

Ronan Keegan CCP4 Group MR Search Models and Assessing the Solution

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Overview

Introduction

- Step-by-step guide to performing Molecular Replacement
- Automatic Molecular Replacement in CCP4
- What to do if Molecular Replacement doesn't work
- Acknowledgements





Introduction: Basics of Molecular Replacement



h	k		F	ф
0	0	1	12.6	123
0	0	2	2.1	12
0	0	3	69.9	287



h	k		F	ф
0	0	1	10.4	113
0	0	2	3.5	18
0	0	3	57.2	265

(slide from Gabor Bunkoczi)

MrBUMP

3



Introduction: Basics of Molecular Replacement



Search model crystal form

Target crystal form

 $\Re \varphi, \psi, \kappa, x, y, z$

(slide from Gabor Bunkoczi)





Introduction: *Basics of Molecular Replacement* (φ, ψ, κ) (x, y, z)

(slide from Gabor Bunkoczi)





Introduction: Molecular Replacement and the PDB

- Today, the vast majority of entries in the PDB have been phased using MR
- Increasing success rate of MR has been due to:
 - Improving methods in software e.g. maximum likelihood in Phaser
 - Increasing availability of suitable search models



(image from Fei Long)





Introduction: *Step-by-step Molecular Replacement*

 Despite this, successfully performing MR depends on attention to detail both before and after running the Molecular Replacement program







Step-by-Step MR: *Examining the data*

• Data issues can have an impact on how well MR will work

Examine the data





Step-by-Step MR: Examining the data

- Data issues can have an impact on how well MR will work
- Things to think about:
 - How many copies in the asymmetric unit?
 - What's the resolution of the data?
 - Self-rotation function signs of NCS?







Step-by-Step MR: Examining the data

- Data issues can have an impact on how well MR will work
- Things to think about:
 - How many copies in the asymmetric unit?
 - What's the resolution of the data?
 - Self-rotation function signs of NCS?
- Potential problems:
 - Pseudo translation?
 - Twinned data?
 - Space group assignment correct?

Examine the data





• A well chosen and optimized search model has two key advantages







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 - 1. Makes it easier for the MR program to find the correct position for that search model and hence sensible starting phases







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 - 1. Makes it easier for the MR program to find the correct position for that search model and hence sensible starting phases
 - 2. Facilitates quicker and easier model building and refinement post MR







• How to find a search model?

 Amino acid sequence similarity often correlates well with structural similarity









- Considerations
 - Target Size:
 - The bigger the structure the more likely there will be sizeable conformational changes
 - Data Resolution :
 - Lower than 2.5 Angstroms search model should represent large fraction of target molecule
 - Between 1.0 and 2.5 Angstroms search model can be small fragment of target – DM and model building can improve phases
 - Better than 1.0 Angstrom search model can be single atom (McCoy et al. 2017, PNAS)
 - Homologue Sequence identity < 30%
 - Suitable search models may exist sequence identity calculation is difficult







• The field of Bioinformatics has given us a wealth of sophisticated and sensitive sequence-based search tools for finding potential homologues from which we can derive search models for MR







• PSI-Blast

- Profile-based searching
- Online server, fast
- Works well at finding suitable homologues down to sequence identities of 30%



C _ blast.ndbi.nlm.nih.gov	/Blast.cgi	
Description 1dtx_A Molecule type amino ac Query Length 59	ngDNE- 🏦 Maxde MXS 1.6 (+ 🗠 Grefit Card Exper 🗅 CCH4 Program 5.6 🦉 Check overage (+ Description : PDB protein database Program : BLASTP 2.2.324 (+ <u>Cataton</u>	COnstante Margari Henorge September Constanting of the second sec
Other reports: > Search Si	ummary [Taxonomy reports] [Distance tree of results] [Multiple alignment] Manual Analyze your query with SmartBLAST	Sequences producing significant alignments with E-value BETTER than threshold Select: All None Selected 0
Show Conserved Domain	5	🛱 Alignments 🖾 Download – GanPept Grasphice Distance tree of results Multicle atlanment
Query seq. Q P R	Putative conserved domains have been detected, click on the image below for detailed results.	Description Max Total Query E score score corer value Mar Accession to the text score score corer value
Specific hits Superfamilies	KU KU superfamily	🗍 Chain A, Crystel Structure Of Alpha-Dendrotoxin From The Green Mamba Venom And Its : 122 122 98% 29-37 100% 1DTX_A 🕏
		Chain A, Proteinase Inhibitor Homologues As Potassium Channel Biockers 113 113 98% 7e-34 93% 1DEM A
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	Mouse-over to show define and scores, click to show alignments	Chain A. Solution Structure Of A Chymotrypsin inhibitor From The Taiwar Cobra 71.2 71.2 96% 1e-17 51% 2M99_A
	Color key for alignment scores	Chain A. Solution Structure Of Bungarus Faciates In: A Kunitz-Type Championsin Inhibitar 67.4 67.4 96% 56-16 53% 1JC6_A
	<40 40-50 50-80 80-200 >=200	Chain K. Hemostatic Effect Of A Monocional Antibody Mab 2021 Blocking The Interaction 1 60.1 60.1 96% 3e-13 46% 4DTG_K
	1 10 20 30 40 50	Chain C, Complex Of The Second Kunitz Domain Of Tissue Factor Pathway Inhibitor With 58.2 58.2 88% 1e-12 48% 1TEX_C
		Chain A, The Solution Structure Of The Second Kunitz Domain Of Tissue Factor Pathway I 58.5 58.5 68% 1e-12 48% 1ADZ A
		Chain E. Thrombin-bound Boophilin Displays A Functional And Accessible Reactive-site 1: 56.6 100 93% 1e-11 45% 200Y E 🛛
		🗉 Chain J. Crystal Structure Of Textilinin-1. A Kuritz-Type Steine Protesses inhibite: From The 53.5 53.5 96% 70-11 44% 3065_1 👻
		🗉 Chain A. Createl Structure Of Textlinin-1. A Kunitz-True Serine Protesses Inhibitor From Th. 53.5 53.5 96% 7e-11 44% 38YB_A 😢
		🗟 Chain A. Calocidudine (Cao) From Green Mamba Dendroaspis Ancuaticeos. Nerr. 15 Struct. 52.4 52.4 77% 20-10 50% 1BEQ_A 🕑
		Chain A. Solution Structure Of Anntoxin 52.0 52.0 88% 3e-10 42% 2KCR_A
		Chain A, A 2.4 Crystal Structure Of Cenkunitzin S1, A Novel Kunitzi Feld Cone Snall Neuro 49.7 49.7 98% 2e-09 41% 1982 A
		Chain A, The Anisotropic Refinement Of Kunitz Type Domain CS AL0.95 Angatron 49.7 49.7 68% 20-09 42% 1KTH_A
		Chain A. Kdt Of Human Tipi In Complex With A Synthesic Peptide 48.9 48.9 91% 5e-09 37% 4800_A





• HHpred

- Hidden Markov Model approach
- Suitable for more difficult cases where no obvious homologue is available









 Once a suitable homologue or set of homologues have been found they need to be prepared for use as search models







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 The closer we can make the homologue to the target in terms of structural similarity the more likely it is to be successfully positioned in Molecular Replacement







 Once a suitable homologue or set of homologues have been found they need to be prepared for use as search models

- The closer we can make the homologue to the target in terms of structural similarity the more likely it is to be successfully positioned in Molecular Replacement
- The sequence alignment generated in the search step can be used as a guide for the pruning of the homologue







• Example target:

 5TPX - Bromodomain from Plasmodium Faciparum Gcn5





- Example target:
 - 5TPX Bromodomain from Plasmodium Faciparum Gcn5
- Homologue:
 - 1E6I chain A
 - 45% sequence identity over 82% coverage of target









Search Model preparation and MR:
All atoms?





• Search Model preparation and MR:

- All atoms?
- Polyalanine?





- Search Model preparation and MR:
 - All atoms?
 - Polyalanine?
 - Mixed model based on sequence alignment
 - Keep common residues
 - Truncate to common atoms on aligned residues





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 - All atoms?
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 - Keep common residues
 - Truncate to common atoms on aligned residues





• Mixed-model generation in CCP4

• Chainsaw & Sculptor







Mixed-model generation in CCP4
Molrep

Input Data Basic O	ptions Advanced Options	
Job title Molrep]
Use data from	n job 7 Import an alignment 🗘 as in	put below
Experimental data	i	
Reflections	must be selected	
R Free R set	must be selected	
Search model Atomic model click for options to view Atom selection Sequence of targe	2 A of 1 of Atomic model imported from 4nec.pdb by jot jobs and files (1890 atoms)	Help
Sequence	is not used	•
The number of mono	omers to search for Auto 🗢	
Fixed Model		
Atomic model	is not used	•
1000		







- Manual pruning in Coot
- Remove anything that is likely to be flexible:
 - External loops
 - Longer side chains on the surface of the molecule (e.g. LYS)
 - Domains if there is evidence of "hinge motion"









• Ensembles: alignment of search models









- Ensembles: alignment of search models
 - 1. Variance across members can guide experimental data weighting in Phaser







- Ensembles: alignment of search models
 - 1. Variance across members can guide experimental data weighting in Phaser
 - 2. Truncation based on alignment variance can identify conserved regions or core









- Ensembles: Generating ensembles in CCP4
 - Ensembler:
 - Align models and truncate based on a variance threshold
 - General alignment tools:
 - Gesamt
 - Superpose
 - Can also be run through CCP4mg and Coot graphical interfaces







Ensembles: CCP4mg & MrBUMP

- Finds homologues using Phmmer
- Runs Chainsaw to prune and create search models
- Aligns models using Gesamt
- Truncation tool to adjust variance threshold









• Multimers as search models

- A single chain search model can be too small if the target has crystallised in multimeric form
- The signal for the correct position is too weak against the background noise of incorrect positions
- Particularly a problem at lower resolutions and crystals with high symmetry








Step-by-Step MR: *Preparation of search model*

• Domains as search models

- A hinge motion may alter the relative orientation of domains within a chain
- Domain models should be isolated from the parent search model and used separately in MR







- CCP4 has several programs for doing Molecular Replacement
 - Amore
 - Manual steps but very fast
 - Molrep
 - Automated MR
 - Several useful features e.g. searching a map
 - Phaser
 - Maximum likelihood approach
 - Accounts for potential model errors
 - Best for difficult cases and for correctly positioning fragment search models













Important points on using Phaser

• Phaser accounts for errors in:







- Phaser accounts for errors in:
 - 1. Model
 - Provide accurate details of AU composition
 - Use RMS value rather than sequence identity and try different values if first attempt doesn't work







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 - 1. Model
 - Provide accurate details of AU composition
 - Use RMS value rather than sequence identity and try different values if first attempt doesn't work
 - 2. Data
 - Provide intensities internally works out amplitudes accounting for experimental errors







- Phaser accounts for errors in:
 - 1. Model
 - Provide accurate details of AU composition
 - Use RMS value rather than sequence identity and try different values if first attempt doesn't work
 - 2. Data
 - Provide intensities internally works out amplitudes accounting for experimental errors
- Phaser performs clever decision making for automation
 - Provide minimal details and let Phaser make it's own decisions e.g. search order, search all possible space groups
 - If it doesn't work take step-by-step approach 1 copy at a time











Phased translation search

- Used when searching for several copies or dealing with a complex
- Available through MOLREP (3 protocols) and Phaser
- Often more successful than standard MR search approach particularly when looking for small domains



• Phased translation search

- From MOLREP (CCP4i):
- 3 Protocols for phased search Try all!

v Averaged Dased Translation Function	,55_monep.de	Hel
Job title fit model to 4n9g		
Do MR Lising Phases		
Lise MAP files for observed data		
	Prouso	View
	DIUWSE	View
Obs not used	1110	
Model 4nec - trun_7_molrep1.pdb	Browse	View
Sequence 4nec -	Browse	View
Fixed 4nec - temp.pdb	Browse	View
Automatic output filename		
Solution 4nec - trun_7_molrep4.pdb	Browse	View
Search Options		
Search protocol SAPTF + Local Phased RF + Phased TF 🛁 💻		
Number of corpions to find = = = 1 = = = = = = = = = = =		
Number of RF peaks to use in TF		
Radius of search model		
Pseudo-Translation: Auto –		
Experimental Data		
Model		
Infrequently used options		
Run - Save or Restore -	Close	•







Phased translation search

• From Phaser (CCP4i):

😣 🗐 🗊 Maximum Likelihood M	olecular Replacen	nent Initial paramete	rs from /home/rmk65,	/Projects/ample/cor
FW1 .			Ullession	IEU
Space group read from mtz fil	e 'P 21 21 21'	Bun Phaser with	mtz space group and	d enantiomorph
Define ensembles (models)	•••••••		na opaoo group an	a entantiennerpri
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Similarity of PDB #1 to the t	arget structure	sequence identity	- 0.2	
	Edit list	- Add supe	erimposed PDB file t	to the ensemble
Ensemble # 2				×
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Similarity of PDB #1 to the t	arget structure	rms difference	- 0.2	
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· · · · · · · · · · · · · · · · · · ·			Edit list -	Add ensemble
Define composition of the asy	mmetric unit			_
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Run	-	Save or Rest	ore —	Close













• How to know if the search model is correctly positioned in the target unit cell







• How to know if the search model is correctly positioned in the target unit cell

 In difficult cases the position may be correct but getting from the MR solution to a complete model may not be straight forward













- Rough guide to MR program scoring
 - Phaser scores
 - LLG scores has it increased by 60 or more after the placement of a new molecule?
 - (resolution and space group dependent)
 - TFZ greater than 8?
 - Few or single solution almost always indicative of success







- Rough guide to MR program scoring
 - Phaser scores
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 - (resolution and space group dependent)
 - TFZ greater than 8?
 - Few or single solution almost always indicative of success
 - Molrep scores
 - RFZ rotation search score greater than 5 is there a clear peak?
 - TFZ translation search score is there a clear peak?







Step-by-Step MR: Assessing the MR solution• Refinement







Refinement

- Look at Rfactor/Rfree
 - are they falling? Is Rfree below 0.5?







• Refinement

- Look at Rfactor/Rfree
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- Use 200 cycles of jelly body refinement option in Refmac post MR







• Refinement

- Look at Rfactor/Rfree
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MrBUMP



Examine the data

Find a search model

MrBUMP

- Refinement
 - Look at Rfactor/Rfree
 - are they falling? Is Rfree below 0.5?
 - Use 200 cycles of jelly body refinement option in Refmac post MR





• Examine solution by eye

• Use Coot to examine positioned models & maps









• Phase Improvement & C-alpha tracing







 Phase Improvement & C-alpha tracing

> SHELXE for MR (resolution better than 2.5Å)



Run Molecular Replacement

Assessing the MR Solution





Phase Improvement & C-alpha tracing

 SHELXE for MR (resolution better than 2.5Å)



Experimental phasing Automated structure solution - CRANK2 phasing and building CRANK2 experimental phasing pipeline Automated structure solution - SHELXC/D/E phasing and building SHELX Experimental phasing pipeline SHELX (run via Crank2) SAD phasing from heavy atom sites - PHASER Complete a heavy atom model and calcula **ACORN - Phase Refinement with Dynamic Density Modification** Un-biased improvement of initial phases for high resolution data (1.5 Angstoms and b ACORN Find HA sites - SHELXC/D Find sites from SAD/MAD/SIR/SIRAS/RIP/RIPAS dat **Density modification - PARROT** Modify the electron density (Parrot, Bioinformatics including model preparation for Molecular Replacement

• ACORN (resolution better than 1.7Å)

Assessing the MR Solution



• SHELXE scoring: CC >= 25 % (average c-alpha fragment length >= 10)

SHELXE scoring tested for 1000 separate MR solutions



(2015) Acta Crystallogr D Struct Biol **71**: 338-343







Automatic model building: Buccaneer & ARP/wARP

- Can be used post-MR for generation of better model and phases for the target
- Rebuilding parts that may not be present in search model
- Useful for assessing whether or not your positioned MR model is true eliminates bias







Automated Molecular Replacement in CCP4

•Several automation pipelines for MR in CCP4:

• MrBUMP – model search, preparation, MR and refinement

• **BALBES** – model search in custom version of PDB database

• *MoRDa* – similar to BALBES





MrBUMP

- Covers all stages from data examination through to initial model building
- Two modes:
 - 1. Model generation only
 - 2. Model generation and Molecular Replacement through to model building
- Can be run from:
 - CCP4i2, CCP4i & CCP4 Online
 - CCP4mg model generation step with graphical view







BALBES/MoRDa

- Covers all stages from data examination through to refinement
- Custom database:
 - 1. Non-redundant version of PDB
 - 2. Basic entry is a domain with definitions for constructing full chains, multimers and ensembles
- Other features:
 - Search all spacegroups
 - Uses Molrep for MR and Refmac for refinement
 - Available inCCP4i & on CCP4 Online







CCP4 Online: MrBUMP, BALBES, MoRDa & more













•Try different search models or different preparation methods for the homologues





- •Try different search models or different preparation methods for the homologues
- •Try more distant homologues
 - Sequence similarity does not always imply structural similarity e.g. S100 family of proteins
 - Distant homologues can be structurally similar e.g. globular enzymes





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- •Try more distant homologues
 - Sequence similarity does not always imply structural similarity e.g. S100 family of proteins
 - Distant homologues can be structurally similar e.g. globular enzymes
- Experimental Phasing
 - MR-SAD Phaser, Crank2 or SHELX
 - Sulpher SAD





• **AMPLE**: Ab initio modelling to generate models for molecular replacement

• Uses programs like *Rosetta* and *Quark* to generate search models from the target sequence

• Can exploit sequence alignment derived residue contact predictions






What to do if Molecular Replacement doesn't work

•Crystal Contaminants

- Always check that you don't have a contaminant present
- Perform mass spectroscopy on your solution and crystals if possible
- Test your data immediately after data collection using SIMBAD or Contaminer

METHODS AND APPLICATIONS

Protein purification and crystallization artifacts: The tale usually not told

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Abstract: The misidentification of a protein sample, or contamination of a sample with the wrong protein, may be a potential reason for the non-reproducibility of experiments. This problem may occur in the process of heterologous overexpression and purification of recombinant proteins, as well as purification of proteins from natural sources. If the contaminated or misidentified sample is used for crystallization, in many cases the problem may not be detected until structures are deter-

Abbreviations: CSGID, Center for Structural Genomics of Infectious Diseases; GNAT, Gon5-related N-acetyltransferase; IMAC, immobilized metal affinity chromatography; MAD, multi-wavelength anomalous diffraction; MR, mdecular replacement; MCSG, Midwest Center for Structural Genomics; NVSGRC, New York Structural Genomics Research Consortium; PDB, Protein Data Bank; RMSD, not mean square deviation; SAD, single-wavelength anomalous dispersion; SEC, size exclusion chromatography; TEV, tobacco etch virus.

Additional Supporting Information may be found in the online version of this article.

Ewa Niedzialkowska and Olga Gasiorowska have contributed equally to this work.

The authors declare that there is no conflict of interest.

Description of Supporting Information material: Summary of data collection and refinement statistics for the deposited structures and the list of deposits used to identify crystallization artifacts by MR. Filename: Supplementary Materials.

Structural data are available in PDB database under accession numbers 4TNN, 4YYC, and 4ZNZ.

This work focuses on a particular difficulty that may occur as a result of accidental purification or contamination of the sample with a



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