Refinement of Macromolecular structures using REFMAC5

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1) Introduction
2) Considerations for refinement
3) Twin
4) Overall parameters
5) TLS
6) Some new features in REFMAC
7) Conclusions
Available refinement programs

- SHELXL
- CNS
- **REFMAC5**
- TNT
- BUSTER/TNT
- Phenix.refine
- RESTRAINT
- RESTRAINT
- MOPRO
Some features of REFMAC

- Simple maximum likelihood restrained refinement
- Twin refinement
- Phased refinement (with Hendrickson-Lattmann coefficients)
- SAD/SIRAS refinement
- Structure idealisation
- Library for more than 10000 ligands
- Covalent links between ligands and ligand-protein
- Rigid body refinement
- NCS local, restraints to external structures, jelly body
- TLS refinement
- Map sharpening
- Occupancy refinement
- etc
Considerations in refinement

• Function to optimise (link between data and model)
  – Should use experimental data
  – Should be able to handle chemical (e.g. bonds) and other (e.g. NCS, structural) information

• Parameters
  – Depends on the stage of analysis
  – Depends on amount and quality of the experimental data

• Methods to optimise
  – Depends on stage of analysis: simulated annealing, conjugate gradient, second order (normal matrix, information matrix, second derivatives)
  – Some methods can give error estimate as a by-product. E.g second order.
Two components of target function

Crystallographic target functions have two components: one of them describes the fit of the model parameters into the experimental data and the second one describes chemical integrity (restraints).

Currently used restraints are: bond lengths, angles, chirals, planes, ncs if available, some torsion angles, reference structures.
Crystallographic refinement

The function in crystallographic refinement has a form:

\[ L(p) = wL_X(p) + L_G(p) \]

Where \( L_X(p) \) is -loglikelihood and \( L_G(p) \) is -log of prior probability distribution – restraints.

\( L_X(p) \) – X-ray part of the function describes fit of the model parameters to the experimental data.

\( L_G(p) \) – Geometry part makes sure that the model is consistent with chemistry: bond lengths, angles, torsions, NCS, known structure etc.
Various forms of X-ray component

- SAD functions uses observed F+ and F- directly without any preprocessing by a phasing program (It is not available in the current version but will be available soon)
- SIRAS – uses native and derivative anomalous data directly
- MLHL - explicit use of phases with Hendrickson Lattman coefficients
- Rice - Maximum likelihood refinement without phase information
- TWIN – Maximum likelihood refinement against twinned data
-loglikelihood

-loglikelihood depends on assumptions about the experimental data, crystal contents and parameters. For example with assumptions that all observations are independent (e.g. no twinning), there is no anomalous scatterers and no phase information available, for acentric reflections it becomes:

\[ L_X(p) = \sum \frac{|F_o|^2 + |F_c|^2}{\Sigma} - \log(I_0(2|F_o||F_c|/\Sigma)) + \log(\Sigma) + \text{const} \]

And for centric reflections:

\[ L_X(p) = \sum \frac{|F_o|^2 + |F_c|^2}{2\Sigma} - \log(\cosh(|F_o||F_c|/\Sigma)) + 0.5\log(\Sigma) + \text{const} \]

All parameters (scale, other overall and atomic) are inside \(|F_c|\) and \(\Sigma\)
Note that these are loglikelihood of multiples of chi-squared distribution with degree of freedom 2 and 1
Twin refinement in REFMAC

Twin refinement in refmac (5.5 or later) is automatic.

- Identify “twin” operators
- Calculate “Rmerge” \((\Sigma |I_h - <I>_{twin}| / \Sigma I_h)\) for each operator. If Rmerge>0.50 keep it: Twin plus crystal symmetry operators should form a group
- Refine twin fractions. Keep only “significant” domains (default threshold is 5%): Twin plus symmetry operators should form a group

Intensities can be used
If phases are available they can be used
Maximum likelihood refinement is used
Likelihood

\[ P(I_0; F) = \int_F P(I_0, F; F_c)dF = \int_F P(I_0; F)P(F; F_c)dF \]
\[ P(I_0; F) = N_0e^{\frac{\sum I_{ij} - \sum \alpha_{ij} |I_{ij}|}{2\sigma_{ij}^2}} \]
\[ P(F; F_c) = N_c \prod e^{\frac{|F - \Delta F_c|^2}{\Sigma}} \]

The dimension of integration is in general twice the number of twin-related domains. Since the phases do not contribute to the first part of the integrant the second part becomes Rice distribution.

The integration is carried out using Laplace approximation.

These equations are general enough to account for: non-merohedral twinning (including allawtwin), unmerged data. A little bit modification should allow handling of simultaneous twin and SAD/MAD phasing, radiation damage.
**Warning**: Usually twin refinement reduces R factors but electron density does not improve much.

“non twin” map

“twin” map

Electron density: 1jrg
Effect on intensity statistics

Take a simple case. We have two intensities: weak and strong. When we sum them we will have four options w+w, w+s, s+w, s+s. So we will have one weak, two medium and one strong reflection.

As a results of twinning, the proportion of the weak and strong reflections becomes smaller and the number of medium reflections increases. It has effect on intensity statistics

In probabilistic terms: without twinning distribution of intensities is $\chi^2$ with degree of freedom 2 and after perfect twinning degree of freedom increases and becomes 4. $\chi^2$ distributions with higher degree of freedom behave like normal distribution
Twin: Few warnings about R values

Rvalues for random structures (no other peculiarities)

<table>
<thead>
<tr>
<th>Twin</th>
<th>Modeled</th>
<th>Not modeled</th>
</tr>
</thead>
<tbody>
<tr>
<td>Yes</td>
<td>0.41</td>
<td>0.49</td>
</tr>
<tr>
<td>No</td>
<td>0.52</td>
<td>0.58</td>
</tr>
</tbody>
</table>
Map calculation

- After refinement programs usually give coefficients for two type of maps: 1) 2Fo-Fc type maps. They try to represent the content of the crystal. 2) Fo-Fc type of maps. They try to represent difference between contents of the crystal and current atomic model. Both these maps should be inspected and model should be corrected if necessary.

- Refmac gives coefficients:

$$2m \ F_o - D \ F_c$$ – to represent contents of the crystal

$$m \ F_o - D \ F_c$$ - to represent differences

$m$ is the figure of merit (reliability) of the phase of the current reflection and $D$ is related to model error. $m$ depends on each reflection and $D$ depends on resolution.

If phase information is available then map coefficients correspond to the combined phases.
Parameters

Usual parameters (if programs allow it)
1) Positions x,y,z
2) B values – isotropic or anisotropic
3) Occupancy

Derived parameters
4) Rigid body positional
   • After molecular replacement
   • Isomorphous crystal (liganded, unliganded, different data)
5) Rigid body of B values – TLS
   – Useful at the medium and final stages
   – At low resolution when full anisotropy is impossible
6) Torsion angles
Bulk solvent
Method 1: Babinet’s bulk solvent correction

At low resolution electron density is flat. Only difference between solvent and protein regions is that solvent has lower density than protein. If we would increase solvent just enough to make its density equal to that of protein then we would have flat density (constant). Fourier transformation of constant is zero (apart from F000). So contribution from solvent can be calculated using that of protein. And it means that total structure factor can be calculated using contribution from protein only.

\[
\rho_s + \rho_p = \rho_T \quad \Rightarrow \quad F_s + F_p = F_T
\]
\[
\rho_s + k\rho_p = c \quad \Rightarrow \quad F_s + kF_p = 0
\]
\[
F_s = -kF_p \quad \Rightarrow \quad F_T = F_p - kF_p = (1-k)F_p
\]

\(k\) is usually taken as \(k_b \exp(-B_b s^2)\). \(k_b\) must be less than 1. \(k_b\) and \(B_b\) are adjustable parameters.
Bulk solvent
Method 2: Mask based bulk solvent correction

Total structure factor is the sum of protein contribution and solvent contribution. Solvent region is flat. Protein contribution is calculated as usual. The region occupied by protein atoms is masked out. The remaining part of the cell is filled with constant values and corresponding structure factors are calculated. Finally total structure factor is calculated using

\[ F_T = F_p + k_s F_s \]

\( k_s \) is adjustable parameter.

Mask based bulk solvent is a standard in all refinement programs. In refmac it is default.
Overall parameters: Scaling

There are several options for scaling:

1) Babinet’s bulk solvent assumes that at low resolution solvent and protein contributors are very similar and only difference is overall density and B value. It has the form: \[ k_b = 1 - k_b e^{-B_b s^2/4} \]

2) Mask bulk solvent: Part of the asymmetric unit not occupied by atoms are assigned constant value and Fourier transformation from this part is calculated. Then this contribution is added with scale value to “protein” structure factors. Total structure factor has a form: \[ F_{\text{tot}} = F_p + s_s e^{-s B s^2/4} F_s \]

3) The final total structure factor that is scaled has a form:
\[ S_{\text{aniso}} S_{\text{protein}} k_b F_{\text{tot}} \]
TLS
Rigid-body motion

General displacement of a rigid-body point can be described as a rotation along an axis passing through a fixed point together with a translation of that fixed point.

\[ \mathbf{u} = \mathbf{t} + D\mathbf{r} \]

for small librations

\[ \mathbf{u} \approx \mathbf{t} + \lambda \times \mathbf{r} \]

D = rotation matrix
\( \lambda \) = vector along the rotation axis of magnitude equal to the angle of rotation
Use of available knowledge

1) NCS local
2) Restraints to known structure(s)
3) Restraints to current inter-atomic distances (implicit normal modes or “jelly” body)
4) Better restraints on B values

These are available from the version 5.6

Note
Buster/TNT has local NCS and restraints to known structures
CNS has restraints to known structures (they call it deformable elastic network)
Phenix has B-value restraints on non-bonded atom pairs and automatic global NCS
Local NCS (only for torsion angle related atom pairs) was available in SHELXL since the beginning of time
NCS: Three approaches

NCS may improve refinement and produce “better” model. There are three different approaches in refinement:

1) Strict NCS
   NCS related molecules are exact copy of each other. You need to give only one independent copy. You need also to give NCS operators.

2) NCS global restraints
   Entire molecules are similar to each other

3) Local NCS
   Molecules are only locally similar
Auto NCS: local and global

1. Align all chains with all chains using Needleman-Wunsh method
2. If alignment score is higher than predefined (e.g. 80%) value then consider them as similar
3. Find local RMS and if average local RMS is less than predefined value then consider them aligned
4. Find correspondence between atoms
5. If global restraints (i.e. restraints based on RMS between atoms of aligned chains) then identify domains
6. For local NCS make the list of corresponding interatomic distances (remove bond and angle related atom pairs)
7. Design weights

The list of interatomic distance pairs is calculated at every cycle
Global RMS is calculated using all aligned atoms.

Local RMS is calculated using $k$ (default is 5) residue sliding windows and then averaging of the results.

\[
\text{Ave}(\text{RmsLoc})_k = \frac{1}{N - k + 1} \sum_{i=1}^{N-k+1} \text{RmsLoc}_i \\
RMS = \text{Ave}(\text{RmsLoc})_N
\]
**Auto NCS: Iterative alignment**

Example of alignment: 2vtu.
There are two chains similar to each other. There appears to be gene duplication

RMS – all aligned atoms
Ave(RmsLoc) – local RMS

<table>
<thead>
<tr>
<th>N</th>
<th>Chain 1</th>
<th>Chain 2</th>
<th>No of aligned</th>
<th>Score</th>
<th>RMS</th>
<th>Ave(RmsLoc)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>J( 131 - 256 ) : J( 3 - 128 ) :</td>
<td>126 :</td>
<td>1.0000 :</td>
<td>5.2409 :</td>
<td>1.6608 :</td>
<td></td>
</tr>
<tr>
<td>2</td>
<td>J( 1 - 257 ) : L( 1 - 257 ) :</td>
<td>257 :</td>
<td>1.0000 :</td>
<td>4.8200 :</td>
<td>1.6694 :</td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>J( 131 - 256 ) : L( 3 - 128 ) :</td>
<td>126 :</td>
<td>1.0000 :</td>
<td>5.2092 :</td>
<td>1.6820 :</td>
<td></td>
</tr>
<tr>
<td>4</td>
<td>J( 3 - 128 ) : L( 131 - 256 ) :</td>
<td>126 :</td>
<td>1.0000 :</td>
<td>3.0316 :</td>
<td>1.5414 :</td>
<td></td>
</tr>
<tr>
<td>5</td>
<td>L( 131 - 256 ) : L( 3 - 128 ) :</td>
<td>126 :</td>
<td>1.0000 :</td>
<td>0.4515 :</td>
<td>0.0464 :</td>
<td></td>
</tr>
</tbody>
</table>
In many cases it could be expected that two or more copies of the same molecule will have (slightly) different conformation. For example if there is a domain movement then internal structures of domains will be same but between domains distances will be different in two copies of a molecule.
Robust estimators

One class of robust (to outliers) estimators are called M-estimators: maximum-likelihood like estimators. One of the popular functions is Geman-Mcclure.

Essentially when distances are similar then they should be kept similar and when they are too different they should be allowed to be different.

This function is used for NCS local restraints as well as for restraints to external structures.

Red line: $x^2$
Black line: $x^2/(1+w \times x^2)$

where $x=(d1-d2)/\sigma$, $w=0.1$
Restraints to external structures
It is done by Rob Nicholls

ProSmart

Compares Two Protein Chains
- Conformation-invariant structural comparison
- Residue-residue alignment
- Superimposition
- Residue-based and global similarity scores

Produces local atomic distance restraints
- Based on one or more aligned chains
- Possibility of multi-crystal refinement
ProSmart Restrain

structure to be refined  known similar structure (prior)

Remove bond and angle related pairs
Restraints to current distances

The term is added to the target function:

\[ \sum_{\text{pairs}} w(|d| - |d_{\text{current}}|)^2 \]

Summation is over all pairs in the same chain and within given distance (default 4.2Å). \(d_{\text{current}}\) is recalculated at every cycle. This function does not contribute to gradients. It only contributes to the second derivative matrix.

It is equivalent to adding springs between atom pairs. During refinement inter-atomic distances are not changed very much. If all pairs would be used and weights would be very large then it would be equivalent to rigid body refinement.

It could be called “implicit normal modes”, “soft” body or “jelly” body refinement.
B value restraints and TLS

Designing restraints on B values is much more difficult. Current available options to deal with B values at low resolutions

1) Group B as implemented in CNS
2) TLS group refinement as implemented in refmac and phenix.refine

Both of them have some applications. TLS seems to work for wide range of cases but unfortunately it is very often misused. One of the problems is discontinuity of B values. Neighbouring atoms may end up having wildly different B values.

In ideal world anisotropic U with good restraints should be used. But this world is far far away yet. Only in some cases full aniso refinement at 3Å gives better R/Rfree than TLS refinement. These cases are with extreme anisotropic data.
Kullback-Leibler divergence

If there are two densities of distributions – \( p(x) \) and \( q(x) \) then symmetrised Kullback-Leibler divergence between them is defined (it is distance between distributions)

\[
\frac{1}{2} \left( \int_{-\infty}^{\infty} p(x) \log\left( \frac{p(x)}{q(x)} \right) dx + \int_{-\infty}^{\infty} p(x) \log\left( \frac{q(x)}{p(x)} \right) dx \right)
\]

If both distributions are Gaussian with the same mean values and \( U_1 \) and \( U_2 \) variances then this distance becomes:

\[
tr(U_1 U_2^{-1} + U_2 U_1^{-1} - 2I)
\]

And for isotropic case it becomes

\[
3\left( \frac{B_1}{B_2} + \frac{B_2}{B_1} - 2 \right) = 3\left( \frac{B_1 - B_2}{B_1B_2} \right)^2
\]

Restraints for bonded pairs have more weights more than for non-bonded pairs. For nonbonded atoms weights depend on the distance between atoms.

This type of restraint is also applied for rigid bond restraints in anisotropic refinement.
Example, after molecular replacement
3A resolution, data completeness 71%

Rfactors vs cycle
Black – simple refinement
Red – Global NCS
Blue – Local NCS
Green – “Jelly” body

Solid lines – Rfactor
Dashed lines - Rfree
Example: 4A resolution, data from pdb 2r6c

Starting R/Rfree = 36.0/35.6

R/Rfree after 40 cycles of refinement

<table>
<thead>
<tr>
<th></th>
<th>None</th>
<th>Ncs local</th>
<th>Jelly body</th>
<th>External structure</th>
</tr>
</thead>
<tbody>
<tr>
<td>R</td>
<td>20.80</td>
<td>21.44</td>
<td>23.72</td>
<td>23.38</td>
</tr>
<tr>
<td>Rfree</td>
<td>32.69</td>
<td>31.79</td>
<td>29.82</td>
<td>28.69</td>
</tr>
</tbody>
</table>
Simple map sharpening may increase signal as well as noise. Sometimes
noise amplification may be very large and mask out the signal
completely.
Noises in electron density: series termination, errors in phases and noises
in experimental data.

Regularised map sharpening tries to reduce noise amplification while
increasing signal

\[
F_{\text{deblurred}} = \frac{e^{-B|s|^2/4}}{e^{-2B|s|^2/4} + \alpha |s|^2} F = K_\alpha (s,B) F
\]
MAP SHARPENING: 2R6C, 4Å RESOLUTION

Original

No sharpening

Sharpening, median B
$\alpha$ 0

Sharpening, median B
$\alpha$ optimised

Top left and bottom:
After local NCS refinement
Example, 2r6c, Electron density

Known structure (2r6a) superimposed to 2r6c structure. There is a helix. Side chains are visible to some degree.
Things to look at

- R factor/Rfree: They should go down during refinement
- Geometric parameters: rms bond and other. They should be reasonable. For example rms bond should be around 0.02
- Map and coordinates using coot
- Logggraph outputs. That is available on the cpp4i interface
- Twin operators and twin fractions
Behaviour of R/Rfree, average Fobs vs resolution should be reasonable. If there is a bump or it has an irregular behaviour then something is wrong with your data or refinement.
What and when

• Rigid body: At early stages - after molecular replacement or when refining against data from isomorphous crystals
• “Jelly” body – At early stages and may be at low resolution
• TLS - at medium and end stages of refinement at resolutions up to 1.7-1.6A (roughly)
• Anisotropic - At higher resolution towards the end of refinement
• Adding hydrogens - Higher than 2A but they could be added always
• Phased refinement - at early and medium stages of refinement
• SAD – at the early stages
• Twin - always (?). Be careful at early stages
• NCS local – always?
• Ligands - as soon as you see them
• What else?
How to use new features

Download refmac from the website
www.ysbl.york.ac.uk/refmac/data/refmac_experimental/refmac5.6_linux.tar.gz
www.ysbl.york.ac.uk/refmac/data/refmac_experimental/refmac5.6_macintel.tar.gz

Download the dictionary:
www.ysbl.york.ac.uk/refmac/data/refmac_experimental/refmac5.6_dictionary_v5.18.gz

Change atom names using molprobity (optional: important if you have dna/rna)
http://molprobity.biochem.duke.edu/

Refmac refmac5 with the new one and you are ready for the new version.
Twin refinement (it works with older version also)
Adding external keywords

• Add the following command to a file:

```plaintext
cnsr local           # automatic and local ncs
ridg dist sigm 0.05  # jelly body restraints
mapcalculate shar    # regularised map sharpening
```

Save in a file (say keyw.dat)
Add external keywords file in refmac interface

**Browse files**
Add external keywords file in refmac interface

Select keywords file
Add external keywords file in refmac interface

![Refmac interface screenshot]

Keywords file

- Include keyword file: `keyw.dat`
Conclusion

- Twin is automatic refinement improves statistics and occasionally electron density
- Use of similar structures should improve reliability of the derived model: Especially at low resolution
- NCS restraints must be done automatically: but conformational flexibility must be accounted for
- “Jelly” body works better than I thought it should
- Regularised map sharpening looks promising. More work should be done on series termination and general sharpening operators
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REFMAC is available from CCP4 or from York’s ftp site:
www.ysbl.york.ac.uk/refmac/latest_refmac.html

This and other presentations can be found on:
www.ysbl.york.ac.uk/refmac/Presentations/