestion

React with Cross-linker

Digestion

LC-MS/MS Xlink Identifier

Xlink Explorer

A-XXXX
B-XXXX
C-XXXX
D-XXXX

Amplitude

00
33
67
100

330. 747.5 1165. 1582.5 2000.

m/z

$\text{LIFAGKQLEDGR}$

$\text{TLSDYNIQKESTLHLVLR}$

$m/z = 1252.0 \ (3+)"

$[\text{M-NO2+3H}]^{3+}$
Low complexity approach using ubiquitin in gel digestion

- IQDKEGI PPDQQR
- LIFAGKQLEDGR

m/z 1049.76 (3+)

m/z

y6 (α)

b7 (α)

y6 (α)

b7 (α)

y7 (β)

y9 (β)

y11 (β)

b6 (α) - y11 (β)

b6 (α) - y10 (β)

y11 (α) - y10 (β)

b12 (α) - y12 (β)

b7 (α)

b5 (α) - b8 (β) - H2O

b5 (α) - b8 (β)

b6 (α) - b8 (β)

b6 (α) - b7 (β)

b7 (α)

b7 (α) - y8 (β)

b7 (α) - y7 (α)

b7 (α) - y6 (α)

b7 (α) - y5 (α)

b7 (α) - y4 (α)

y2 (α)

y3 (α)

y4 (α)

y5 (α)

y6 (α)

y7 (α)

y8 (α)

y9 (α)

y10 (α)

y11 (α) - b9 (β)

b10 (α)

y11 (α) - y10 (β)

y12 (β)

y13 (α) - y12 (β)

b11 (β)

b12 (α) - y10 (β)

y11 (β)

y10 (β)

y9 (β)

y8 (β)

y7 (β)

y6 (β)

y5 (β)

y4 (β)

y3 (β)

y2 (β)

b3 (α)

b4 (α)

b5 (α)

b6 (α)

b7 (α)

b8 (α)

b9 (β)

b10 (β)

b11 (β)

b12 (β)

[M - NO2 + 3H]+3+
Cross-linker and enrichment agent maintain typical CID fragmentation

Unmodified

LIFAGKQLEDGR

m/z = 673.87 (2+)

Dead-end cross-linking before enrichment

LIFAGK^QLEDGR

After CLICK enrichment

LIFAGK^QLEDGR

Amplitude

m/z
Automated identification of enriched inter cross-linked peptides by Xlink-Identifier

Click enrichment
Inter cross-linked peptide
CID

LIFAGKQLEDGR
LIFAGKQLEDGR
m/z = 854.40 (4+)

ETD

m/z = 854.40 (4+) CID

Click enrichment
Inter cross-linked peptide

LIFAGKQLEDGR

Automated identification of enriched inter cross-linked peptides by Xlink-Identifier
Cross-linking results consistent with previously published crystal structures

Inter cross-linked peptides
- LIFAGK^QLEDG--- TLSDYNIQK^ESTLHLVLR
- LIFAGK^QLEDG--- LIFAGK^QLEDGR
- LIFAGK^QLEDG--- IQDKEGIPPDQQR

Intra cross-linked peptide
- AK^IQDK^EGIPPDQQR

Dead-end
- TLSDYNIQK^ESTLHLVLR
- LIFAGK^QLEDGR
- IQDKEGIPPDQQR
Summary – Part 1

Initial characterization of enrichment-based cross-linking strategy

• Small molecule cross-linker with optional enrichment capability
• Software developed to aid analysis
• Fragmentation spectra remained interpretable for cross-linked and enriched peptides
• Studies with higher complexity protein samples currently in progress
Part 2: *Salmonella* virulence study to discover interacting partners

Targeted 3 virulence-associated proteins with known binding partners

- STM 0982 (HimD) is known to stably interact with STM 1339 (HimA)
- STM 1231 (PhoP) is known to transiently interact with STM 1230 (PhoQ)
- PduB (STM 2039) is expected to interact with other members of the Pdu operon

Example of known interacting system (www.mcgill.ca/microimm/lemoual)
Pull down approach with HBH tag and formaldehyde cross-linking

**Advantages**
- Cell permeable and nonspecific
- Inactivate the enzyme immediately
- *In vivo* and in functional context
- Strong denaturing conditions can be used

**Disadvantages**
- Close proximity (~2Å)
- Non-specific modification in proteins

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Modified HBH-cross-linking strategy

**Bead control**
- STM cells
  - 1% Formaldehyde
  - Lyse
  - Proteins
  - Affinity Purification
  - Wash and Digest
  - Peptides
  - LC-MS/MS
  - Bead interacting

**Bait control**
- STM cells (HBH-bait)
  - 0% Formaldehyde
  - Lyse
  - Proteins
  - Affinity Purification
  - Wash and Digest
  - Peptides
  - LC-MS/MS
  - Bait
    - + bead interacting
    - + non-specific binding

**Cross-link**
- STM cells (HBH-bait)
  - 1% Formaldehyde
  - Lyse
  - Proteins
  - Affinity Purification
  - Wash and Digest
  - Peptides
  - LC-MS/MS
  - Bait
    - + bead interacting
    - + non-specific binding
    - + specific binding
Reproducibility study with HimD-HBH

- 3 independent biological experiments:
  - one using 0.5% formaldehyde
  - two using 1% formaldehyde

- Two controls for each experiment:

Results highly reproducible and consistent with known stable interactions
Lists of identified interactions for 3 virulence-related proteins

HimD (STM0982)

PduB (STM2039)

PhoP (STM 1231)

Interaction diagrams with known or expected interactors labeled
Summary – Part 2

Tandem affinity tag and cross-linking applied to identify interactions related to *Salmonella* pathogenesis

- A modified HBH tag and formaldehyde cross-linking strategy used to differentiate background, non-specific, and specific interactions
- *In vivo* application to three *Salmonella* virulence proteins identified known and novel protein-protein interactions
- Currently, a few interactions are being validated with other methods
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